SI Appendix

MB07811, (2R,4S)-4-(3-chlorophenyl)-2-[(3,5-dimethyl-4-(4-hydroxy-3-isopropylbenzyl)phenoxy)methyl]-2-oxido-[1,3,2]-dioxaphosphonane, MB07344, (3,5-dimethyl -4-(4'-hydroxy-3'-*iso*-propylbenzyl)-phenoxy)methylphosphonic acid, KB-141, 3,5-dichloro-4-(4-hydroxy-3-isopropylphenoxy)phenylacetic acid (1) and the glutathione conjugate **2** (2) were synthesized at Metabasis Therapeutics. $[^{125}I]$-T_3 was purchased from PerkinElmer. T3 and clotrimazole were purchased from Sigma-Aldrich (St. Louis). Male SD rats and male C57BL/6 mice were purchased from Harlan (San Diego, CA). Male thyroidectomized SD rats and male LDLR$^{-/-}$ mice were purchased from Charles River Laboratories (Wilmington, MA) and Jackson Laboratory, respectively. Lutrol F68 NF was purchased from BASF.

Receptor Binding Affinity. *Preparation of recombinant TR heterodimers.* cDNAs encoding TRα (IMAGE: 2961613) and TRβ (ATCC ID: 67244) were purchased from the American Type Culture Collection. cDNA for RXRα was purchased from Stratagene. The Bac-to-Bac baculovirus expression system was purchased from Invitrogen.

Using the above-mentioned cDNAs as templates and the *pfu* enzyme (Stratagene), cDNA fragments of TRα, TRβ and RXRα were generated by PCR with use of the following primers designed to flank the coding region for each receptor:

Table 2. Primers for TRs and RXR

Receptor	End	Sequence
TRα	5'	acg tgg atc cac cat gga aca gaa gcc aag caa
TRα	3'	acg tgc ggc cgc ttt aga ctt cct gat cc
TRβ	5'	acg tgg atc cat ggc aga aaa tgg cct tac agc
TRβ	3'	atg cct cga gct aat cct cga aca ctt cc

RXRα	5'	acg tga att cca cca tgg aca cca aac att tc
RXRα	3'	aaa gat cgc ggc cgc

The cDNA fragment encoding full-length TRα was ligated into the pFastBac HT B vector; the TRβ fragment was ligated into pFastBac HT A; the RXRα fragment was inserted into pFastBac 1. In each construct, nucleotides encoding a His-tag were fused in frame to the 5'-ends of the coding regions of the receptors. Sequences of the cDNAs in the vectors were verified. Baculoviruses expressing the receptors were generated according to the instructions enclosed in the Bac-to-Bac baculovirus expression kit.

Thyroid hormone receptor scintillation proximity assays. Cells (sf9) were cultured with Sf-900 II SFM medium in an Erlenmeyer flask (Corning, Corning, NY) at 27°C and 123 rpm. When a density of 1×10^5 cells/ml was reached, the cells were coinfected with TRα and RXRα baculoviruses, or TRβ and RXRα baculoviruses. The TR to RXRα baculovirus ratio was 1:1; the baculovirus-to-cell ratio was ≈10:1. Three days later, the infected cells were lysed in SPA buffer (50 mM NaCl, 10% glycerol, 20 mM Tris, pH 7.6, 2 mM EDTA, 5 mM β-mercaptoethanol, 1.25% CHAPS, 200 mM glycine). After centrifugation (10,000 rpm, 30 min, 4°C, SS34 rotor) using a Sorvall RC5B centrifuge (DuPont, Wilmington, DE), the supernatants were collected, divided into aliquots, and stored at -80°C. The lysates were diluted ≈20-fold before use.

Displacement studies were conducted by first incubating 0.25 nM [^{125}I]-T3 and TRα/RXRα or TRβ/RXRα with different concentrations of test compound (0.005 to 10,000 nM) at 4°C for 30 min in the wells of 96-well plates (final volume of 50 μl). A 50 μl suspension of SPA beads (2 μg/μl; Ysi Copper His-Tag SPA beads, Amersham Pharmacia, Piscataway, NJ) in SPA buffer was then added to each well. After a 30-min incubation at 4°C, the samples were counted by means of a TRI-LUX MicroBeta plate reader (Perkin-Elmer, Finland). Displacement curves were generated by means of a nonlinear regression program (SigmaPlot, Systat, CA). The four parameter logistic equation used is as follows:

$$y = \min + \frac{\max - \min}{1 + 10^{(\log EC_{50} - x) * hillslope}}$$

Where x = concentration of compound, y = response, min = minimal response plateau, max = maximal response plateau, and hillslope = describes slope of curve.

K_i(apparent) values were calculated from IC_{50} values using the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[L^*]}{K_d}}$$

Where K_i = apparent inhibition constant of compound, $[IC_{50}]$ = the concentration of compound which displaces 50% of the specific binding of radioligand, $[L^*]$ = concentration of radioligand, and K_d = dissociation constant of the radioligand.

Prodrug Activation Assays. Initial velocities were determined by incubating MB07811 (20 and 200 μM) with SD rat liver microsomal protein concentrations of 0.5, 1, and 2 mg/ml. The reaction mixtures consisted of 100 mM potassium phosphate pH 7.4, 10 mM glutathione, 2 mM NADPH, and dimethyl sulfoxide (DMSO) to yield a final concentration of 2% (v:v). All reaction components (except the NADPH) were preincubated for 5 min at 37°C using a Thermomixer R (Eppendorf, Westbury, NY) set to 800 rpm. Aliquots (80 μl) were removed from the reaction mixtures before the addition of NADPH, and after 2.5, 5, 7.5, and 10 min of incubation (under the aforementioned conditions) after the addition of NADPH. The aliquots were quenched in 120 μl of 100% methanol in a 96-well microtiter plate. The plate was centrifuged in a GLC-4 clinical centrifuge (Sorvall Instruments, Asheville, NC) set to maximum rpm. The resulting supernatants were transferred into HPLC vials and placed in a Type 37900 Culture Incubator (Thermolyne, Dubuque, IA) set at 50°C for 4 h to generate the glutathione

conjugate of the byproduct of MB07811 activation, 2. The samples were analyzed for 2 by HPLC-UV analysis as described below.

Kinetic assays. The kinetic studies were performed by incubation of MB07811 (12.5, 25, 50, 100 and 200 μM) at a microsomal protein concentration of 1 mg/ml. The reaction mixture consisted of 100 mM potassium phosphate pH 7.4, 10 mM glutathione, 2 mM NADPH and DMSO adjusted to yield a final concentration of 2% (v:v). The reactions were essentially performed and quenched as described above. A negative control (DMSO, n = 1) was also included. Using the HPLC-UV methods described below, samples were analyzed for MB06588 and MB07344.

Quantification of ***2*** *by reversed phase HPLC-UV analysis*. HPLC-UV analysis of MB06588 was performed on an Agilent 1100 series instrument (Agilent Technologies, Palo Alto, CA) equipped with a 96-well microtiter plate autosampler (Catalog no. G1367A), a sample temperature control unit (Catalog no. G1330B), a column temperature control unit (Catalog no. G1316A), a quaternary pump (Catalog no. G13311A), a solvent degasser (Catalog no. G1322A) and a diode array UV/Visible detector (Catalog no. G1315B). Chromatographic separation was accomplished with an Ultrasphere C18 column (5 μm 4.6 × 250 mm; Catalog no. 235329, Beckman Coulter, Fullerton, CA), equipped with an Econosphere C18 guard column (5 μm 4.6 × 7.5 mm Catalog no. 96121, W. R. Grace & Co., Columbia, MD). The column was equilibrated with 10% acetonitrile in 20 mM potassium phosphate pH 6.2 and eluted with a linear gradient of 10–60% acetonitrile over 10 min at a flow rate of 1.0 ml/min and at a column temperature of 40°C. The UV absorbance was monitored at 245 nm. The retention time of **2** was ≈6.3 min. **2** was quantified by comparison to single or multiple point standards diluted in the appropriate matrix. The lower limit of quantitation was not determined.

Quantification of MB07344 by reversed phase HPLC-UV analysis. HPLC-UV analysis of MB07344 was performed on an Agilent 1100 series instrument (Agilent Technologies, Palo Alto, CA). Chromatographic separation was accomplished with an Ultrasphere C18 column (5 μm; 4.6 × 250 mm; Catalog no. 235329; Beckman Coulter, Fullerton, CA)

equipped with an Econosphere C18 guard column (5 μm, 4.6 × 7.5 mm Catalog no. 96121, W. R. Grace & Co., Columbia, MD). The column was equilibrated with 20% acetonitrile in 20 mM potassium phosphate pH 6.2 and eluted with a linear gradient of 20–80% acetonitrile over 20 min at a flow rate of 1.5 ml/min at a column temperature of 40°C. The UV absorbance was monitored at 280 nm. The retention time of MB07344 was ≈7.4 min. MB07344 was quantified by comparison to single or multiple point standards diluted in the appropriate matrix.

Determination of V_{max}, K_m and CL_{int} values. The Enzyme Kinetics module of Sigma Plot software was used to determine V_{max} and K_{m} kinetic values by fitting the kinetic data to the Henri-Michaelis-Menten equation (version 9.01, Systat Software, Point Richmond, CA). Sigma Plot was used to generate double-reciprocal data plots. CL_{int} values were calculated by dividing V_{max} values by K_{m} values. Statistical significance was determined by the unpaired Student's *t* test using the GraphPad InStat software (version 3.01 for Windows 95/NT; GraphPad Software, San Diego, CA) and *P* values less than 0.05 are considered significant. V_{max}, K_{m} and CL_{int} values are reported as mean values and the standard error of the mean (SEM).

Clotrimazole inhibition studies. The evaluation of the inhibition of CYP3A-mediated activation of MB07811 by clotrimazole was performed in mixed pools of male SD rat liver microsomes. The reaction mixtures contained 50 mM potassium phosphate pH 7.4, 8 μM magnesium chloride, 10 μM MB07811, 10 mM glutathione, 2 mg/ml rat liver microsomes, a NADPH-regenerating system consisting of 5 mM glucose-6-phosphate and 1.5 units/ml of glucose-6-phosphate dehydrogenase, and clotrimazole (ranging in log increments from 10 pM to 10 μM). Control reaction mixtures lacked clotrimazole. After preincubation of the mixtures at 37°C for 2 min, the reactions were initiated by the addition of 4 mM NADPH and incubated at 37°C for an additional 30 min. Aliquots were removed at 0 and after 30 min of incubation and extracted with 1.5 volumes of ice- cold methanol and mixed by vortexing. The extracts were clarified by centrifugation (Eppendorf Microfuge, 14,000 rpm, room temperature, 1 min) and the supernatants analyzed for MB07344 by the HPLC-UV method described above. The amount of

MB07344 formed was plotted versus the clotrimazole concentration and a K_i value calculated using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + [S]/K_m)$

Rat Hepatocyte Assays. Rat hepatocytes were suspended at 1 million cells per ml in *InVitro*GRO HI (incubation) media (In Vitro Technologies, Baltimore, MD). The cells were maintained in a shaking water bath at 37°C under an oxygenated atmosphere. Stock solutions of MB07811 and MB07344 were prepared in dimethyl sulfoxide (DMSO). Each test article at a final concentration of 10 µM was added to cell suspensions in duplicate and gently mixed. Aliquots (200 µl) were removed from each tube at the following time points: 0, 15, 30, 60, 90, and 120 min. Samples were centrifuged (Eppendorf microfuge, 14,000 rpm, 2 min, room temperature) through 50 µl of oil (composed of 1 volume of mineral oil and 4 volumes of silicone oil) to separate medium from the cells. The medium (top layer) was removed and an aliquot was added to 3 volumes of 100% methanol. The oil was aspirated and 300 µl of methanol was added to the cell pellet. The cell pellet was extracted by sonication in a water bath sonicator (2 min, room temperature). Methanol-extracted medium and cell samples were clarified by centrifugation (Eppendorf microfuge, 14,000 rpm, 20 min, room temperature) and the resulting supernatants analyzed by LC-MS/MS for MB07811 and MB07344 content as describe below.

ADME Studies. *Oral bioavailability studies*. Three groups ($n = 5$ per group) of catheterized, male, Sprague–Dawley rats (250-300 g, 6-8 weeks old) were dosed intravenously (i.v.) with MB07811 at 3 mg/kg in 100% propylene glycol or orally (*per os*) with MB07811 at 3 or 10 mg/kg in 100% polyethylene glycol-400. Another set of rats was administered an i.v. bolus of 5 mg/kg of MB07344 (in pH 7 adjusted isotonic saline). Animals were fasted for 3 h before oral administration and were refed 1 h after dosing. Animals had free access to food before and during the i.v. evaluation. Blood samples were taken predose and at 5, 20, and 40 min and 1, 1.5, 2, 3, 5, 8, 12, and 24 h after i.v. administration of MB07811 and MB07344 or at 0.5, 1, 2, 3, 4, 5, 6, 8, 12, and 24 h after oral administration of MB07811. Plasma was prepared from blood samples by centrifugation and analyzed by liquid chromatography tandem mass spectrometry (LC-

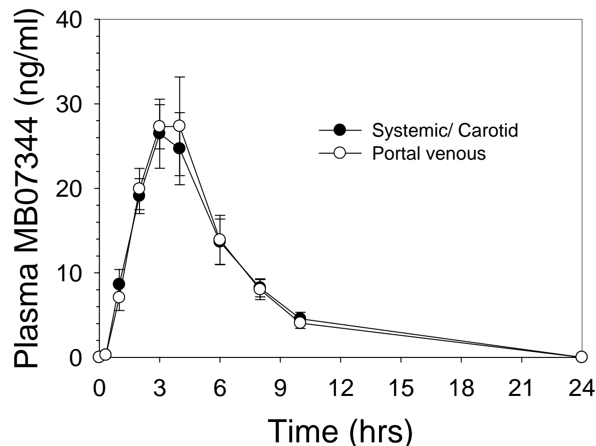
MS/MS) for MB07811 and MB07344 levels as described below. The temporal profile of MB07811 and MB07344 concentrations in plasma was analyzed by noncompartmental methods (WinNonLin, Pharsight, Mountain View, CA). The relative oral bioavailability of MB07811 was calculated by comparison of the dose-normalized area under the curve (AUC) values of the plasma concentrations of MB07811 after oral administration of MB07811 with the AUC values of MB07811 after i.v. administration of MB07811. The relative oral bioavailability of MB07344 was estimated by dividing the plasma AUC values of MB07344 after oral dosing of MB07811 by the plasma AUC values of MB07344 after i.v. administration of the prodrug MB07811.

Quantification of MB07811 and MB07344 by LC-MS/MS analysis. Plasma, bile, or cell extracts were analyzed for MB07811 and MB07344 levels using an LC-MS/MS (API 4000; Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 binary pump and a LEAP injector. Ten microliter injection volumes were analyzed on a Luna C8 column (5 μm, 2 × 50 mm, Phenomenex, Torrance, CA) fitted with a SecurityGuard C18 guard column (5 μm, 4.0 × 3.0 mm, Phenomenex), and eluted with a gradient from mobile phase A [20 mM N,N-dimethylhexylamine (DMHA) and 10 mM propionic acid in 20% methanol] to B (20 mM DMHA and 10 mM propionic acid in 80% methanol) at a flow rate of 0.3 ml/min (0 min, 60% B; 0-1 min, 60-100% B; 1-6 min, 100% B; 6-6.1 min, 100–60% B; 6.1-9 min, 60% B). The injector temperature was set at 10°C. Elution times for MB07344 and MB07811 were ≈4.1 and 5.6 min, respectively. MB07811 and MB07344 were detected by using the MS/MS mode (513/63.1 for MB07811 and 363.3/63.1 for MB07344) and quantified by comparison of peak areas to standard curves obtained by spiking known concentrations of the analytes into blank rat plasma, bile, or hepatocyte extracts. Calibration curves ranging from 10 to 3000 ng/ml of MB07344 and MB07811 were generated. The limit of quantitation for both MB07344 and MB07811 was ≈10 ng/ml.

Hepatic extraction. Male SD rats (n = 4 - 5) were catheterized at the carotid and portal vein and were administered a dose of 3 mg/kg of MB07811 (suspension in 0.5% CMC and 1% Lutrol F68) by gavage. Blood samples were taken from the carotid (systemic

concentration) and portal vein at predose, 0.5, 1, 2, 4, 8, 12, and 24 h after administration, heparinized, and centrifuged to obtain plasma. Plasma samples were extracted with methanol and the resulting supernatants were analyzed for MB07811 and MB07344 by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The area under the curve (AUC) of the plasma concentration-time profile of MB07811 and MB07344 from the portal vein (AUC_{pv}) and carotid artery (AUC_{sys}) were determined by noncompartmental analysis to the last measurable time point. Hepatic extraction (E_H) of MB07811 was determined from the equation $E_H = (AUC_{pv} – AUC_{sys}) / AUC_{pv}$ (3). The mean plasma AUC value of MB07811 as sampled systemically and from the portal vein was 0.062 ± 0.011 and 0.129 ± 0.004 mg·hr/L, respectively. The plasma AUC value of MB07344 was 0.151 ± 0.52 and 0.155 ± 0.041 mg·hr/L from the systemic and portal blood samples, respectively. The E_H of the prodrug in this study was estimated to be 0.52. The systemic and portal vein plasma concentrations of MB07344 after oral dosing of 3 mg/kg MB07811 are shown in the figure below. The systemic MB07344-to-MB07811 AUC ratio was two-fold greater than the value observed portally (2.4 versus 1.2), an indication of first pass prodrug extraction and metabolism. Similar preand postliver exposure to MB07344 may be attributed to the potential bidirectional transport of the metabolite in the liver, its limited distribution to extrahepatic tissues, and its almost exclusive clearance by bile.

Fig. 6. MB07344 Levels after Oral Administration of MB07811.



Tissue distribution. Male SD rats (n = 8, ≈250 g, ≈ 7 weeks of age) were administered an oral dose of 5 mg/kg of ^{14}C-radiolabeled MB07811 (≈250 μCi/kg, 0.5% CMC/ 1% Lutrol F68). Groups of rats (n = 4 per time point) were killed at 3 and 24 h after dose administration and the following tissues were harvested: cervical lymph nodes, thyroid gland, testes, epididymal fat, urinary bladder, prostate, spleen, pancreas, stomach, mesenteric lymph nodes, small intestine, large intestine, liver, adrenal glands, kidneys, thymus, heart, lungs, bone marrow, quadriceps muscle, eyes, brain, pituitary gland, skin, blood, plasma, and bone (femur). For the stomach, small intestine and large intestine, the luminal contents and a wash were also collected. The tissues were rinsed in water, dissolved by the addition of Soluene-350, decolorized as necessary, and then analyzed for radioactive content by liquid scintillation counting. Liquid samples (washes, bone marrow, blood, and plasma) were decolorized with sodium hypochlorite and analyzed directly by liquid scintillation counting.

Selected tissues were also homogenized in 4 volumes of 60% acetonitrile in water for radiolabeled metabolite identification. The analysis of the radiolabeled tissue extracts was performed on a Hewlett Packard HP1050 HPLC (Palo Alto, CA). The mobile phase was degassed by helium sparging. The samples were analyzed on an Beckman Ultrasphere C18 column (5 μm, 4.6 × 250 mm) (Cat no.235329) (Alltech, Deerfield, IL) equipped with an All-Guard cartridge containing an Econosphere C18 (5 μm, 4.6 × 7.5 mm) guard column (Cat no.96121) (Alltech). The column was equilibrated with 20% acetonitrile and 80% 20 mM potassium phosphate pH 6.2 and eluted with a linear gradient of 20–80% acetonitrile over 20 min at a flow rate of 1.5 ml/min. The column temperature was ambient (≈22°C) and not controlled. The UV absorbance was monitored at 280 nm and radioactivity was monitored by a Radiometric Series A-100 detector (open channel) by Packard (Madison, CT) connected through an Agilent 35900E interface. The ratio of liquid scintillant Ultima-FLO M or Ultima-FLO AP (Packard) to HPLC eluent was 3-to-1. The retention times of MB07344 and MB07811 were ≈8.4 and 20.4 min, respectively. The retention time of MB06588 was ≈4 min in the UV chromatograms. Authentic ^{14}C-MB07811 and ^{3}H-MB07344 and unlabeled standards were used to confirm the

identification of the prodrug and active metabolite, respectively, in the incubation mixtures.

Table 3. Tissue and fluid concentrations of ^{14}C-MB07811 and metabolites after oral administration of 5 mg/kg ^{14}C-MB07811 to SD rats (Mean ± SD)

Tissue	3 Hour (nmol/g)	24 Hour (nmol/g)
lymph(c)	0.12 ± 0.18	0.03 ± 0.00
thyroid	0.20 ± 0.13	0.03 ± 0.02
testes	0.03 ± 0.02	0.04 ± 0.02
fat	0.15 ± 0.10	0.55 ± 0.96
bladder	0.18 ± 0.12	0.05 ± 0.02
prostate	0.12 ± 0.09	0.03 ± 0.02
spleen	2.57 ± 4.81	0.01 ± 0.00
pancreas	0.25 ± 0.11	0.25 ± 0.05
stomach	76.96 ± 48.75	0.22 ± 0.09
stom. C&W	24.52 ± 10.88	0.06 ± 0.02
lymph(m)	16.61 ± 17.57	0.05 ± 0.01
small int.	28.56 ± 22.73	0.40 ± 0.22
sma. C&W	39.69 ± 30.16	0.20 ± 0.03
large int.	1.23 ± 1.12	0.64 ± 0.21
larg. C&W	4.68 ± 2.85	2.50 ± 0.89
liver	6.67 ± 3.54	3.67 ± 0.64
adrenal	0.77 ± 0.46	0.09 ± 0.02
kidneys	0.48 ± 0.37	0.14 ± 0.01
thymus	0.09 ± 0.05	0.01 ± 0.00
heart	0.36 ± 0.36	0.05 ± 0.01
lungs	0.31 ± 0.21	0.04 ± 0.00
marrow	0.06 ± 0.09	0.01 ± 0.00
muscle	0.07 ± 0.05	0.01 ± 0.00
eyes	0.04 ± 0.02	0.01 ± 0.00
brain	0.03 ± 0.01	0.01 ± 0.00
pituitary	0.09 ± 0.12	0.02 ± 0.00
skin	0.08 ± 0.05	0.15 ± 0.10
blood	0.12 ± 0.07	0.05 ± 0.01
plasma	0.15 ± 0.10	0.05 ± 0.01
bone	0.14 ± 0.14	0.02 ± 0.01

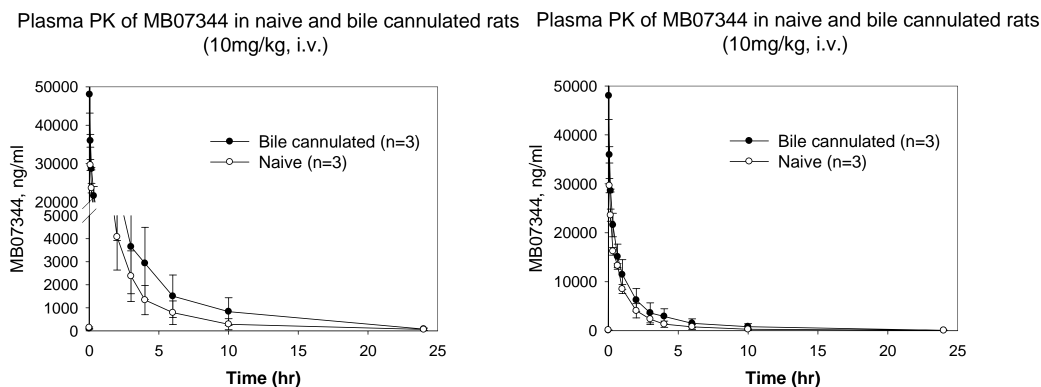
Mass balance. After i.v. bolus administration (via the tail vein) of 2 mg/kg of ^{3}H-radiolabeled MB07344 (in neutralized isotonic saline) to male SD rats (n = 6, ≈250 g, ≈7 weeks of age), urine (plus cage wash) and feces were collected over the course of 4 days

for the periods 0-12 h, 0-24 h, 0-48 h, 0-72 h, and 0-96 h. The urine and cage wash samples were analyzed for radioactivity by liquid scintillation counting without dilution. The fecal samples were extracted with 50% (vol/vol) acetonitrile in water, decolorized with sodium hypochlorite at 60°C, and then analyzed for radioactivity by liquid scintillation counting.

Biliary excretion. Bile duct-cannulated rats (*n* = 3) and naïve rats (*n* = 3) were administered MB07344 (iv) at 10 mg/kg. For bile duct-cannulated rats, bile was collected over the following periods: 0-1, 1-3, 3-6, 6-9 and 9-24 h. For both bile duct-cannulated and naïve rats, blood samples were collected at 0, 2, 5, 10, 20 and 40 min, and at 1, 2, 3, 4, 6, 10 and 24 h after administration of MB07344, and plasma AUCs of MB07344 in both rats were evaluated. MB07344 was extracted from plasma and bile samples with methanol and analyzed with LC-MS/MS.

Plasma MB07344: there was no difference in plasma PK profiles of MB07344 between naïve and bile-diverted animals. These results indicate that MB07344 is not subject to enterohepatic recirculation. Note, the data are plotted with and without expanding the *y* axis at the lower concentrations of MB07344 for clarity.

Fig. 7. MB07344 Plasma Levels



mRNA Expression Analysis. Adult SD rats (Harlan, San Diego) 62-73 days of age were used for all gene expression studies. For the T3 experiment, groups of animals (*n* = 6)

were dosed (*per os*; 5 ml/kg) with vehicle (water) or T3 (dissolved in water and adjusted to neutrality with NaOH) at 12, 36 or 120 μg/kg and killed 24 h later. On a separate day, 4 additional groups of animals were similarly treated and sets from each group (n = 6) were killed 3 or 8 h later. The KB-141 and MB07811 experiments were done on separate days. Groups of animals were dosed (5 ml/kg; *per os*) with water (KB-141 experiment) or KB-141 at 0.05, 0.15, or 0.5 mg/kg or vehicle (0.5% CMC/1% Lutrol F68 for the MB07811 experiment) or MB07811 at 0.4, 1.2, and 4 mg/kg. Sets of animals (n = 6) from each group were killed 3, 8 or 24 h after dosing. At necropsy, animals were anesthetized with isoflurane (Hospira, Lake Forest, IL) at 2.5%, the abdominal cavity was opened, the diaphragm was cut, and the heart quickly removed and freeze-clamped in liquid nitrogen. The following tissues were also removed and either freeze-clamped or snap-frozen in liquid nitrogen: the pituitary, thyroid gland, lungs, liver, spleen, kidneys, adrenal glands, soleus muscle and extensor digitalis longus muscle. All samples were stored at -80°C until processed for mRNA analysis.

Frozen tissues (10-50 mg) from rats treated with T3, KB-141 or MB07811 were transferred to Lysing Matrix D tubes containing 1.4 mm ceramic spheres (QBiogene, Irvine, CA). After addition of 1 ml of Trizol (Invitrogen), the tissues were homogenized with a FastPrep tissue disruptor (QBiogene, Irvine, CA). Chloroform (200 μl) was added to each sample and mixed thoroughly. The samples were then centrifuged at 12,000 rpm for 15 min in an Eppendorf table-top centrifuge. The resulting RNA-containing supernatants were carefully transferred to new tubes and mixed with 70% ethanol (400 μl). Total RNA was further purified by means of RNeasy RNA purification reagents (Qiagen, Valencia, CA) used according to the instructions provided by the manufacturer. After incubation with RNase-free DNase I (Invitrogen) for 15 min at 25°C, the RNA samples were used as templates to synthesize first strand cDNA with reagents from Invitrogen used according to the manufacturer's instruction. The RT-PCRs contained 5 ng of cDNA, 200 nM of each primer, and 5 μl of SYBR supermix. The total volume of the reaction was 10 μl. The following qRT-PCR protocol was used: 1 cycle at 95°C for 3 min followed by 40 cycles at 95°C for 15 seconds and 60°C for 45 seconds. All reactions were carried out in an iCycler IQ RT- PCR Detection System (Bio-Rad Laboratories,

Hercules, CA). The RT-PCR threshold numbers were adjusted based on the actual curves and the ratios between vehicle and compound treated samples were calculated by using the $2^{-\Delta\Delta Ct}$ method (4).

Primers for RT-PCRs were as follows:

Table 4. Primers for selected TR-sensitive genes

Gene	Sequences
CYP7a	5'-GTTTCGACATGCTCTCGCTAT-3' (forward) and 5'-GACCAGAATAACCTCAGACTC-3 (reverse)
MHCβ	5'-CAGGCCAAGCGCAACCACCTG-3' (forward) and 5'-ACTCTGGAGGCTCTTCACTTG-3' (reverse)
Iodothyronine deiodinases I	5'-GTGGACACAATGCAGAACCAG-3' (forward) and 5'-ACTTCCTCAGGATTGTAGTTC-3' (reverse)
TSHβ	5'-AGGAGAGAGTGTGCCTACTGC-3' (forward) and 5'-GGTATTTCCACCGTTCTGTAG-3' (reverse)
Malic enzyme	5'-GCTCTATCCTCCTTTGAATAC-3' (forward) and 5'-ATAATTAGTGCTGTACATCTG-3' (reverse)
SREBP-1c	5'-GGAGCCATGGATTGCACATT-3' (forward) and 5'-AGGAAGGCTTCCAGAGAGGA-3' (reverse)
UCP3	5'-TGTGCTGAGATGGTGACCTA-3' (forward) and 5'-TCGGGTCTTTACCACATCCAC-3' (reverse)
Cyclophilin	5'- CCAGGATTCATGTGCCAGG-3' (forward) and 5'-CGCTCCATGGCTTCCACAATG-3' (reverse)
PEPCK	5'-AGGTCACTCAGGAATCCAG-3' (forward) and 5'-ACACATATATACAGCTCTTC-3' (reverse)
SREBP-2	5'-CAAGTCCTGCAGCCTCAAGTG-3' (forward), and 5'-CCGGGACCTGCTGCACCTGTG-3' (reverse)
LDLR	5'-TGACGGGCTGGCGGTAGACTG-3' (forward) and 5'-AGTGTGATGCCATTTGGCCAC-3' (reverse)

HMGR	5'- CTGTAGCTGACAGAACTTCTG-3' (forward) and 5'- ATTCTTCCATGAAGAAGTAGG-3' (reverse)

Fig. 8. Expression of D1 in the hearts of rats treated with T3, KB-141 and MB07811, respectively.

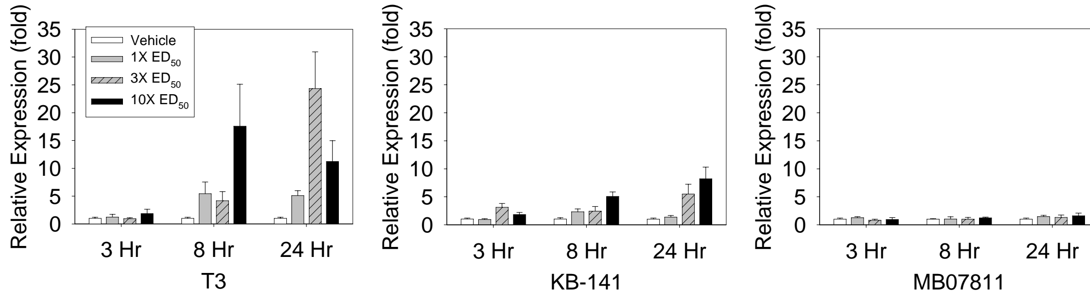


Fig. 9. Expression of MHCβ in the hearts of rats treated with T3, KB-141 and MB07811, respectively.

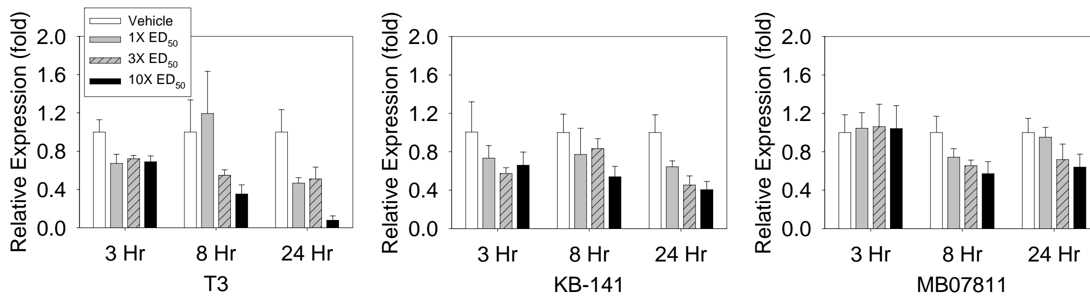


Fig. 10. Expression of D1 in the kidneys of rats treated with T3, KB-141 and MB07811, respectively.

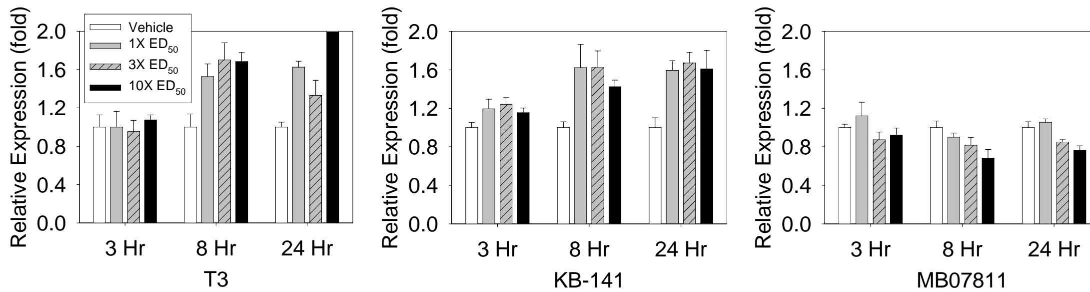


Fig. 11. Expression of CYP7a in the livers of rats treated with T3, KB-141 and MB07811, respectively.

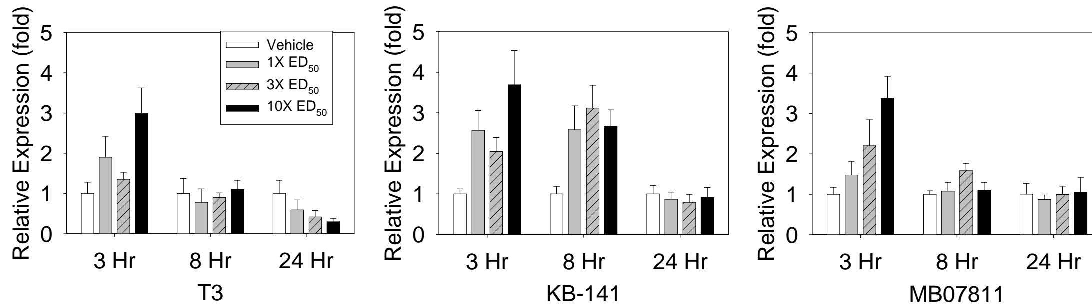


Fig. 12. Expression of SREBP-1c in the livers of rats treated with T3, KB-141 and MB07811, respectively.

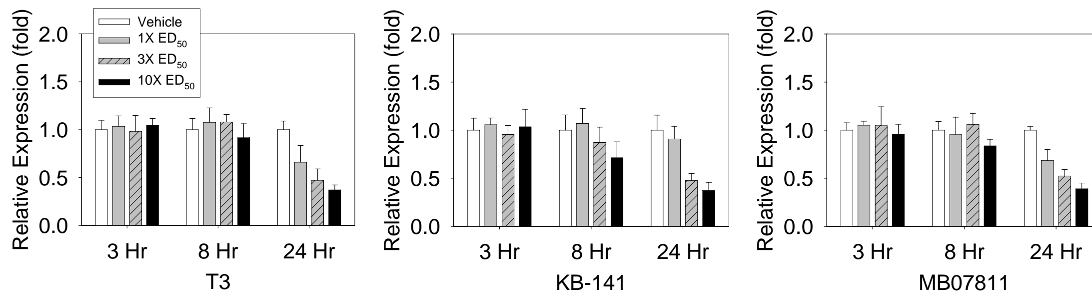


Fig. 13. Expression of D1 in the livers of rats treated with T3, KB-141 and MB07811, respectively.

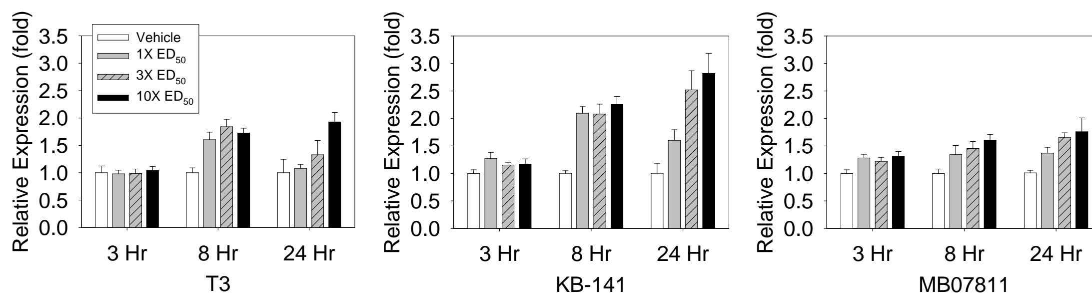


Fig. 14. Expression of malic enzyme in the livers of rats treated with T3, KB-141 and MB07811, respectively.

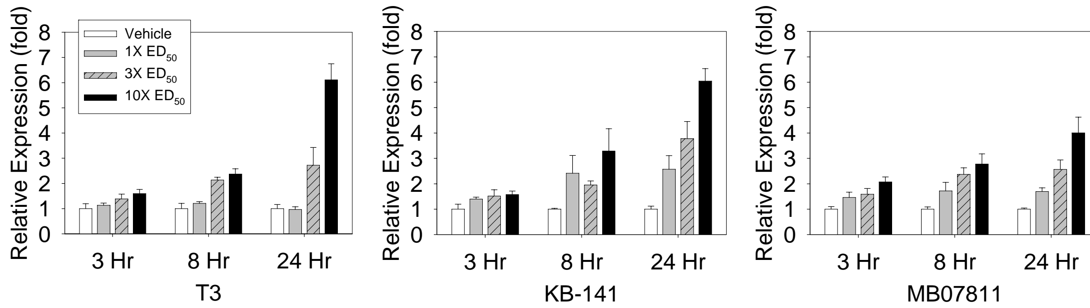


Fig. 15. Expression of TSHβ in the pituitaries of rats treated with T3, KB-141 and MB07811, respectively.

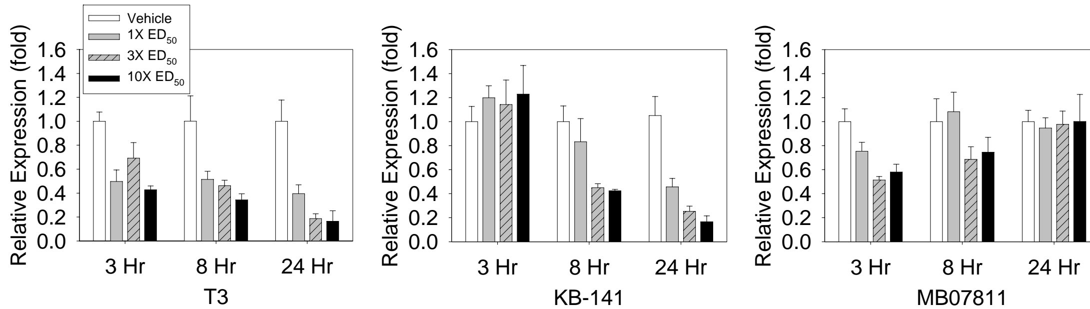


Fig. 16. Expression of UCP3 (uncoupling protein-3) in the SMs of rats treated with T3, KB-141 and MB07811, respectively.

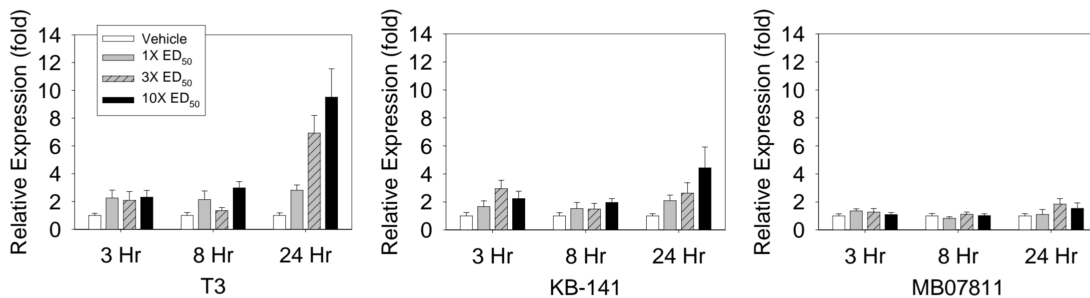


Fig. 17. Expression of D1 in the spleens of rats treated with T3, KB-141 and MB07811, respectively.

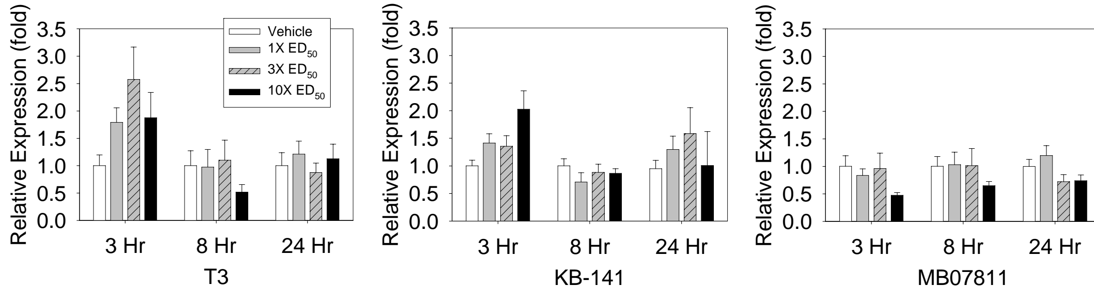


Fig. 18. Expression of D1 in the thyroid glands of rats treated with T3, KB-141 and MB07811, respectively.

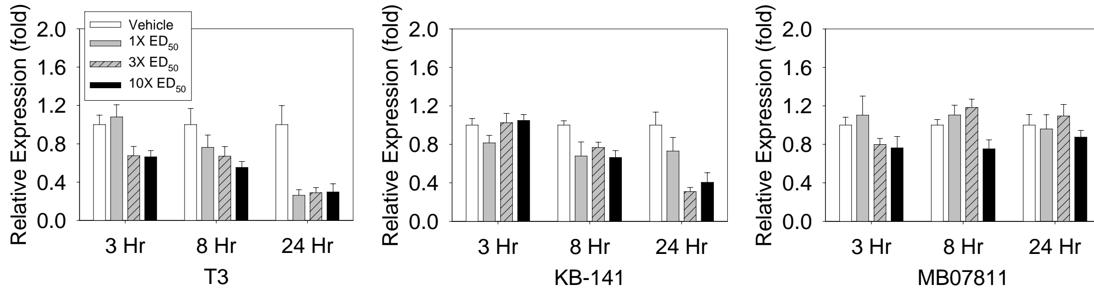


Fig. 19. Expression of LDLR (LDL receptor) in the livers of rats treated with T3, KB-141 and MB07811, respectively.

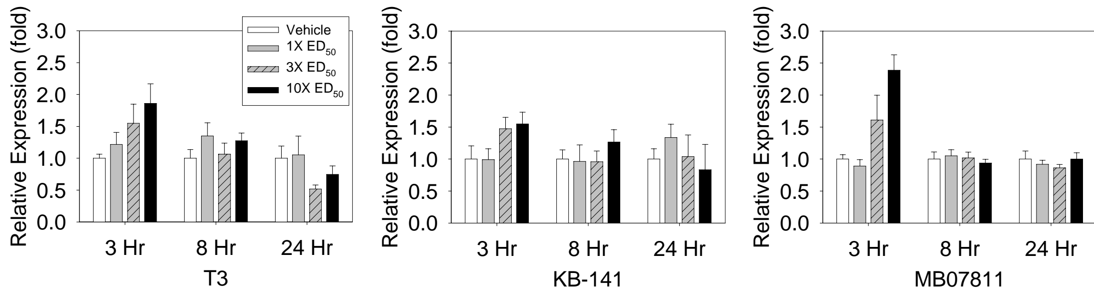


Fig. 20. Expression of SREBP-2 in the livers of rats treated with T3, KB-141 and MB07811, respectively.

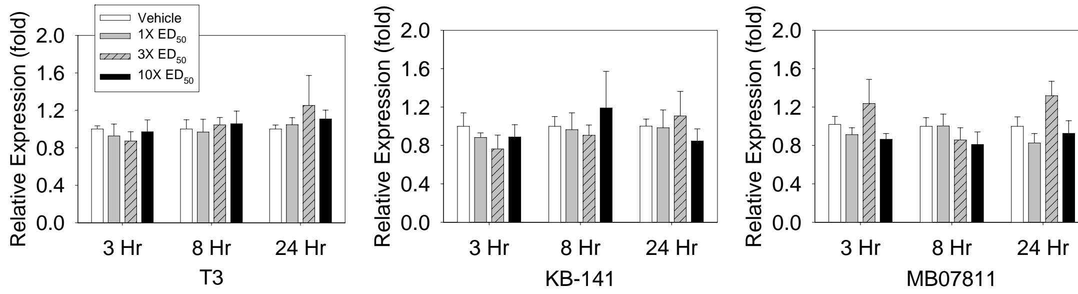


Fig. 21. Expression of HMGR (HMG CoA reductase) in the livers of rats treated with T3, KB-141 and MB07811, respectively.

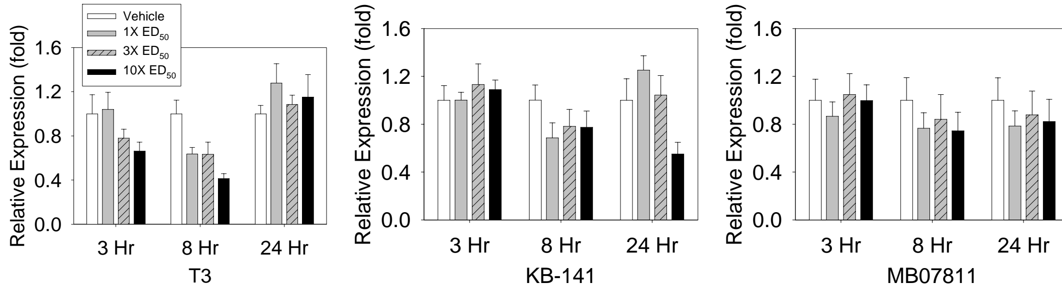
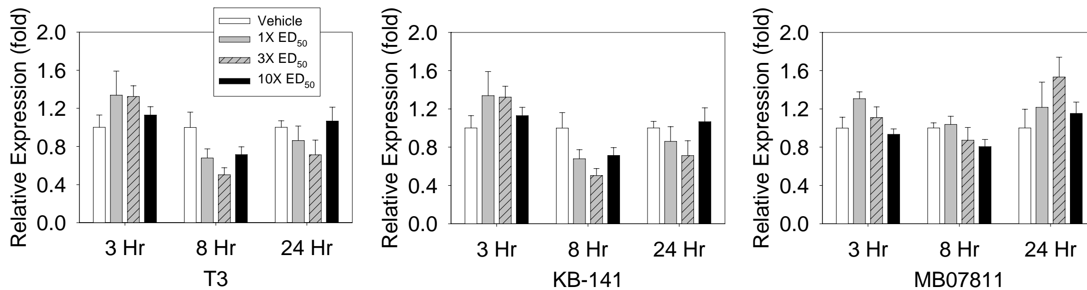


Fig. 22. Expression of phosphoenolpyruvate carboxykinase in the livers of rats treated with T3, KB-141 and MB07811, respectively.



Cholesterol-Fed Rat Dose-Response Studies. Male SD rats (6 weeks of age) were fed a normal chow diet (Harlan 7001) supplemented with 1.5% cholesterol and 0.5% cholic acid (wt/wt) for at least 10 days before initiation of treatment. Rats were divided into groups of 6 and gavaged with either vehicle (polyethylene glycol-400 or 0.1% CMC in deionized water), MB07811, KB-141 or T_3. MB07811 was administered as a solution in polyethylene glycol-400. KB-141 was administered as a solution in water adjusted to neutrality by dropwise addition of 0.1 M NaOH. T_3 was administered as a solution in water adjusted to neutrality with NaOH as described for KB-141. The dosing volumes were 1 ml/kg body weight. Average body weights at the initiation of treatment were ≈325 g. Blood samples were obtained from the tail vein before dosing and 24 h after the first dose for analysis of plasma cholesterol levels. Cholesterol was assayed by means of an Infinity cholesterol reagent used according to the manufacturer's instructions (Thermo Electron Corporation; Waltham, MA). Cholesterol levels were expressed as a percentage of change from the predose levels and the ED_{50} values were calculated by using the four-parameter logistic curve fit described above. T_3, KB-141 and MB07811 lowered total plasma cholesterol levels with calculated ED_{50} values of 0.012 mg/kg, 0.05 mg/kg, and 0.4 mg/kg, respectively (based on results averaged from repeated experiments, data not shown).

Safety Pharmacology Studies. *Cardiac function*. Male SD rats were treated once-daily with vehicle (polyethylene glycol-400), KB-141 (1 mg/kg/d, *per os*), or MB07811 (0.1, 0.3, 1, 5, 10, 30, 50 mg/kg/d, *per os*) for 7 days (n = 6 per group) at a volume of 1 ml/kg/d. All drugs were freely soluble in polyethylene glycol-400. In a separate study, male SD rats were treated once daily with KB-141 (0.01, 0.03, 0.1, 0.33, 1, 5 mg/kg/d *per os*) or T3 (0.0065, 0.0195, 0.0651, 0.195, 0.651 mg/kg/d *per os*) (n = 3-6 animals per group). T3 was administered as a solution in water adjusted to neutrality by dropwise addition of 0.1 M NaOH. All animals were dosed in the morning over 7 days with animals killed and cardiovascular (CV) recordings made on day 8, ≈24 h after the last dose. For each animal, liver tissue was collected and frozen for determination of drug levels. After euthanasia, the heart was excised, blotted dry, trimmed of vessels and weighed.

Cardiac function was evaluated in animals anesthetized with isoflurane 2.5% (O_2 carrier) and placed in a dorsal recumbent position. Body temperature was maintained with a circulating-water heating pad at 37°C. Needle electrodes were placed s.c. to allow continuous recording of lead I electrocardiogram (Gould Instrument Systems). The ECG signal was processed with a Gould Biotach amplifier generating heart rate measurements on a beat to beat basis and expressed as beats per minute (bpm). The right common carotid artery was exposed via a midline incision, ligated distally, and cannulated with a pressure-calibrated 2.5F Millar catheter tip manometer interfaced to a Transducer amplifier (Gould). The pressure signal was further processed by using a Differential amplifier (Gould) allowing continuous recording of the LV dP/dt. The catheter-tip was advanced across the aortic valve into the left ventricle as determined from pressure waveforms. Aortic pressure was recorded immediately after LVP and LV dP/dt measurements, by retracting the pressure catheter into the aortic arch. All analog signals were digitally acquired at 400 hz using the CODAS acquisition system (Dataq Inc), and average values (heart rate, LVP, LV dP/dt, systolic/ diastolic aortic pressure) determined in the playback mode. Heart weights were determined after excision and trimming to remove the atria and great vessels. Average values (± SEM) for each experimental group were calculated by using standard Excel spreadsheet software and are depicted in the figures. One animal (MB07811 30 mg/kg/d group) died on day 5 apparently to intratracheal dosing. Thus, the average data in this group consisted of only five animals.

Thyroid hormone axis analysis. Male SD rats at 225-250 g were housed two per cage under a controlled temperature of 22 ± 2°C and a 12 h:12 h, light:dark cycle with lights on at 7:00 a.m. Pelleted 7001 chow (NEWCO distributors, Rancho Cucamonga, CA) and water were provided *ad libitum*. Dosing was initiated after 1 week of acclimation in-house.

Animals were weighed and sorted into 21 groups (5 treatment groups with sets of animals to be killed after 7, 14, 28, and 49 days of treatment) with similar body weight. The treatment (5 ml/kg; *per os*; QD in the morning) groups were: vehicle (0.5% CMC/1%

Lutrol F68), MB07811 (3 and 30 mg/kg), KB-141 (0.1 and 1 mg/kg). Eight animals were killed the day the study was initiated to get baseline values of parameters to be measured. Animals were weighed 1-2 times per week and the dosing volume per animal was adjusted accordingly.

Eight animals were killed the day the study was initiated to get baseline values of parameters to be measured. Groups of animals from each treatment set (*n* = 5-6 per group) were killed after 1, 2, 4 or 7 weeks of treatment with the exception of the KB-141 1 mg/kg 7-week group that was killed early after 4 [1/2] weeks of treatment (animals stopped gaining BW). When killed, animals were decapitated without anesthesia to minimize effects of stress on measures of the THA. Trunk blood was obtained, allowed to clot at 4°C, and centrifuged at 20,000 rpm for 15 min. Serum was collected from the samples and stored at -80°C. The pituitary was quickly removed and frozen on dry ice, and stored at -80°C.

Samples of serum from all of the baseline and treated groups were sent to the Diagnostic Center for Population and Animal Health (Michigan State University Lansing, MI) for measurement of free T_3, free T_4, total T_3 and total T_4 levels. Other samples of serum were used to measure TSH levels using the Rat TSH [^{125}I] Biotrak Assay System with Magnetic Separation (Amersham Pharmacia Biosciences, Piscataway, NJ; cat. no. RPA554).

THA pilot study/method validation. Male Sprague–Dawley rats (age 6-8 weeks; ≈220 g) were purchased from Charles River Laboratories (Wilmington, MA). The rats were housed two per cage under standard vivarium conditions and were fed normal chow. After 5 days of acclimation, rats (*n* = 6 per group) were gavaged daily for 7 days with T3 at doses ranging from 3 to 1000 μg/day. T3 was dissolved in water and adjusted to pH ≈8.0 by addition of 1 N NaOH. The dosing volume was 2 ml/kg. Twenty-four hours after the final dose, animals were anesthetized and blood was obtained from the inferior vena cava. Plasma was prepared and analyzed for total T3 and T4 levels by LC-MS/MS (5) and serum was prepared and analyzed for TSH levels as described above in *mRNA*

Expression Analysis. Pituitaries were removed and analyzed for TSHβ mRNA as described in *Materials and Methods*. Results (mean values ± standard deviations) are shown below.

Table 5. THA effects of T_3.

T3 μg/kg/day (7 days)	T_3, ng/ml	T_4, ng/ml	TSHβ mRNA, relative levels	TSH, relative levels
Vehicle (aq.)	0.55 ± 0.09	41.1 ± 2.01	1.0	1.0
3	0.49 ± 0.02	37.3 ± 2.61	0.57 ± 0.06	0.93 ± 0.13
30	0.42 ± 0.02	4.2 ± 0.27	$1.39x10^{-2} \pm 3.3\ x10^{-3}$	0.14 ± 0.02
300	4.06 ± 0.75	1.43 ± 0.33	$3.1\ x10^{-3} \pm 2.1\ x10^{-3}$	0.0978 ± 0.02
1000	16.45± 5.1	8.4 ± 5.2	$1.365\ 10^{-3} \pm 7.3810^{-4}$	0.0810 ± 0.03

Glycemic control. Thirty male SD rats were housed under a 12 h light/dark cycle (lights on at 7 a.m.) and randomly assigned to five treatment groups (n = 6 per group); vehicle, T_3 (650 μg/kg/day), or MB07811 (1, 10, or 50 mg/kg/day). Dosing with vehicle or MB07811 (in polyethyleneglycol-400) was *per os*; T3 (in water adjusted to neutrality with NaOH) was dosed s.c. Animals were dosed (1 ml/kg) for 8 days at ≈4:00 p.m. each day. Food was removed from animals each day at ≈8:00 a.m. and blood samples were obtained by a tail vein nick just before dosing (≈4:00 p.m.) on days 1, 4, 5, 6 and 8 for the measurement of fasting blood glucose. Additional blood samples were collected from the tail vein into heparinized Eppendorf tubes at ≈4:00 p.m. on days 1 and 5. Food was returned after dosing each day. Plasma was prepared from these samples by centrifugation (Eppendorf microfuge, 14,000 rpm, 2 min, room temperature) for analysis of free fatty acid and insulin levels.

Oral glucose tolerance was assessed after 6 days of dosing. Rats were fasted for 6 h (7:30 a.m. to 1:30 p.m.) before glucose administration. Glucose (2 g/kg) was administered orally. Blood samples were obtained by means of a tail vein nick just before glucose

administration and at 15, 40, 75, 110, and 150 min thereafter for the measurement of blood glucose. Additional blood samples were collected from the tail vein into heparinized Eppendorf tubes just before glucose administration and at 15, 40, and 150 min thereafter. Plasma was prepared from these samples as described above and analyzed for insulin. The AUC for the change in blood glucose during the oral glucose tolerance test was calculated by using linear trapezoidal interpolation.

Blood glucose was measured by using a OneTouch blood glucose meter and disposable test strips (Lifescan, Milpitas, CA) with results reported as glucometer readings. Plasma free fatty acids were analyzed by using the nonesterified fatty acid kit from Wako Chemicals (Catalog number 994-75409, Richmond, VA). Plasma insulin was analyzed by using the Mercodia Rat Insulin ELISA kit from American Laboratory Products Company (Catalog number 10-1124-10 Windham, NH).

Table 6. Glycemia parameters

Treatment	Δ BW, g	Food Intake, % BW/24 h	Δ Blood glucose, mg/dl	Free fatty acids, mM	Insulin, ng/ml
Vehicle (*per os*)	47.2 ± 1.0	15.3 ± 0.4	4.7 ± 3.0	0.397 ± 0.045	0.439 ± 0.042
T_3 (650 μg/kg/d, s.c.)	4.3 ± 5.1*	16.2 ± 0.4	25.0 ± 3.4*	0.937 ± 0.138*	0.334 ± 0.023
MB07811 (mg/kg/d, *per os*)					
1	42.8 ± 2.8	15.1 ± 0.6	9.0 ± 3.7	0.454 ± 0.043	0.424 ± 0.055
10	37.9 ± 2.8	14.6 ± 0.4	11.8 ± 4.2	0.541 ± 0.055	0.342 ± 0.030
50	49.3 ± 2.9	15.6 ± 0.6	-0.5 ± 5.5	0.524 ± 0.134	0.402 ± 0.034

DIO Mouse Study. Male C57BL/6 mice at 32 days of age were caged four mice per cage, and housed under a reversed 12 h:12 h, light:dark cycle with lights off at 11:00 a.m. Pelleted (high fat chow, 60% fat by kcal) D12494 (Research Diets, New Brunswick, NJ) and water were provided *ad libitum*. Dosing was initiated after 5.5 months of acclimation. Mice were 6.5 months old at the start of the study. Eighty adult male DIO mice were assigned to ten groups based on pretreatment cholesterol levels and body weights so that the group average levels and associated standard errors were as comparable as possible for all treatment groups. Animals were dosed daily with vehicle (0.5% CMC/1% Lutrol F68), MB07811 (0.3, 1, 3, 10 and 30 mg/kg), or KB-141 (0.03, 0.1, 0.3 and 1 mg/kg). Dosing occurred at *ca.* 9:30 a.m. Dosing volume for all treatments was 5 ml/kg. Conscious mice were bled weekly using a tail nick. Blood glucose was measured immediately and an additional sample taken in a heparinized glass capillary tube for separation of plasma by centrifugation. Blood glucose, plasma cholesterol and triglycerides levels were measured weekly during the study before dosing.

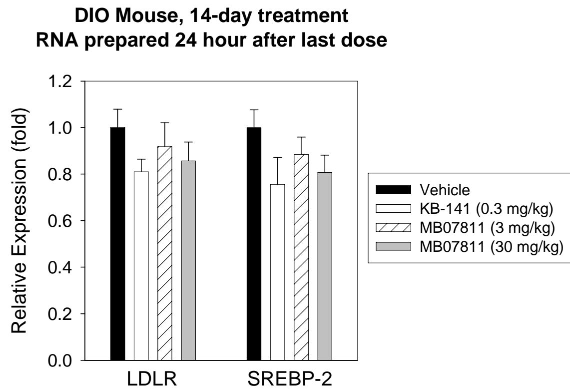
Animals were killed by cervical dislocation. Blood was collected via cardiac puncture and placed in plasma and serum separator tubes (Becton Dickinson, catalog no.36573). Serum was isolated by centrifugation (Eppendorf Microcentrifuge, 14,000 rpm, 10 min, room temperature) and stored at -80°C before analysis. The liver was removed, weighed, and a portion freeze-clamped for triglyceride analysis. The heart was removed and weighed.

Blood glucose was measured by using a OneTouch blood glucose meter and disposable test strips (Lifescan, Milpitas, CA). Total plasma cholesterol was measured by using the Infinity cholesterol reagent and a standard curve prepared by using a 300 mg/dl cholesterol standard (catalog no. TR1341-1L and TR1391-030, respectively, Thermo Electron Corporation, Waltham, MA). Total plasma triglycerides were measured by using the Infinity triglyceride reagent and a standard curve prepared by using a 200 mg/dl triglycerides standard (catalog no. 2780-500 and TR2291-030, respectively, Thermo Electron Corporation, Waltham, MA). Briefly, a small portion of frozen liver (100-200 mg) was homogenized in saline (1 mg liver: 5 µl saline). The homogenate was treated with a mixture of chloroform:methanol (2:1) and thoroughly mixed. Additional saline and chloroform were added to facilitate phase separation. The lower organic phase was collected, evaporated, and dissolved in a small volume (50 µl) of *t*-butyl alcohol:methanol:Triton X-100 (3:1:1). The triglyceride content in the organic extract was measured by using the Infinity triglyceride reagent and a standard curve prepared by using a 200 mg/dl triglyceride standard (catalog no. 2780-500 and TR2291-030, respectively, Thermo Electron Corporation, Waltham, MA). The triglyceride content per weight of liver was calculated from the weight of the starting material. Frozen serum samples were shipped to the Diagnostic Center for Population and Animal Health (Michigan State University Lansing, MI) for total T_4, total T_3, free T_4, free T_3 measurements. The diagnostic lab was blinded to the treatment assignments.

Effects of KB-141 and MB07811 on LDLR and SREBP-2 mRNA levels in DIO mice. Liver was harvested from mice 24 h after the final dose of the 14-day treatment with KB-

141 or MB07811 described in Fig. 5. RT-PCR analysis of mRNA prepared from the tissue showed no change in LDL receptor (LDLR) or SREBP-2 gene expression. The methods for this analysis are described above in *mRNA Expression Analysis*.

Fig. 23. Effects of KB-141 and MB07811 on LDL receptor mRNA levels in DIO mice.



A separate study was conducted to provide insight into the mechanism responsible for the decrease in cholesterol observed with the 14 day MB07811 treatment in the DIO mouse model. DIO mice, which had been fed the high fat diet as described above for 190 days, were treated with a single oral dose of vehicle (0.5% CMC/ 1% Lutrol F68), T3 (0.15 mg/kg), or MB07811 (10 mg/kg), n = 6 per timepoint, and livers were harvested at 3 and 8 h after dosing for LDLR mRNA quantitation by RT-PCR. The methods for this analysis are described above in *mRNA Expression Analysis*.

Table 7. Early temporal effects of T3 and MB07811 on LDL receptor mRNA levels in DIO mice

	LRLR mRNA (Fold Relative Units)	
Treatment	3 hr	8 hr
vehicle	1.00 ± 0.23	1.00 ± 0.13
MB07811 (10 mg/kg)	1.23 ± 0.31	2.01 ± 0.28
T3 (0.15 mg/kg)	2.04 ± 0.18	1.39 ± 0.33

Thyroidectomized Rat Study. Thyroidectomized, male SD rats (7 weeks of age; 200 g) were purchased from Charles River Laboratories (Wilmington, MA). The rats were housed 2 per cage under standard vivarium conditions and were fed normal chow. After 7 days of acclimation, rats (n = 6 per group) were administered a single dose of vehicle, T3 (0.5 mg/kg), or MB07811 (5 mg/kg). Drugs were administered in polyethyleneglycol-400 in a dosing volume of 2 ml/kg. After 24 h of treatment, rats were anesthetized and their livers collected and immediately frozen in liquid nitrogen. The liver RNA fractions were isolated and mRNA for the LDL receptor quantified as described in above in *mRNA Expression Analysis*.

Average values ± SEM were calculated and are shown below.

Table 8. Effects of T3 and MB07811 on LDL receptor mRNA levels in thyroidectomized rats

	Relative mRNA levels
Vehicle	1.070 ± 0.120
T3	6.644 ± 0.520
MB07811	21.063 ± 5.087

Both T3 and MB07811 treatment (24 h) resulted in marked increases in mRNA levels for the LDL receptor.

Comparative Efficacy of T3, KB-141, and MB07811 in Wild-type and LDL Receptor-Deficient Mice. Male LDL receptor-deficient ($LDLR^{-/-}$) and wild-type C57BL/6 mice were purchased at 6 weeks of age from The Jackson Laboratory (Bar Harbor, MN). Body weights ranged from 20 to 24 g. Mice were housed 3 per cage under standard vivarium conditions and, after 2 days of acclimation, were provided a high-

cholesterol diet (1.5% cholesterol/0.5% cholic acid). Mice were maintained on the high-cholesterol diet for two weeks before initiation of treatment and throughout the remainder of the study. At initiation of treatment, mice were divided into groups (*n* = 6 per group) with matched baseline cholesterol levels. Groups of $LDLR^{-/-}$ and wild-type mice were gavaged with vehicle, T3 (0.5 mg/ kg/day), KB-141 (0.5 mg/ kg/day) or MB07811 (5 mg/kg/day) once daily for 7 days. All compounds were administered in 0.5% carboxymethylcellulose (CMC) at a dosing volume of 5 ml/kg. Blood samples were obtained from the tail vein and analyzed for total cholesterol levels by means of a standard assay kit (Infinity; Electron Corporation, Louisville, CO).

The percentage difference in cholesterol levels on day 7 relative to the vehicle-treated group was calculated for each treatment group. The data were expressed as mean values ± standard deviations. Statistical analysis was performed by means of the Dunnett's post hoc test and the Tukey-Kramer test.

As shown in the table below, all three drug treatments resulted in similarly reduced cholesterol levels in wild-type mice. In contrast, no significant reductions were observed for any of the treatments in $LDLR^{-/-}$ mice.

Table 9. Effects of T3, KB-141 and MB07811 on LDL receptor mRNA levels in wild-type and LDL receptor-deficient mice

Treatment	Wild-type mice	*P*	$LDLR^{-/-}$ Mice	*P*
Vehicle (CMC)	0 ± 7.9^{a}		0 ± 3.1	
T3	-42.4 ± 11.5^{b}	0.0014	9.5 ± 4.9	0.5559
KB-141	-55.8 ± 4.2^{b}	0.0005	-10.6 ± 9.8	0.7693
MB07811	-31.8 ± 6.0^{b}	0.0258	-10.8 ± 6.1	0.7583

Superscripts reflect the results of the Tukey-Kramer test. Groups with a common letter are not significantly different.

Statistics. Results are expressed as mean ± SEM unless otherwise indicated. All analyses were performed by using JMP 5.0-6.0 (SAS Institute, Cary NC). Data obtained at multiple timepoints in the same animals were analyzed by using a two-way ANOVA with repeated measures on time. If a significant effect of either treatment or the interaction of treatment and time was found, data were analyzed by using the method for endpoint data. Endpoint data were analyzed by using a one-way ANOVA followed by a Dunnett's post hoc test, with the vehicle-treated group as the control, or the unpaired Student's *t* test as indicated. *P* values of less than 0.05 were considered statistically significant.

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