Supporting information for:

Monoalkylglycerol ether lipids promote adipogenesis

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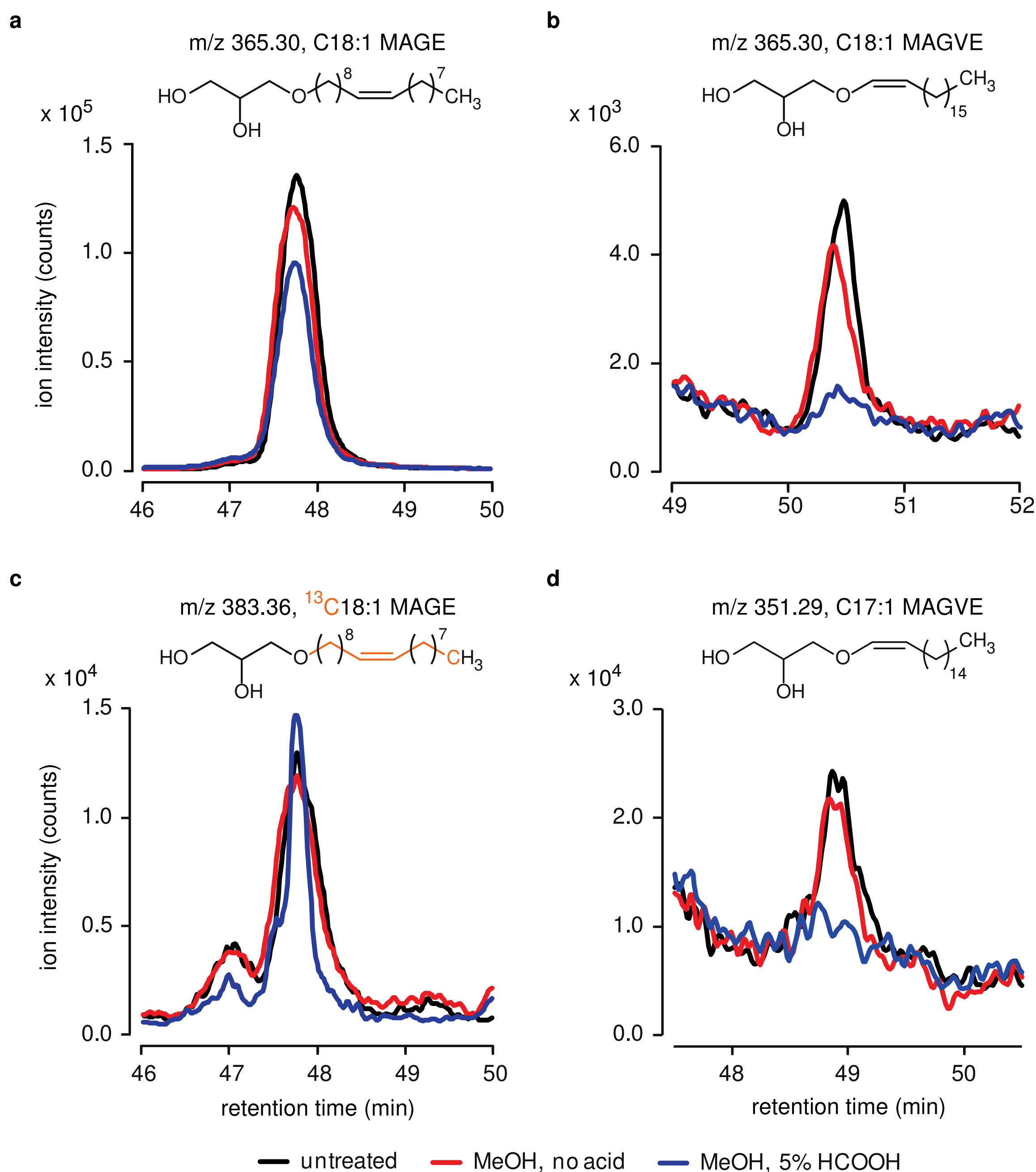


Supporting Information Figure 1. Effect of acid treatment on lipid stability. MAGEs and MAGVEs were distinguished by their susceptibility to acid hydrolysis. Cellular pellets from 3T3-L1 cells were extracted with a 2:1:1 mixture of chloroform (containing 1 nmol ^{13}C18:1 MAGE and 100 pmol C17:1 MAGVE), methanol and phosphate buffered saline. Lipid extracts were left untreated or incubated in methanol with or without 5% formic acid for 60 min at 40 ℃, upon which the residue was analyzed by LC-MS. Presented are ion chromatograms of the m/z windows corresponding to the $[M+Na]^+$

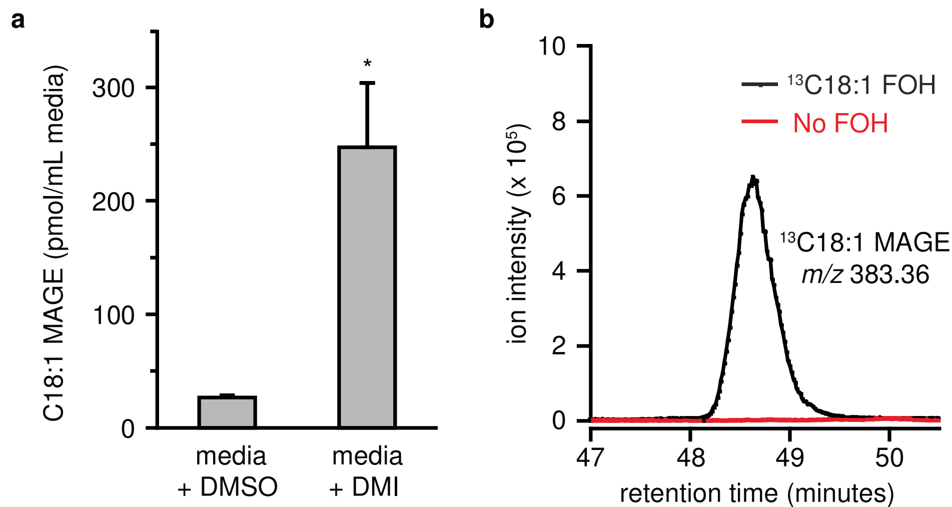
adducts of a) C18:1 MAGE, b) C18:1 MAGVE, c) ^{13}C18:1 MAGE, and d) C17:1 MAGVE.

Lipid Class Acyl/Alkyl chain	Fold Day 2 / Day 0	P-value (n = 3)
MAGE		
C16:0	7.4	> 0.001
C18:1	7.3	> 0.001
C18:0	4.9	0.016
MAGVE		
C16:1	7.2	0.001
C18:2	7.3	> 0.001
C18:1	9.0	> 0.001
MAG		
C16:0	2.8	0.005
C18:1	3.1	0.011
C18:0	0.9	0.78
DAG		
C34:1	2.2	0.007
C36:2	1.8	0.008
C36:1	1.6	0.022
TAG		
C52:2	0.7	0.016
C54:3	0.7	0.003
C54:2	0.6	0.005
FFA		
C16:1	3.2	0.010
C16:0	0.8	0.24
C18:2	0.4	0.006
C18:1	1.8	0.007
C18:0	0.6	0.011
C20:4	0.2	0.001
Phospholipids		
C18:1 LPC	1.4	0.15
C18:1 LPE	0.8	0.10
C34:1 PA	1.4	0.005
C34:1 PC	0.5	> 0.001
C34:1 PE	0.5	0.005
C34:1 PS	0.7	0.018
Sterols		
Cholesterol	0.4	0.039

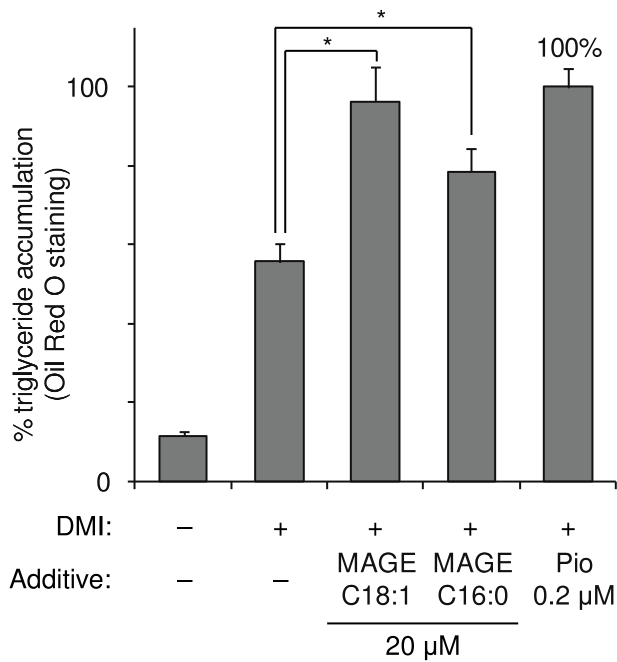
Abbreviations: MAGE, monoalkylglycerol ether; MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; FFAs, free fatty acids; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Supporting Information Table 1. Relative levels of representative lipids measured by metabolite profiling in 3T3-L1 cells at days 0 and 2. Data represent the ratio in normalized mass ion intensity from the most abundant ion adduct. Lipid extracts were

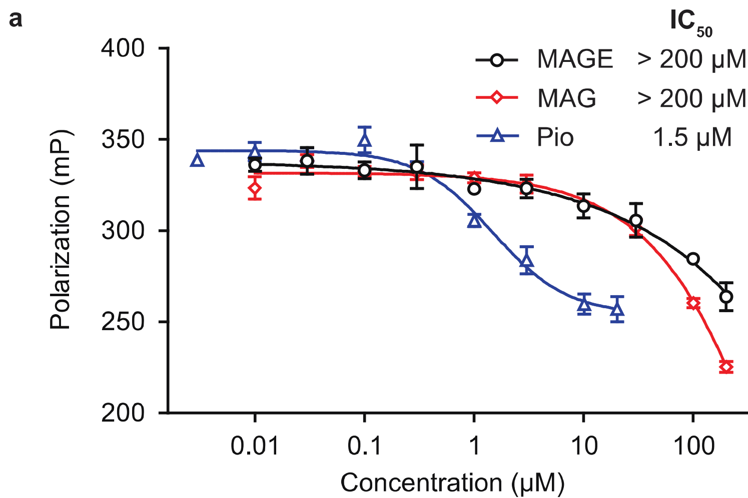
prepared from 3 independent wells of cultured cells and analyzed by LC-MS. Statistical significance values were calculated using an unpaired two-tailed Student's *t* test.

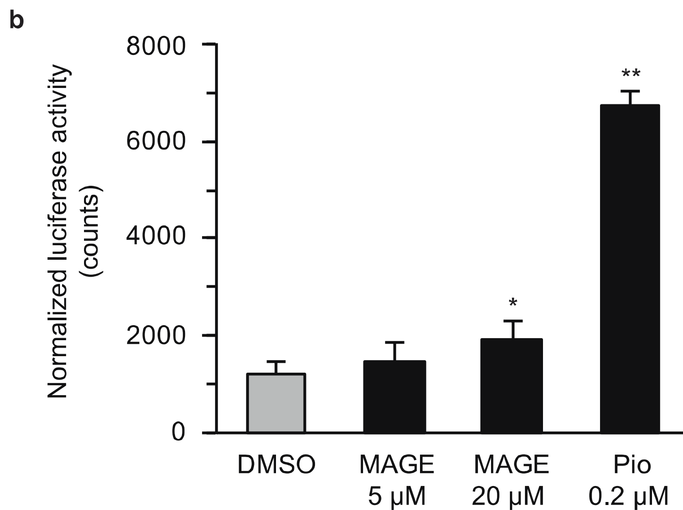


Supporting Information Figure 2. Intracellular production of MAGE in 3T3-L1 cells. a) Medium conditioned for two days over untreated or DMI-treated cells was extracted with 2 parts chloroform (containing 1 nmol ^{13}C18:1 MAGE) and 1 part methanol. Concentration of C18:1 MAGE was quantified by comparison with the [^{13}C]-labeled internal standard (n = 3; *, $p < 0.05$). b) 3T3-L1 cells were induced to differentiate with DMI containing 20 ⌈M all-[^{13}C]-oleyl alcohol. After 48h, the presence of ^{13}C18:1 MAGE in cellular lipid extracts, which indicated that the cells possessed the capacity to produce MAGE, was measured by LC-MS.



Supporting Information Figure 3. 1-Oleylglycerol (C18:1 MAGE) or 1-palmitylglycerol (C16:0 MAGE) was added to the DMI induction medium of 3T3-L1 cells cultured in 6-well multiwell plates. On day 2 the culture medium was replaced with medium supplemented only with 5 µg/ml insulin, which was removed at day 4. Cells were fixed and stained on day 8, and absorbance of extracted Oil Red O stain was measured ($n = 3$; *, $p < 0.01$).





Supporting Information Figure 4. MAGE does not bind PPARγ directly. a) PPARγ binding was tested in a competitive fluorescence polarization assay with the PPARγ ligand binding domain. Decrease in polarization indicated displacement of the fluorophore-coupled PPARγ agonist by increasing concentrations of MAGE, MAG, or pioglitazone. IC_{50} values were calculated by nonlinear curve-fitting (n = 3). b) PPARγ activity was tested in a cell-based reporter assay. HEK293T cells were transfected with PPARγ LBD-GAL4 DBD hybrid construct, pUASx4-TK-luciferase reporter construct and pCMV-β-galactosidase as a transfection control. Transfected cells were stimulated with DMSO, MAGE or pioglitazone in media containing delipidated serum. The next day, cell lysates were assayed for luciferase and β-galactosidase activity. Data are presented as

the normalized ratio of luciferase/β-galactosidase activity (n = 4; *, $p < 0.05$; **, $p < 0.01$; Student's *t* test compared to DMSO control).

SUPPLEMENTARY METHODS

Materials and Reagents. Dexamethasone, insulin, 3-Isobutyl-1-methylxanthine, were purchased from Sigma (St. Louis, MO). Pioglitazone was purchased from Axxora LLC (San Diego, CA). 1-Oleylglycerol, 1-([^{13}C]-oleyl)-glycerol, and [^{13}C]-oleyl alcohol were chemically synthesized as previously described (Chiang 2006).

Cell Culture and Induction of Adipogenesis. HEK-293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone). 3T3-L1 cells provided by Dr. H. Green (Harvard Medical School, Boston, MA) were cultured as described (Green 1975). Briefly, 1.8×10^5 cells were seeded on 100-mm dishes and maintained in DMEM supplemented with 10% bovine calf serum (BCS, Hyclone). Two days after confluence, the medium was changed to DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5 μg/mL insulin. Forty-eight hours after induction, the medium was replaced with DMEM containing 10% FBS and 5 μg/mL insulin. Forty-eight hours later, cells were fed maintenance medium (DMEM plus 10% FBS), which was replenished every 2 days.

Lipid isolation. Cells were scraped into PBS from 100-mm culture dishes and cell pellets were resuspended in 1 mL of PBS. A small aliquot of cells was removed prior to lipid extraction for determination of protein concentration by BCA assay. The cell suspensions were added to 2:1 chloroform:methanol (3 mL) and were Douce-homogenized. The homogenate was centrifuged at 2000 x *g* for 5 min at 4 ℃, which cleanly separated the organic and aqueous layers. The organic layer was transferred to a clean glass vial and concentrated under an N_2 stream. The extract was dissolved in 120 μL $CHCl_3$ prior to analysis by LC-MS with injection volumes of 40 μL. Absolute quantitation of MAGE levels was accomplished by isotope dilution mass spectrometry using a synthetic ^{13}C18:1 MAGE (100 pmol/sample) as an internal standard in the chloroform:methanol extraction medium.

LC-MS analysis. A Gemini C18 reversed phase column (5 µm, 4.6 x 50 mm, Phenomonex) and a C18 reversed phase guard column (3.5 µm, 2 mm x 20 mm, Western Analytical) was used for LC-MS analysis in negative mode. In positive mode, a Luna C5 reversed phase column (5 µm, 4.6 x 50 mm, Phenomonex) was used together with a C4 reversed phase guard column (3.5 µm, 2 mm x 20 mm, Western Analytical). Mobile phase A consisted of a 95:5 water:methanol mixture and mobile phase B consisted of 60:35:5, 2-propanol:methanol:water. In negative mode 0.1% ammonium hydroxide was added to the mobile phases and in positive mode 0.1% formic acid plus 5 mM ammonium formate were added. An Agilent 1200 series binary pump system was used for chromatographic analysis. The flow rate for each profiling run was 0.1 mL/min for the first 5 min followed by 0.4 mL/min for the remainder of the gradient. At 5 min, concomitant with the increase in flow rate, the gradient was increased from 0% B to 20% B. The gradient increased linearly to 100% B at 65 min, followed by a 20 minute wash with 100% B before re-equilibrating the column with 0% B for 10 min. MS analysis was performed using an Agilent 6220 ESI-TOF fitted with an electrospray ionization (ESI) source. The capillary voltage was set to 3500 kV and the fragmentor voltage to 100 V. The drying gas temperature was set to 350°C at a flow rate of 10 L/min with a nebulizer pressure set to 45 psi. Untargeted data were collected using a mass range of 100-1500 Da.

Data analysis. Data analysis with XCMS was used to identify changing metabolites between samples. Raw data files were converted to mzXML files using the program mzStar for subsequent XCMS analysis. Samples were compared in a pairwise fashion (i.e., day 2 vs day 0) and differences were ranked according to statistical significance. The data was then filtered based on a peak size (> 5e4 counts) and statistical significance (p-value < 0.05) prior to visual inspection and manual integration of the remaining ions to ensure that the differences identified by XCMS were reflected in the raw data. Peak areas were normalized to total cellular protein in order to account for differences in sample mass and these numbers were used to calculate relative changes. For the volcano plot, displayed data were filtered based on mass range (m/z 150-950), retention time range (15-75 min) and abundance (> 5e4 counts).

Oil Red O Staining and Analysis. Differentiated 3T3-L1 cells in 6-well plates (35-mm) were washed with PBS and fixed with 10% formaldehyde/PBS for 1 h at room temperature. After washing with distilled water and allowing plates to dry, cells were stained for 30 min with an Oil Red O solution (0.6% Oil Red O in 3:2 isopropanol:water) that was pre-filtered through a PES filter (0.2 µm). Cells were washed once with 20% isopropanol/water, then twice with distilled water before photography or microscopy. After imaging, the stained cells were allowed to dry, after which the Oil Red O dye was extracted with 2 mL isopropanol (30 min). The Oil Red O extract was added to a 1 cm cuvette, diluted with isopropanol (1:2), and neutral lipid accumulation was quantified by monitoring the absorbance at 510 nm.

RNA Preparation and Real-Time PCR Assays. Total RNA was isolated from cultured adipocytes at the indicated timepoints using the RNeasy Mini Kit (Qiagen) and reverse transcription of total RNA was carried out using QuantiTect Reverse Transcription Kit (Qiagen), both according to the manufacturer's instructions. The mRNA for genes of interest was quantitated with Quantitect SYBR Green PCR kit (Qiagen) performed with an MX3000P thermal cycler (Stratagene). The relative amount of mRNA was normalized using 36B4 as the reference gene, and was calculated using the comparative Ct method. Primer sequences (Waki 2007): 36B4 fwd., 5′-AGA TGC AGC AGA TCC GCA T-3′; 36B4 rev., 5′-GTT CTT GCC CAT CAG CAC C-3′; adiponectin fwd., 5′-CCG GAA CCC CTG GCA G-3′; adiponectin rev., 5′-CTG AAC GCT GAG CGA TAC ACA-3′; aP2 fwd., 5′-CAC CGC AGA CGA CAG GAA G-3′; aP2 rev., 5′-GCA CCT GCA CCA GGG C-3′.

Analysis of structure activity relationship (SAR). Post-confluent 3T3-L1 cells in 6-well culture plates were treated with MAGE, MAG, or MAGVE (all at 10 µM) pre-mixed with the normal differentiation medium, DMI in DMEM containing 10% FBS. Pioglitazone (0.2 µM) and DMSO were used for positive and negative controls. The medium was replaced after 2 days with DMEM containing 10% FBS and 5 µg/mL insulin alone. The differentiated cells at day 8 were stained by Oil red O solution to quantify the extent of triglyceride accumulation.

PPARγ binding assay. The binding of MAGE or MAG to PPARγ was directly measured with the PolarScreen™ PPAR competitor assay kit (Invitrogen, CA, USA) according to the manufacturer's instructions. Briefly, 20 μL of 2× MAGE or MAG solution (from 20 nM to 400 μM) was added to a 384-microwell plate, then to each well was added 20 μL of 2× PPARγ-LBD/Fluormone™ PPAR Green complex. The plate was covered to protect the reagents from light and was incubated at room temperature for 2 hrs. The fluorescence polarization value of each well was measured by a Victor^3 V multilabel plate reader (PerkinElmer Life Sciences, MA). Fluorescence polarization was monitored using a 485 nm excitation wavelength and a 535 nm emission wavelength.

PPARγ activation assay. HEK-293T cells (1×10^5 cells) were plated in 24-well plates and maintained in DMEM containing 10% charcoal dextran-stripped (CS)-FBS. The transfection study was performed using Fugene 6 transfection reagent (Roche, IN, USA) according to the manufacturer's instructions. Preconfluent cells were transfected with a PPARγ-LBD-GAL4-DBD construct, pUASx4-TK-luc reporter construct and pCMV-β-galactosidase as a transfection control (Ziouzenkova 2003), using antibiotic-free and phenol red-free DMEM containing 1% CS-FBS as the transfection medium. The next day, the culture medium was replaced with DMEM containing 10% CS-FBS plus DMSO or the stimulating compounds, pioglitazone (0.2 μM) and MAGE (20 μM). Cells were incubated at 37 ºC overnight. After 24 hr, the cells were lysed and directly assayed for luciferase (Promega, WI, USA) and β-galactosidase (Promega, WI, USA) activity in lysates prepared from the transfected cells. The relative activity of luciferase reporter was normalized to β-galactosidase activity.

References for Supporting Information

(1) Chiang, K. P.; Niessen, S.; Saghatelian, A.; Cravatt, B. F. *Chem Biol* **2006**, *13*, 1041.

(2) Green, H.; Kehinde, O. *Cell* **1976**, *7*, 105.

(3) Waki, H.; Park, K. W.; Mitro, N.; Pei, L.; Damoiseaux, R.; Wilpitz, D. C.; Reue, K.; Saez, E; Tontonoz P. *Cell Metab* **2007**, *5*, 357.

(4) Ziouzenkova, O. et al. *Proc Natl Acad Sci USA* **2003**, *100*, 2730.

Full Author List for Reference 19

19. Yamauchi, T.; Kamon, J.; Waki, H.; Terauchi, Y.; Kubota, N.; Hara, K.; Mori, Y.; Ide, T.; Murakami, K.; Tsuboyama-Kasaoka, N.; Ezaki, O.; Akanuma, Y.; Gavrilova, O.; Vinson, C.; Reitman, M. L.; Kagechika, H.; Shudo, K.; Yoda, M.; Nakano, Y.; Tobe, K.; Nagai, R.; Kimura, S.; Tomita, M.; Froguel, P.; Kadowaki, T. *Nat Med* **2001**, *7*, 941.