Supplemental Material and Data

An LXR agonist promotes GBM cell death through inhibition of an EGFR/AKT/SREBP-1/LDLR-dependent pathway

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Western Blotting

Western blotting was done as previously described and shown in supplemental materials (1). Cultured cells or snap-frozen tissue samples were lysed and homogenized using buffer containing 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 10 µg/mL leupeptin, 60 µg/mL aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride. Equal amounts of protein extracts were separated by using 8% or 10% SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking for 1 hour in a Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk, the membrane was probed with various primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase. The immunoreactivity was revealed by use of an ECL kit (Amersham Biosciences Co., Piscataway, NJ).

Immunohistochemical assay

Paraffin-embedded tissue blocks were sectioned using the UCLA Pathology Histology and Tissue Core Facility. Immunohistochemical staining (IHC) was performed as previously described and shown in supplemental materials (2,3). Slides were counterstained with hematoxylin to visualize nuclei. Paraffin-embedded tissue sections underwent immunohistochemical analysis in which the results were scored independently by two pathologists who were unaware of the findings of the molecular analyses. Quantitative image analysis to confirm the pathologists' scoring was also performed with Soft Imaging System software (4). We have previously demonstrated the utility of this quantitative method for measuring drug-specific effects in paraffin-embedded tissue samples from GBM patients enrolled in clinical trials with targeted agents (2, 3, 5).

Tissue Microarrays

Tissue microarrays (TMAs) were used to analyze p-EGFR Tyr1086, p-Akt Ser473, nuclear SREBP-1, LDLR, p-MET, p-PDGFR Tyr579 immunohistochemical staining in 140 GBM patient samples. Tissue microarrays (TMAs) enable tumor tissue samples from hundreds of patients to be analyzed on the same histological slide. We constructed two GBM TMAs by using a 0.6 mm needle to extract 252 representative tumor tissue cores and 91 adjacent normal brain tissue cores from the paraffin-embedded tissue blocks of 140 primary GBM patients. These cores were placed in a grid pattern into two recipient paraffin blocks, from which tissue sections were cut for immunohistochemical analysis of p-EGFR, p-Akt, nuclear SREBP-1, LDLR, p-Met, p-PDGFR. These TMAs have been used for other studies (4-6).

Xenograft Model

Isogenic human malignant glioma cells (U87, U87-EGFRvIII) and human primary GBM model GBM39 were implanted into immunodeficient SCID/Beige mice for subcutaneous (s.c.) xenograft studies. SCID/Beige mice were bred and kept under defined-flora pathogen-free conditions at the AALAC-approved Animal Facility of the Division of Experimental Radiation Oncology, UCLA. For s.c. implantation, exponentially growing tumor cells in culture were trypsinized, enumerated by Trypan Blue exclusion, and resuspended at 1×10^6 cells/ml in a solution of dPBS and Matrigel (BD Biosciences). Tumor growth was monitored with calipers by measuring the perpendicular diameters of each s.c. tumor. U87 and U87-EGFRvIII cell lines were implanted s.c. on opposite sides of the mouse abdomen (n=6), and U87-EGFRvIII xenograft mice were treated with GW3965 by oral gavage administration method (40 mg/kg daily). Mice were euthanized if tumors reached 14 mm in maximum diameter, or animals showed signs of illness. All experiments were conducted after approval by the Chancellor's Animal Research Committee of UCLA (5).

Immunohistochemistry and image analysis-based scoring

Tissue sections were cut from blocks of formalin-fixed paraffin tumor tissue from TMA or glioblastoma patients treated with lapatinib. Tumor specimens were obtained according to a protocol approved by the Institutional Review Board of UCLA. The first set of paired pre- and post-treatment tumor tissues for lapatinib trial were examined. Five-micron tissue sections were stained with polyclonal antibodies directed against p-EGFR Tyr1086, p-Met Tyr1349, p-PDGFR Tyr579, p-AKT Ser473, SREBP-1 and LDLR for sections of lapatinib trial and tissue microarray; Digital scores for p-EGFR, p-AKT, and LDLR were based on absolute staining intensity of tumor cells as quantified following false-color conversion. Sections were photographed using a Colorview II camera mounted on an Olympus BX41 microscope at 20× magnification. 5 images were captured per slide from representative regions of the tumor. Borders between individual cells were approximated using a separator function of the Soft Imaging Software (with the parameter of Smooth and Fine/Coarse, 2 and 10 respectively). Quantitative analysis was done using HSI color algorithm based on hue,

saturation and intensity. Saturations of the separated cell in the images were quantified in the red-brown hue range to exclude the negative staining area with hematoxylin nuclear staining. To compare the staining intensity of all slides, mean saturation of total cells on each image was quantified and calculated. 1500 to 2000 cells per case (on average) were measured for each slide and statistical comparisons were performed using R software, using an approach previously described (2,5). For SREBP-1 staining scoring, separated cells were quantified with red brown hue range (cells with positive nuclear staining) and total hue range (all cells) after cell border separation and proportion of positive cells was calculated based on these numbers (5).

TUNEL Staining

In vitro TUNEL staining

Three thousand U87 and U87vIII cells were plated on eight chamber glass slides (BD) in 5% FBS. After 24 hours, the media was changed to 1% LPDS with DMSO or GW3965 at 3 uM or 5 uM. After two days, cells were fixed in 4% paraformaldehyde (in PBS, pH 7.4) for one hour at room temperature, washed in PBS and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice for 2 minutes. Slides were then washed twice in PBS before incubation with TUNEL Reaction Mixture (Roche, *in situ* Cell Death Detection Kit, TMR Red) at 37ºC for one hour in the dark. Slides were washed three times in PBS and cover-slipped with ProLong Gold anti-fade reagent with DAPI (Invitrogen). Slides were then analyzed using a TRITC filter (570-620nm wavelength) fluorescence microscope (Lecia) and imaged by cold CCD camera.

Paraffined tissue TUNEL staining

Paraffin sections were deparaffinized and subjected to graded rehydration as with the immunohistochemical method. Peroxidase activity was quenched with 3% hydrogen peroxide in water. TUNEL staining was performed using digoxigenin-conjugated dUTP and HRP-conjugated anti-digoxigenin antibodies following its protocol (Roche). Visualization for staining was performed with NovaRed substrate (Vector Laboratories) and tissues were then counterstained with hematoxylin (5).

Cell Proliferation and Death Assays

Cells were seeded in 96 wells and were treated after 24 hours with different drugs indicated in each experiment in medium containing 1% FBS or lipoprotein deficient serum (LPDS). Relative proliferation was determined using Cell Proliferation Assay Kit (Chemicon). Cells were incubated 1.5 hrs after adding tetrazolium salt WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfo-phenyl)-2H-tetrazolium, monosodium salt] (Chemicon) at 5% CO_2, 37ºC and the absorbance of the treated and untreated cells were measured using a microplate reader (Bio-Rad) at 420 to 480 nm. Cells seeded in 12 well plates were counted using a hemocytometer, and dead cells were assessed using trypan blue exclusion assays (Invitrogen).

ShRNA assay

5×10^4 cells were seeded in 12 well plates and maintained for 24 hrs, after which medium was replaced with fresh 5% FBS medium including 5 ug/ml Polybrene (Sigma), and then shRNA lentivirus was added to cells, followed by incubation for 24 hrs. For Western blot analysis, infected cells were incubated in fresh 5% FBS medium for another 24 hrs, then lysed. For cell proliferation or cell death assays, infected cells were subcultured into 96 well plates or 12 well plates, maintained in 5% FBS medium for 24 hrs, then changed to 1% FBS or LPDS medium with 2 ug/ml puromycin for 4 to 5 days.

Reference

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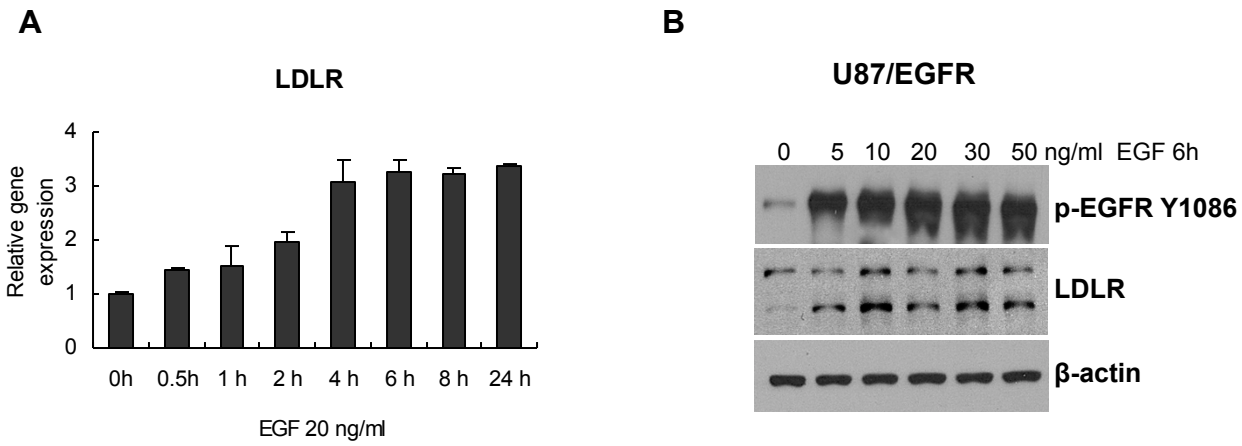
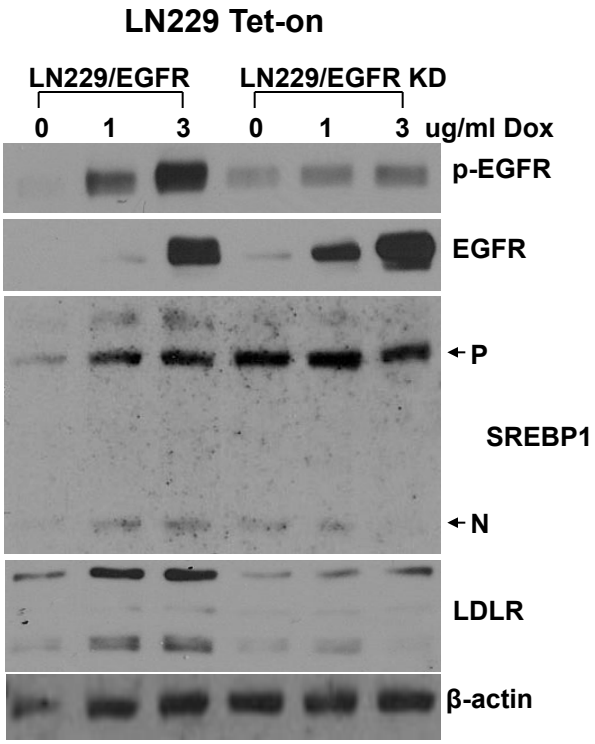


Supplemental Figure 1. EGF stimulation promotes LDLR expression.
A) EGF stimulation promotes LDLR gene expression in a time-dependent manner. U87/EGFR cells were treated with EGF 20 ng/ml in serum free conditions for the indicated times. Total cellular RNA was extracted by Trizol (Invitrogen). LDLR gene expression was quantified by real-time PCR (Bio-Rad). **B**) EGF stimulation promotes LDLR protein expression. U87/EGFR cells were treated with EGF at a range of doses as indicated for 6 hrs. Cell lysates were checked by Western blot using indicated antibodies. The result shows that EGF stimulation increases LDLR protein levels in association with activation of EGFR.



Supplemental Figure 2. Activation of EGFR increases SREBP-1 N-terminal form and LDLR levels. LN229/EGFR and LN229/EGFR KD (kinase dead) tet-on cell lines were treated with doxycycline at 1 and 3 ug/ml dose for 24 hr. Cell lysates were detected by Western blot, which showed p-EGFR levels dramatically increased in LN229/EGFR tet-on cells, but not in LN229/EGFR KD tet-on cells. Total EGFR levels were similarly induced in both cell lines treated by doxycycline. In correlation with p-EGFR levels, SREBP-1 N-terminal cleaved form and LDLR levels increased only in LN229/EGFR tet-on cell line. These results indicate that p-EGFR levels correlate with SREBP-1/LDLR signaling.

A

		LDLR	
		+	-
p-PDGFR	+	58	6
	-	50	40
Percentage in PDGFR tumor		90.60%	
P value		*P*=0.000001	

B

		p-Akt/SREBP1/LDLR	
		+	-
p-PDGFR	+	47	11
	-	44	43
Percentate in PDGFR tumor		81.03%	
P value		*P*=0.000148	

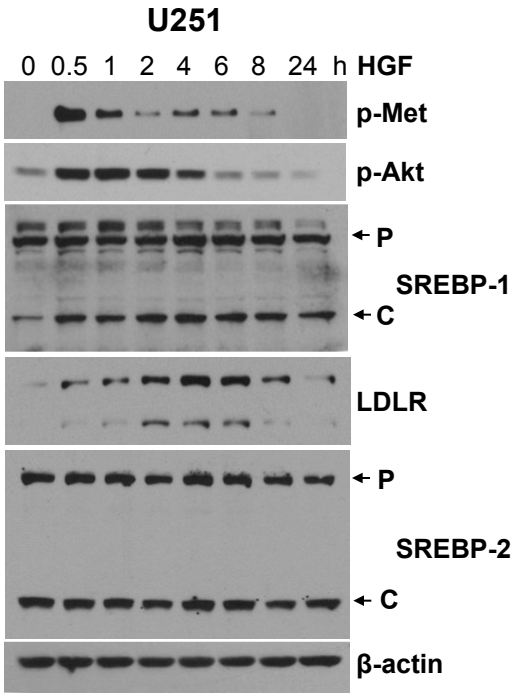
C

		LDLR	
		+	-
p-Met	+	62	6
	-	49	41
Percentage in Met tumor		91.18%	
P value		*P*=0.0000001	

D

		p-Akt/SREBP1/LDLR	
		+	-
p-Met	+	53	14
	-	36	42
Percentate in Met tumor		79.10%	
P value		*P*=0.000039	

E



Supplemental Figure 3. P-PDGFR and p-Met are associated with p-Akt/SREBP-1/LDLR signaling in glioblastoma patients and Met activation can promote p-Akt/SREBP-1/ LDLR signaling in glioblastoma cells *in vitro.*

A) GBM patient tissue microarrays (TMA) were stained using different antibodies by IHC. Correlation analysis shows LDLR significantly correlates with p-PDGFR-beta. **B**) Correlation analysis shows p-PDGFR-beta significantly correlates with p-Akt/SREBP-1/LDLR signaling in GBM patients.**C**) Correlation analysis shows LDLR significantly correlates with p-Met in GBM patients. **D**) Correlation analysis shows p-Met significantly correlates with p-Akt/SREBP-1/LDLR signaling in GBM patients. **E**) Western blot analysis shows activation of Met signaling using HGF (60 ng/ml) promotes p-Akt/SREBP-1/LDLR signaling in U251 GBM cell line.

A

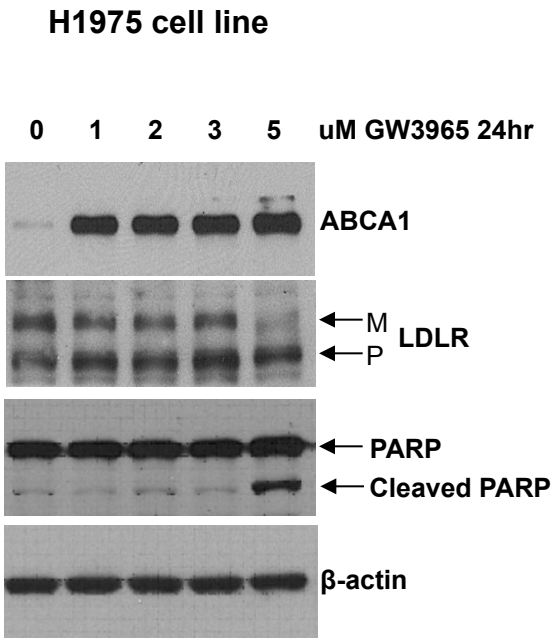
LDLR positive tumor cores	129
p-EGFR (+)/p-Met (-)/p-PDGFRβ (-)	17
p-Met (+)/p-EGFR (-)/p-PDGFRβ (-)	9
p-PDGFRβ (+)/p-EGFR (-)/p-Met (-)	8
p-EGFR (+)/p-Met (+)/p-PDGFRβ (-)	15
p-Met (+)/p-PDGFRb (+)/p-EGFR (-)	16
p-EGFR (+) and p-PDGFRb(+)/p-Met (-)	10
p-EGFR (+)/p-Met (+)/p-PDGFRb(+)	19
p-EGFR (-)/p-Met (-)/p-PDGFRb(-)	35

LDLR tumor negative cores	13
p-EGFR (+)/p-Met (-)/p-PDGFRβ (-)	0
p-Met (+)/p-EGFR (-)/p-PDGFRβ (-)	3
p-PDGFRβ (+)/p-EGFR (-)/p-Met (-)	1
p-EGFR (+)/p-Met (+)/p-PDGFRβ (-)	0
p-Met (+)/p-PDGFRb (+)/p-EGFR (-)	0
p-EGFR (+) and p-PDGFRb(+)/p-Met (-)	0
p-EGFR (+)/p-Met (+)/p-PDGFRb(+)	0
p-EGFR (-)/p-Met (-)/p-PDGFRb(-)	9

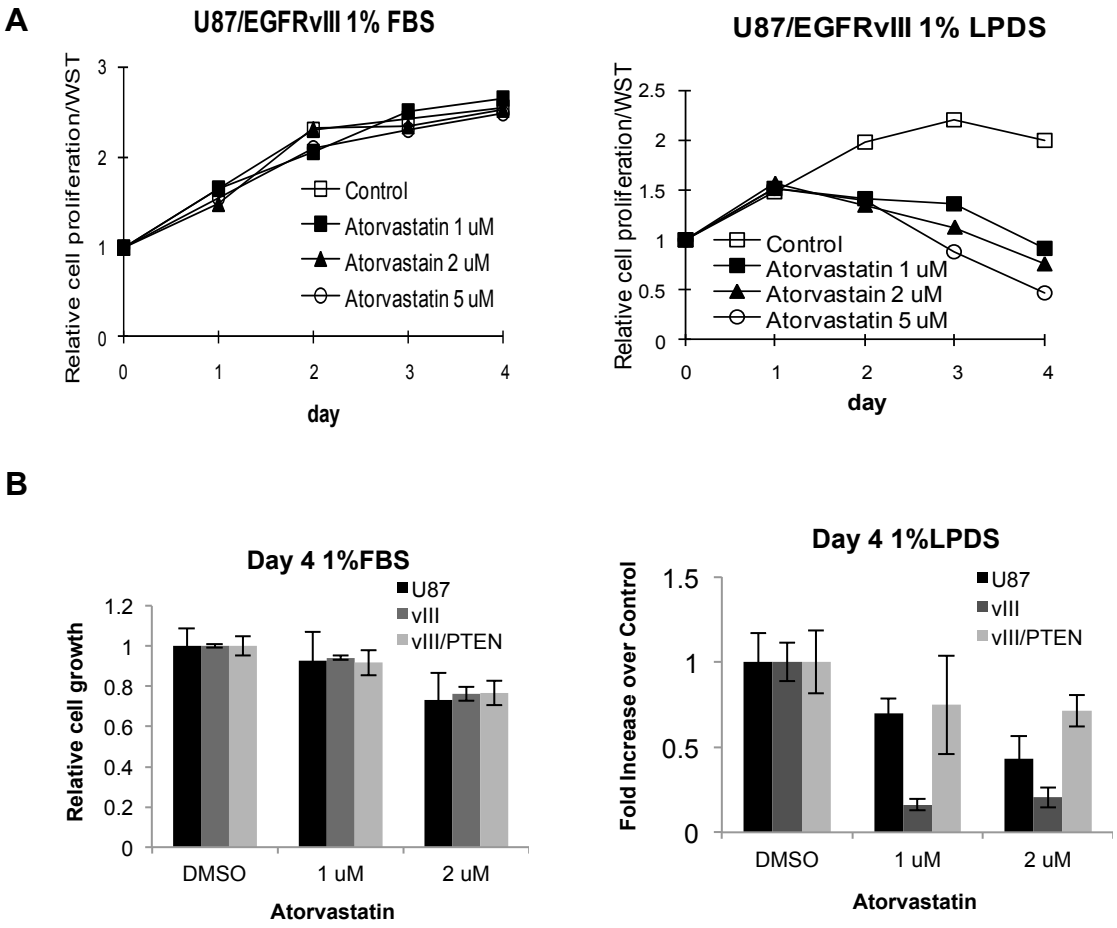
B

		LDLR	
		+	-
p-EGFR, p-MET	+	94	4
or p-PDGFRβ	-	35	9
Percentage in LDLR positive tumor		**72.9%**	
P value		*P*=0.0034	

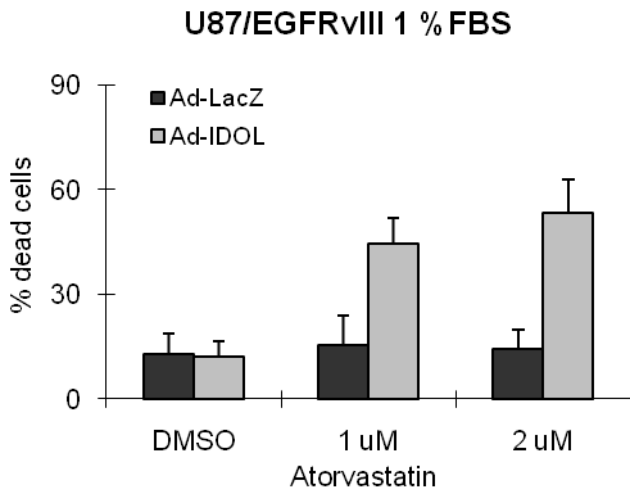
Supplemental Figure 4. LDLR levels correlate with activation of multiple receptor tyrosine kinases (RTKs) in GBM patients. A) GBM patient tissue microarrays (TMA) were stained using different antibodies by IHC. Correlation analysis shows the phosphorylation status of three RTKs, EGFR, Met and PDGFRβ in LDLR positive and negative tumors. **B**) p-EGFR, p-Met and p-PDGFRβ staining alone, or in combination significantly correlates with LDLR in GBM patients. p-EGFR, p-MET, and/or p-PDGFR positively alone, or in combination, was detected in 72.9% of LDLR positive tumors.



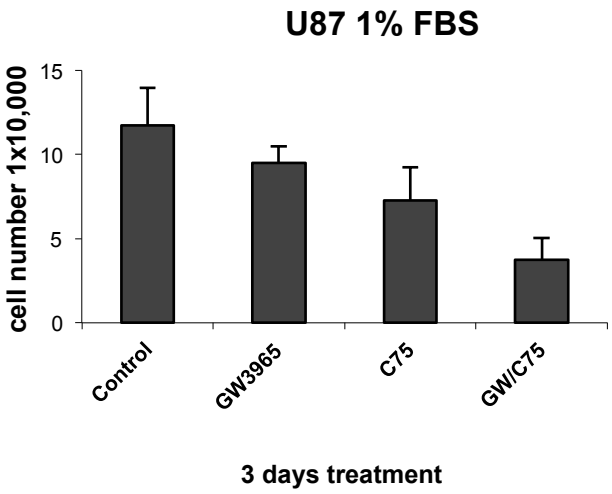
Supplemental Figure 5. GW3965 treatment up-regulates cholesterol transporter gene ABCA1 and reduces LDLR levels in lung cancer cell line.
Non-small cell lung cancer cell line H1975 cells were treated with GW3965 for 24 hrs at indicated doses, and cells were lysed and probed by Western blot using antibodies indicated.



Supplemental Figure 6. GBM cells are sensitive to atorvastatin treatment in the absence of extracellular cholesterol. A) U87/EGFRvIII cells were treated with atorvastatin at indicated doses in 1% FBS or 1% LPDS for 4 days. Lipoprotein deficient serum (LPDS) lacks choelsterol. The results show atorvastatin significantly inhibits GBM cells growth only in LPDS condition. **B**) GBM cell lines U87, U87/EGFRvIII and U87/EGFRvIII/PTEN were treated with atorvastatin in 1% FBS or 1% LPDS for 4 days. The results show EGFRvIII expression sensitizes GBM cells to atorvastatin treatment in LPDS condition, and PTEN overexpression partially rescues its growth.

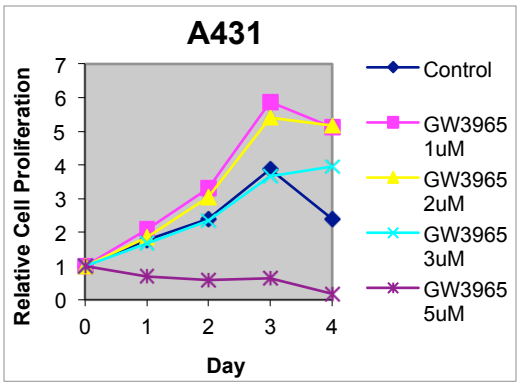


Supplemental Figure 7. IDOL infection and atorvastatin combination treatment induces GBM cell death. U87/EGFRvIII cells were infected by Ad-LacZ or Ad-IDOL for 8 hrs, then treated with atorvastatin for 4 days in 1% FBS. Cells were counted using trypan blue assays. The results show Ad-IDOL infection, combined with atorvastatin treatment, markedly induces GBM cell death.



Supplemental Figure 8. Activation of LXR in combination with inhibition of fatty acid synthesis exerts an additive effect in suppressing GBM cell growth. U87 cells were treated with GW3965 2uM, C75 10ug/ml (fatty acid synthase inhibitor) or a combination for 3 days in 1% FBS. Cells were counted using trypan blue assays. The results show inhibition of fatty acid synthesis enhances the effect of LXR agonist GW3965 on suppression of GBM cell growth.

A



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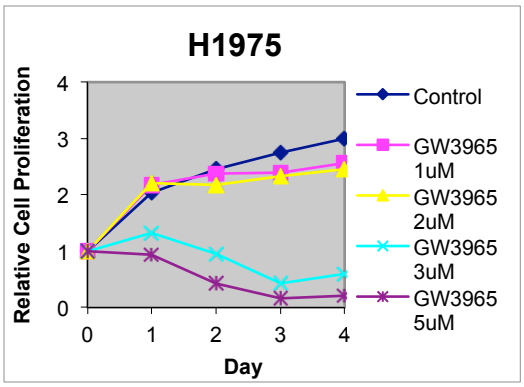

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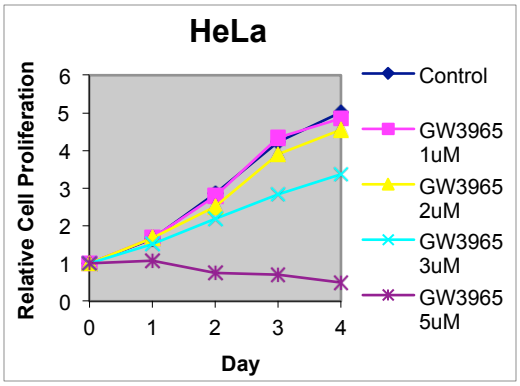

Supplemental Figure 9. Multiple tumor types cell lines are sensitive to GW3965 treatment. **A**) A431 epidermoid carcinoma cells, **B**) H1975 lung cancer cells and **C**) HeLa cervical carcinoma cells were treated with the LXR agonist GW3965 at indicated doses for 4 days. The proliferation rate of these three tumor types cell lines was measured with WST-1 kit (Chemicon) showing a dose-dependent response to GW3965 drug.