Supporting Information

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SI Text

Biomedical Relevance. The present study not only offers new possibilities for the treatment of glioma but also brings an innovative approach to overcome the therapeutic limitations in the treatment of various cancers. This work shows the proof of principle (efficacy in a humanized animal model of glioma) and proof of relevance (superiority with respect to any existing treatment) for a pathological process with unmet clinical needs. In addition, it is provided a comprehensive mechanism of action that covers from the first molecular events to the last cellular consequences of treatments with the synthetic fatty acid, 2-hydroxyoleic acid (2OHOA). Finally, this work provides relevant information about the use of GFAP, glutamine synthetase (GS), dihydrofolate reductase (DHFR), and sphingomyelin (SM) as potential biomarkers for the diagnosis of glioma and assessment of the therapeutic response and further indicates that sphingomyelin synthase (SMS) is a new anticancer drug target. The present results demonstrate that 2OHOA is a first-in-class compound.

As any other new discovery, the new knowledge brings numerous questions. First, further studies would be necessary to fully understand the molecular consequences of SM increases in the nucleus and plasma membrane. In addition, further investigation about molecular mechanism of interaction between PKC or Ras (two proteins whose cellular localization has been shown here to be altered by 2OHOA treatments) with bilayers of different SM and 2OHOA content. From the clinical point of view, and considering the current experience with 2OHOA in humans and animal models of cancer, it can be expected a full response to treatment in a significant proportion of cases from various types of cancers; however, the limited amount of data available makes it difficult to foresee the precise percentage of patients that could fully respond to treatment. In this scenario, it will be of great value to determine the molecular bases underlying full, partial, or no response upon 2OHOA treatment. In any case, the safety of this compound, its efficacy, its dual-mode mechanism of action, and the other data shown here suggest that in those cases that full response is not obtained, 2OHOA could be a very convenient companion in combination therapies using other drugs.

Finally, the signaling cascades covered in the present study are complex, and future studies would be required to shed further light on some of the molecular events here described. Thus, the induction of autophagy, which has been indicated to be related to the retinoblastoma protein phosphorylation status and the activities of Akt and $p27^{Kip1}$ (1), could imply intermediate states of ER stress. Most of these questions and additional issues of interest are currently under investigation in our laboratory. Nevertheless, only the results obtained in advanced phases of the clinical trials could determine the real relevance of this innovative compound. This specific and efficacious fatty acid, which constitutes the first member of a new family of anticancer compounds with no relevant toxicity at therapeutic doses, has a mechanism of action that presents several unusual features that had been indicated to be of potential interest in oncology (e.g., induction of differentiation and autophagy) (1, 2).

Extended Methods. ***Cell Lines and Culture.*** The human glioma cell lines U118, A172 and T98G were obtained from the European Collection of Human Cell Cultures, and SF767 cells were obtained from the Brain Tumor Research Center Tissue Bank (University of California-San Francisco, Department of Neurological Surgery). The cells were cultured at 37 °C in 5% CO_2 and DMEM (SF767) or RPMI 1640 (U118, A172 and T98G), supplemented with 10% fetal bovine serum (v/v), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B. Media and other culture reagents were obtained from Sigma-Aldrich (Madrid, Spain).

Cell Proliferation Assays. Glioma cell growth was determined using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide) assay. Cells were cultured in 96-well plates at a density of $2.5 - 5 \times 10^3$ cells per well and incubated overnight. They were then incubated in the presence or absence of 2OHOA or temozolomide (TMZ) (concentrations and durations indicated in the figures) and, finally, in medium containing MTT (0.5 mg/mL, Sigma-Aldrich, Spain) for 2 h at 37 °C and 5% CO_2. Upon removal of MTT, 150 μl of DMSO was added to each well, and the absorbance was determined at 590 nm using a microplate reader (FLUOstar Omega, BMG LABTECH, Germany). To determine the correspondence between absorbance values and the number of viable cells, duplicate plates cultured in parallel under the same conditions but with increasing cell densities were counted using the trypan blue (0.2%) method.

Microscopy Studies. For confocal microscopy experiments, human glioma cells were cultured as indicated above in NUNC Lab-Tek II chambered slides (Nunc-Thermo Fisher Scientific, Denmark) and in the presence or absence of 2OHOA (150 μM, 10 min and 24 h), they were washed with Tris-buffered saline (TBS) buffer [137 mM NaCl, 2.7 mM, KCl, 25 mM, Tris-HCl (pH 7.4)] and fixed with 4% paraformaldehyde for 30 min at 4 °C. After washing twice with TBS buffer, cells were incubated with 5% normal horse serum in TBS buffer for 1 h at room temperature and then immediately incubated overnight at 4 °C with a monoclonal anti-Ras antibody (1:50, BD Transduction Laboratories, Heidelberg, Germany) in TBS buffer supplemented with 2% horse serum. Finally, the cells were washed with TBS buffer, incubated for 1 h with the secondary antibody (Alexa Fluor 488-labeled goat anti-mouse IgG, 1:200, Molecular Probes; excitation at 488 nm and detection at 510–550 nm), and washed with TBS buffer. Images were acquired on a Leica TCS SP2 spectral confocal microscope with 630x optical magnification and 8x digital magnification (approximately 5,000x total magnification), and they were analyzed with the manufacturer's software. To detect lysosomes, cells were cultured as above in the presence or absence of 2OHOA or palmitate (150 μM, 48 hours). The cells were then incubated for 1 h with the LysoSensor Green DND-189 probe pH Indicator (2 μM, pH 4.5–6, Invitrogen) to detect autophagosomes, and for 5 min with Hoecht 33342 (trihydrochloride trihidrate, 40 μg/mL, Invitrogen), to stain the nuclei. Samples were observed on a Nikon Eclipse TE2000-S fluorescence microscope at 400x magnification. The fluorescence induced by the acidic vesicles was quantified in photomicrographs of live cells using Image J 1.38x public software (Wayne Rasband, National Institutes of Health; rsb.info.nih.gov).

Electron microscopy experiments were performed in triplicate and, for each incubation time and concentration used, a total of 300 control or treated cells were analyzed giving a total of 3600 cells. For this purpose, SF767 cells were seeded at a density of 4×10^4 cells/well in 4-well Lab-Tek chamber slides (Nalge Nunc Int., Naperville, IL) and fixed in 3.5% glutaraldehyde for 1 h at 37 °C. The cells were then postfixed in 2% OsO_4 for 1 h at room temperature and stained with 2% uranyl acetate in darkness for 2 h at 4 °C. Finally, cells were rinsed in 0.1 M sodium phosphate buffer (pH 7.2), dehydrated in ethanol, and infiltrated overnight with

Araldite (Durcupan, Fluka, Buchs SG, Switzerland). Following polymerization, embedded cultures were detached from the chamber slide and glued to araldite blocks. Serial semithin (1.5 μm) sections were cut with an Ultracut UC-6 microtome (Leica, Heidelberg, Germany), mounted onto slides, and stained with 1% toluidine blue (optical microscopy). Selected sections were glued (Super Glue, Loctite) to araldite blocks and detached from the glass slide by repeated freezing (in liquid nitrogen) and thawing. Ultrathin (0.06–0.09 μm) sections were prepared on the Ultracut microtome and stained with lead citrate. Finally, photomicrographs were obtained using a transmission electron microscope (FEI Tecnai G2 Spirit Biotwin) coupled to an Olympus digital camera.

Electrophoresis, Immunobloting and Protein Quantification. Cells were cultured in 6-well plates at a density 1.5×10^5 cells per well. After incubation in the presence or absence of 2OHOA at the indicated concentrations and times, 150 μl of protein extraction buffer [10 mM Tris-HCl (pH 7.4)] containing 50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1% SDS, 5 mM iodoacetamide, and 1 mM PMSF) was added to each well. The contents of two wells were subjected to identical treatments, pooled, and further processed. Cell suspensions were subjected to ultrasound (70% cycle) for 10 s at 50 w using a Braun Labsonic U (probe-type) sonicator, and 30 μl aliquots were prepared for protein quantification. For PKC translocation experiments, this cell suspension was subsequently centrifuged as described elsewhere (3) and PKCα levels were determined by immunoblotting in the membrane and cytosolic fractions.

Tumors derived from immunosuppressed mice injected with SF767 cells were frozen in liquid nitrogen and ground in a glass mortar. The resulting powder was homogenized using a tissue blender (Ultra-Turrax; Janke & Kunkel) in ice-cold protein extraction buffer (1:10 w/v). The homogenate was incubated for 30 min at room temperature and then subjected to ultrasound as described above. Samples were centrifuged for 15 min at 1,000×g and 4 °C, and 30-μl aliquots of the resulting supernatant was used to determine the total protein content. The remaining volume (about 270 μl) was mixed with 30 μl of 10 x electrophoresis loading buffer [120 mM Tris-HCl (pH 6.8)], 4% SDS, 50% glycerol, 0.1% bromophenol blue, and 10% β-mercaptoethanol) and boiled for 3 min.

For immunoblotting, 30 μg of total protein from the cell lysates was resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Schleicher & Schüell). After immunoblotting, nitrocellulose membranes were blocked for 1 h at room temperature in TBS containing 5% nonfat dry milk and 0.1% Tween 20 (blocking solution). The membranes were then incubated overnight at 4 °C in blocking solution containing one of the following primary antibodies: anti-Ras, anti- MAP kinase kinase (anti-MEK), anti-MEKP, anti-extracellular, signal-regulated kinase (anti-ERK), anti-ERKP, antiepidermal growth factor receptor (anti-EGFR), anti-EGFRP, anti-$p21^{Cip1}$, anti-LC3B, anti-Atg5, anti-Raf or anti-RafP (1:1000, Cell Signaling, Danvers, MA); anti-PKC, anti-Cyclin D3, anti-E2F1 (1:1.000, BD Transduction Laboratories, Heidelberg, Germany); anti-GFAP, anti-$p27^{Kip1}$ (1:1000, Abcam, Cambridge, UK); anti-glutamine synthetase (1:2000, Amersham, Billerica, MA); and anti-α tubulin (1:14,000, Sigma-Aldrich, St. Louis MO). After incubation with the primary antibody, membranes were washed three times for 10 min with TBS and incubated for 1 h at room temperature in fresh blocking solution containing horseradish peroxidase-linked goat anti-mouse or donkey anti-rabbit IgG (1:2,000, Amersham Pharmacia). Antibody binding (i.e., protein level quantification) was assessed by Enhanced Chemiluminescence Detection (ECL; Amersham Pharmacia) followed by exposure to ECL hyperfilm (Amersham Pharmacia). The α-tubulin content in the samples was determined by the same procedure and used as a loading control. The films were scanned at a resolution of 600 dpi (41.6 μm resolution), and problem sample bands were interpolated in standard curves of integrated optical density vs. protein content to quantify the relative levels of a given protein with reference to four control samples of different protein content loaded on the same gel. The concentration measured for a given protein was divided by the α-tubulin content of the same sample. The results shown in SF767 cells correspond to mean ± SEM values from triplicate samples measured in 6–8 independent experiments (for a total of 18–24 measurements for each protein). For studies in mouse tumors, the number of animals used is indicated in the corresponding section.

Membrane Lipid Analysis. Cell membrane lipids were extracted directly from the frozen monolayer of cells using the n-hexane: 2-propanol (3:2, v/v) extraction method with slight modifications. Briefly, SF767 cells were cultured as described above and maintained for 72 h in the presence or absence or 2OHOA (200 μM). Frozen cells were washed with PBS, lipids were extracted by the direct addition of 2.2 mL of 2-propanol, and the cells were subsequently removed from the plate using a Teflon cell scraper. The total protein concentration was then determined using the bicinchoninic acid assay (Thermo scientific, Rockford). Then, 6 mL of hexane was added to the mixture and removed. The cell dish was rinsed with another 2.2 mL of 2-propanol that was combined with the first hexane/2-propanol mixture. Cell extracts were then centrifuged at 1000×*g*, and the pellet containing denatured proteins and other cellular debris was discarded. The lipid-containing organic phase was decanted and stored under a N_2 atmosphere at −80 °C until analysis. Individual phospholipid classes and neutral lipids were separated by thin layer chromatography (TLC) or (high performance) thin layer chromatography [(HP) TLC], respectively, on Whatman silica gel-60 plates (20 × 20 cm, 250 μm or 10 × 10 cm, respectively) that were heat-activated at 110 °C for 1 h, and the samples were streaked onto the plates. Phospholipids were separated using chloroform/methanol/acetic acid/water (55:37.5:3 : 2 v/v/v/v), and the phospholipid mass was determined by measuring the lipid phosphorus content of individual lipid classes separated by TLC. Neutral lipids were separated in petroleum ether/diethyl ether/acetic acid (75:25 : 1.3 v/v/v), and the lipid fractions were identified using lipid standards (Larodan, Sweden). After development, plates were air dried, sprayed with 8% (w/v) H_3PO_4 containing 10% CuSO4 (w/v), and charred at 180 °C for 10 min. Lipids were then quantified by image analysis.

Near Infrared Spectroscopy Immunofluorescence. For some immunocytochemical studies, mouse tumors ($N = 10$) were fixed with 4% paraformaldehyde in 100 mM phosphate buffer (pH 7.4), embedded in paraffin, and sections were cut serially (7 μm) using a microtome. Tissue sections were deparaffinized with xylene, and they were rehydrated with ethanol. The sections were then placed in an antigen retrieval solution (Dako A/S, Glostrup, Denmark) for 10 min at 95 °C and rinsed in 100 mM Tris-buffered saline (pH 7.4, TBS). Subsequently, the sections were incubated for 1 h in TBS containing 0.1% Triton X-100 and 5% horse serum followed by overnight incubation at 4 °C with a specific primary antibody against human GFAP (1:100, Abcam, Cambridge, UK). Following four 15 min washes with TBS containing 0.05% Tween 20 (TBST), the tissue sections were incubated for 1 h at room temperature with IRDye™ 800CW-conjugated donkey anti-rabbit IgG (1:8,000, Li-Cor, Lincoln, Nebraska) in blocking solution. The tissue sections were washed four times in TBST. Following a brief rinse with water, sections were allowed to air dry for at least 1 h before fluorescence was detected using the Li-Cor Odyssey Near Infrared Scanner (21 μm resolution, 1 mm offset with highest quality). Channel sensitivity was optimized for each set of stained sections, and the tumor areas were

defined and the integrated intensities were determined with Odyssey software.

In addition to the above ex vivo measurements, in vivo determination of glutamine synthetase expression was performed by near infrared spectroscopy. Accordingly, IRDye 800CW was covalently bound to an anti-glutamine synthetase antibody (Abcam) using the reagents provided by the manufacturer (High MW protein labeling kit, Li-Cor). For in vivo determinations of this enzyme, nude mice were infected with SF767 cells except that the treatments (p.o., 600 mg/kg 2OHOA or 80 mg/kg TMZ or vehicle; $N = 10$ per group) commenced 15 d after cell inoculation and lasted only seven days. IRDye 800CW-anti-glutamine synthetase (50 μg) was then injected intravenously through the tail vein and fluorescence was acquired in vivo using the Li-Cor Odyssey Near Infrared Scanner at 72–144 h postinjection. During this postinjection time (144 h), treatments were maintained as above.

Animals, Tumor Grafts and Treatments. Male NUDE (Swiss) Crl:NU (Ico)-Foxn1^{nu} mice (five week-old, 30–35 g, Charles River Laboratories, Paris, France) were maintained in a thermostat cabinet (28 °C, EHRET, Labor-U-Pharmatechnik) with a sterile air flow at a relative humidity of 40–60% and a 12 h dark/light cycle.

For xenograft tumors derived from human glioma (SF767) cells, 7.5×10^6 cells were inoculated subcutaneously into the animal dorsal area and after one week, tumors were already visible. Animals were randomly divided into groups with a similar mean tumor volume, and they received daily oral treatments with the vehicle alone (water), 2OHOA (600 mg/kg, except in the dose-dependence studies), TMZ (80 mg/kg), or 2OHOA + TMZ (same doses) for 50 ($N = 20$ per group, line graphs in Fig. 1) or 60 days ($N = 15$ per group, bar graphs, line graphs in Fig. 1). In vivo near infrared studies ($N = 10$) and studies of the efficacy of the free acid or the salts ($N = 5$) were performed over seven and 14 days, respectively. Tumor volumes were calculated as $v = w^2 \times L/2$ where w is the tumor width and L its length.

After the final tumor volume measurements, mice were sacrificed by decapitation, the tumors were removed and immediately frozen in liquid nitrogen before being stored at 80 °C for molecular studies or maintained in 10% formaldehyde for histological studies. All experiments were carried out in accordance with the animal welfare guidelines of the European Union and the Institutional Committee for Animal Research of the University of the Balearic Islands.

For orthotopic SF767 grafts in nude mice, cells were collected from cultures at 80% confluence, resuspended in PBS containing 0.05% trypsin and 0.02% EDTA, and centrifuged at $600 \times g$ for 5 min at room temperature. Cell pellets were then resuspended in fresh culture medium and rapidly counted in a Burker chamber. After a second centrifugation in the same conditions, the cells were resuspended in serum-free medium and used to infect mice. Male NUDE (Swiss) Crl:NU(Ico)-Foxn1^{nu} mice were anesthetized with ketamine (60 mg/kg, i.p.) and diazepam (7.5 mg/kg, i.p.). Animals received approximately 3×10^5 SF767 cells (>90% viability) in a volume of 3 μl stereotactically injected in the right caudate nucleus: bregma (anatomical point on the mouse skull at which the coronal suture is intersected perpendicularly by the sagittal suture) 0.5 mm; lateral, 1.75 mm. The needle was initially advanced to a depth of 4 mm and then withdrawn to a depth of 3 mm to limit reflux up the needle tract during injection of cells. After six weeks, the animals were anesthetized and perfused with 4% paraformaldehyde in phosphate buffered saline to fix the brain. Vibratome sections (50 μm) were obtained and immunochemical detection was performed using the HuNu antibody [Millipore, 1:400) and the biotin-avidin-peroxidase complex method (Vector)] visualizing the antibody binding with diaminobenzidine (DAB; Vector). For each animal, quantitative estimates of the total number of grafted cells were determined stereologically using the optical fractionator. The rostral and caudal limits of the reference volume were determined from the first and last frontal sections that contained grafted cells. The sample sites were systematically and automatically generated by the computer and examined using a 60x objective on a Nikon Eclipse TE 300 microscope.

Data Analysis. The data are expressed as the mean ± SEM values from 6–8 independent experiments involving triplicate samples and the number of animals indicated. Experimental groups were compared using one-way ANOVA followed by the Bonferroni multiple-comparison test or the two-tailed t test where appropriate. The differences between experimental groups were considered statistically significant at $P < 0.05$. For statistical significance was also taken as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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3. Martínez J, et al. (2005) Membrane structure modulation, protein kinase C alpha activation, and anticancer activity of Minerval. *Mol Pharmacol* 67:531–540.

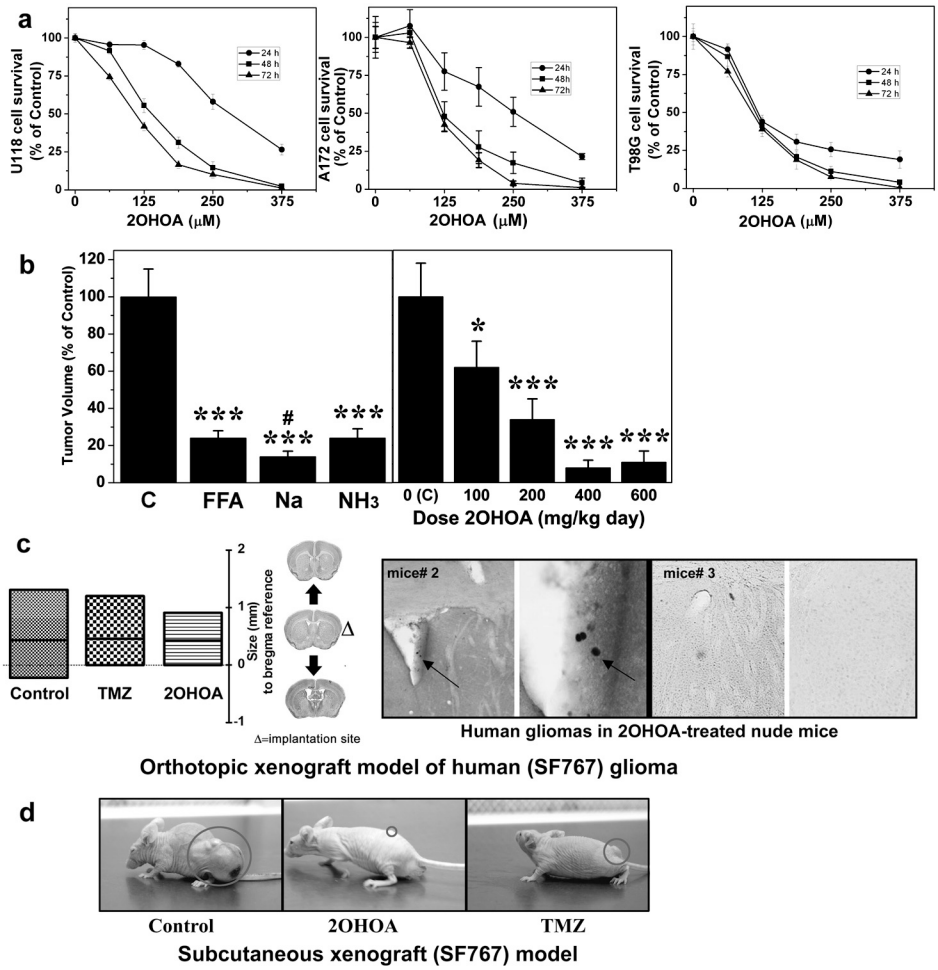

Fig. S1. (*A*) Efficacy of 2OHOA against various types of human glioma cells. Time and concentration dependent inhibition of human glioma cells (U118, *left*; A172, *center*; T98G, *right*) by 2OHOA. (*B*), Effects of the vehicle alone (control, *C*), the free fatty acid form of 2OHOA (FFA), and the sodium (Na) and ammonium (NH_3) salts of 2OHOA, on the SF767-derived tumor volume after 14 days of treatment (600 mg/kg, p.o., $N = 5$). Dose-response effect of 60-day 2OHOA treatment (second graph, $N = 10$). *$P < 0.05$, ***$P < 0.001$, with respect to Control; #, $P < 0.05$ with respect to other treatments. (*C*), Distribution of $HuNu^+$ cells in the brain of mice (*left*, mm to bregma reference, orthotopic model). Additional images showing the effect of oral 2OHOA treatment on the brain of nude mice infected with human glioma cells (*right* panels). In one of the mice (number 2) only a few glioma cells remained (*arrows*); whereas, in the other three mice (mice number 3 is shown as an example) no glioma cells remained. $N = 5$ for all experimental groups. (*D*), Representative photographs of mice infected with SF767 cells and treated with the vehicle alone (Control), 2OHOA (600 mg/kg, p.o., 60 days), and TMZ (80 mg/kg, p.o., 60 days).

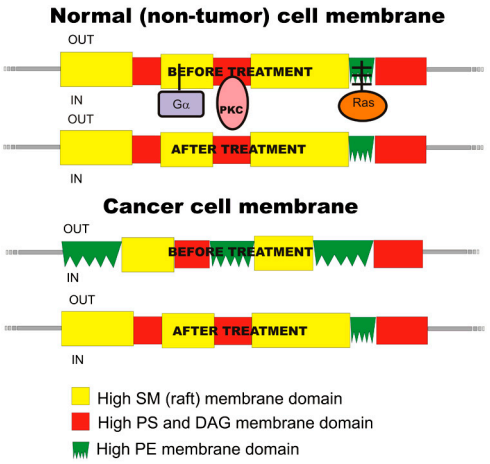

Fig. S2. *Upper Scheme*, Distribution of the distinct membrane domains in normal cells and examples of proteins bound to SM-rich (yellow), diacylglycerol (DAG)-rich (red), and phosphatidylethanolamine (PE)-rich (green) membrane microdomains before and after treatment with 2OHOA. *Lower Scheme*, Distribution of microdomains in glioma (and other cancer) cells before and after 2OHOA-induced increases in SM and DAG and decreases in PE.

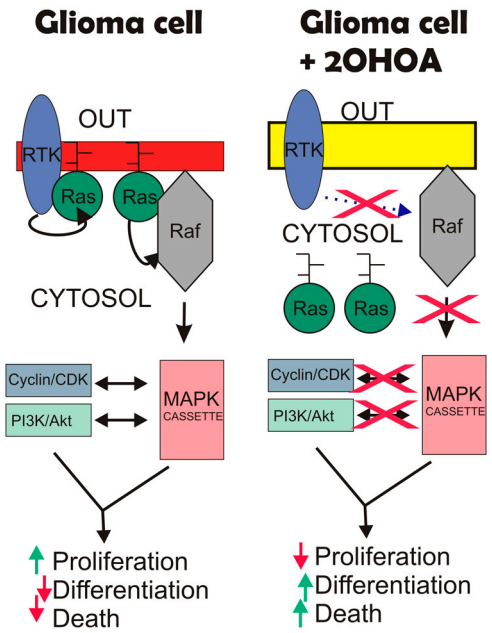

Fig. S3. Scheme of Ras-associated signaling in human glioma cells before (*left*) and after treatment with 2OHOA (*right*). Signals that induce cell proliferation, loss of differentiation, and survival are often propagated through Receptor Tyrosine Kinases [(RTK), e.g., EGFR] that activate Ras that in turn activates Raf. Then, Raf activates MEK, which finally phosphorylates and activates mitogen-activated protein kinase (MAP kinase). The Ras/MAPK signaling cassette regulates positively the PI3K/Akt and Cyclin/CDK pathways. In the presence of 2OHOA, Ras translocates to the cytoplasm, which prevents RTK-Ras and Ras-Raf interactions that only occur at the plasma membrane and impairs the signaling and cross-talk events mentioned above.

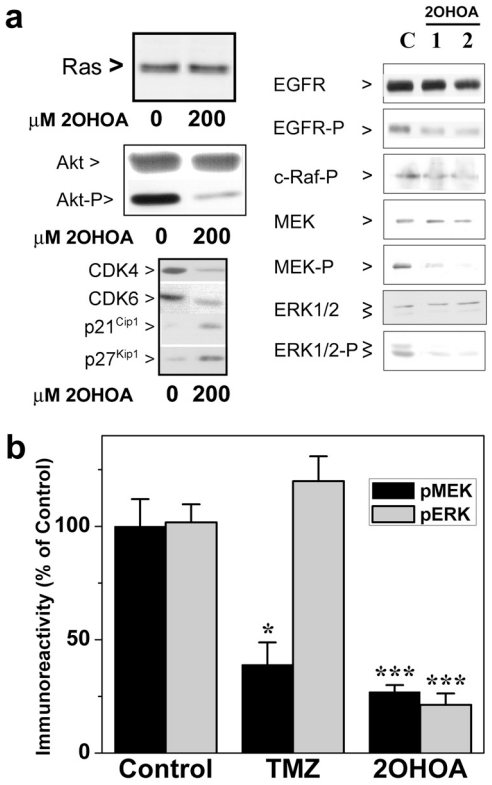

Fig. S4. *A*), Representative immunoblots of the various proteins (or phosphoproteins, -P) whose levels have been quantified in SF767 cells treated in the presence or absence (control, *C*) of 150 (1) or 200 μM (2, 200) 2OHOA (see Figs. 3 and 4 in the main text). (*B*), Effect of 2OHOA on the levels of phospho-MEK and phospho-ERK in SF767-derived tumors. Nude mice bearing tumors were treated with vehicle, TMZ (80 mg/kg, p.o., daily) or 2OHOA (600 mg/kg, p.o., daily) for 50 d. Then, animals were killed and the levels of these proteins measure by quantitative immunoblotting.

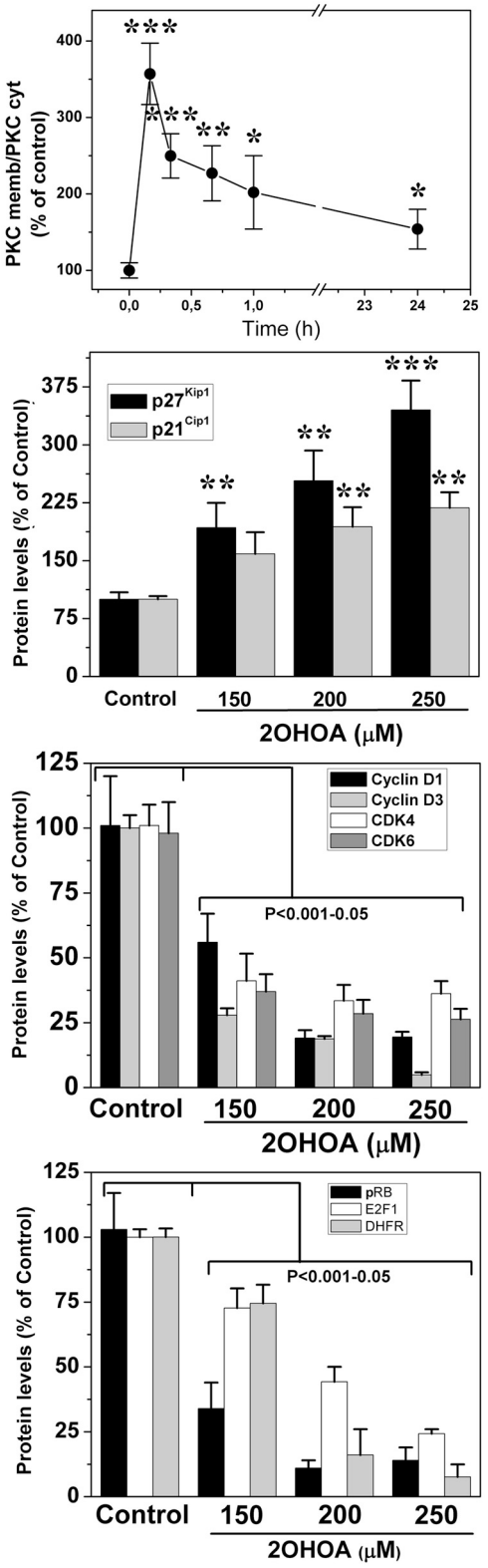

Fig. S5. Effects of 2OHOA on cell (SF767) cycle proteins. *Upper panel*: Incubation with 2OHOA (200 μM) for varying lengths of time induces PKCα translocation to the membrane (ratio of membrane to cytosolic PKCα, as determined by quantitative immunoblotting. Effects of 2OHOA treatment (48 h at the concentrations indicated) on $p21^{Cip1}$ and $p27^{Kip1}$ (*second panel*), Cyclin D1, Cyclin D3, CDK4 and CDK6 (*third panel*), and (*C*) Effect of 2OHOA (48 h at the concentrations indicated) on phosphorylation of the retinoblastoma protein (pRB/RB ratio), E2F-1 and DHFR (*bottom panel*). Quantitative immunoblotting ($N = 6$–8) was used in all cases.

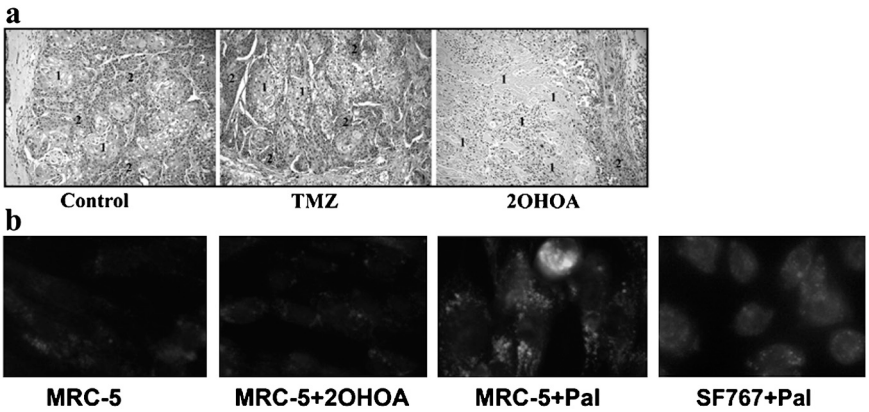

Fig. S6. 2OHOA induces tumor cell death, in vivo, and ER stress-autophagy, in vitro. (*A*) Hematoxylin-eosin staining of tumor sections from mice treated with the vehicle alone (Control), TMZ (80 mg/kg, p.o., 50 d) or 2OHOA (600 mg/kg, p.o., 50 d), showing areas with dead (1) and living (2) cells. (*B*) Fluorescence microscopy of lysosomes/autophagosomes labeled with Lysosensor in nontumor MRC-5 cells in the presence or absence (MRC-5) of 2OHOA (MRC − 5 + 2OHOA: 150 μM for 48 h) or palmitic acid (MRC − 5 + Pal, 150 μM for 48 h). The effects of Pal on SF767 is also shown (SF767 + Pal). Palmitic acid is a known inducer of ER stress and autophagy. $N = 400$ cells from five independent experiments.