An LXR Agonist Promotes Glioblastoma Cell Death through Inhibition of an EGFR/AKT/SREBP-1/LDLR–Dependent Pathway
**ABSTRACT**

Glioblastoma (GBM) is the most common malignant primary brain tumor of adults and one of the most lethal of all cancers. Epidermal growth factor receptor (EGFR) mutations (EGFRvIII) and phosphoinositide 3-kinase (PI3K) hyperactivation are common in GBM, promoting tumor growth and survival, including through sterol regulatory element-binding protein 1 (SREBP-1)–dependent lipogenesis. The role of cholesterol metabolism in GBM pathogenesis, its association with EGFR/PI3K signaling, and its potential therapeutic targetability are unknown. In our investigation, studies of GBM cell lines, xenograft models, and GBM clinical samples, including those from patients treated with the EGFR tyrosine kinase inhibitor lapatinib, uncovered an EGFRvIII-activated, PI3K/SREBP-1–dependent tumor survival pathway through the low-density lipoprotein receptor (LDLR). Targeting LDLR with the liver X receptor (LXR) agonist GW3965 caused inducible degrader of LDLR (IDOL)–mediated LDLR degradation and increased expression of the ABCA1 cholesterol efflux transporter, potently promoting GBM cell death in an in vivo GBM model. These results show that EGFRvIII can promote tumor survival through PI3K/SREBP-1–dependent upregulation of LDLR and suggest a role for LXR agonists in the treatment of GBM patients.

**SIGNIFICANCE:** This study reveals that GBM cells have devised a mechanism to subvert the normal pathways for feedback inhibition of cholesterol homeostasis via EGFRvIII and PI3K-dependent activation of SREBP-1. We show that an LXR agonist causes IDOL-mediated LDLR degradation and increases expression of the ABCA1 cholesterol efflux transporter, potently promoting GBM cell death in vivo. These results suggest a role for LXR agonists in the treatment of GBM patients. *Cancer Discovery; 1(5): OF1–OF15. ©2011 AACR.*

**INTRODUCTION**

Glioblastoma (GBM) is the most common malignant primary brain tumor and one of the most lethal of all cancers (1, 2). GBM is also among the most chemo- and radiation-resistant types of cancer, with a median patient survival of 12 to 15 months from initial diagnosis, despite aggressive therapy (3). Therefore, new treatment strategies are needed (4). The phosphoinositide 3-kinase (PI3K) signaling pathway is a potent regulator of cellular growth and survival, and constitutive PI3K activation has been shown to be oncogenic (5). PI3K signaling is hyperactivated in nearly 90% of GBMs, commonly as a consequence of epidermal growth factor receptor (EGFR) amplification and activating mutation (EGFRvIII), as well as loss of the PTEN tumor suppressor protein, a negative regulator of PI3K signaling (6–8). Despite the compelling nature of EGFR as a drug target in GBM, the EGFR inhibitors gefitinib and erlotinib have failed to demonstrate efficacy (9–11), in large part as a consequence of persistent PI3K signaling due to PTEN loss and/or coactivation of other receptor tyrosine kinases (RTK; refs. 9, 10, 12).

Recent work from our group suggests an alternative approach to treating EGFR/PI3K-activated GBMs, based on targetable differences in the molecular circuitry regulating tumor cell metabolism (13–15). Cancer cells preferentially metabolize glucose by aerobic glycolysis, a phenomenon known as the Warburg effect. Although less efficient at generating ATP, aerobic glycolysis facilitates uptake and incorporation of glycolytic intermediates into nucleotides, amino acids, and lipids, thus meeting the enhanced biosynthetic demand imposed by proliferating cancer cells (16–19). PI3K signaling may be central to linking the common genetic perturbations of cancer, such as RTK mutations and PTEN loss, with altered metabolic processes, including the “lipogenic phenotype” common to many cancers, such as GBM (13, 14, 17, 20). We recently showed both that mutant EGFRvIII-expressing GBMs promote lipogenesis through PI3K-dependent activation of the master transcriptional regulator sterol regulatory element-binding protein 1 (SREBP-1) and that this signal was required for tumor survival in vivo (14). Therefore, blocking specific enzymes in lipogenic circuitry may potentially yield synthetic lethal interactions (19, 21–23), providing an alternative approach for treating tumors with PI3K pathway–activating mutations.
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tive and thus to the increased shop flux. The stimulation of the EGFRvIII receptor activates wild-type EGFR molecules, which promote LDLR expression in GBMs. Consequently, the EGFRvIII signaling can promote LDLR expression in GBMs in vivo.

**EGFRvIII Promotes LDLR Expression through a PI3K/Akt-Mediated, SREBP-1–Dependent Pathway**

EGFRvIII is a constitutively active, truncated form of EGFR lacking the ligand-binding domain of the receptor (9, 33). Although this mutant receptor may differ from the wild-type receptor in potentially important ways, it has been shown that stimulation of wild-type EGFR activates many of the same pathways as does EGFRvIII, including PI3K signaling (9, 13, 14, 29). To establish the kinetics of EGFR-mediated LDLR upregulation, and to assess whether it is a function of EGFR signal strength, we stimulated U87MG cells stably overexpressing wild-type EGFR with EGF and assessed the time course of LDLR expression. Increased LDLR mRNA was detectable within 30 minutes of EGF stimulation (Supplementary Fig. S1A), resulting in increased LDLR protein expression detectable 2 hours after stimulation (Fig. 1E). To assess the effect of EGFR signal strength on LDLR expression, we treated U87-EGFR GBM cells with a range of EGF doses and performed immunoblot analysis. As shown in Supplementary Fig. S1B, EGF stimulation led to a dose-dependent increase in LDLR expression. To further confirm the relationship between EGFR activation and LDLR expression, we infected another GBM cell line, LN229, with EGF, or a kinase-dead version of EGFR, under the control of a doxycycline-regulatable promoter. Doxycycline treatment produced a dose-dependent increase in EGFR expression, phosphorylation, and LDLR expression. These changes were not seen in LN229 cells expressing kinase-dead EGFR (Supplementary Fig. S2). The results show that signal flux through EGFR can promote LDLR expression in GBM cells.

We used a pharmacologic approach to determine whether PI3K signaling downstream of EGFRvIII/EGFR was required for regulation of LDLR expression. Erlotinib (10 μM), LY294002 (10 μM), and Akt 1/2 (5 μM) treatment, targeted to block EGFR, PI3K, and Akt signaling respectively, each produced potent suppression of EGFR-mediated SREBP-1 cleavage and LDLR expression (Fig. 1F). In contrast, the allosteric mTOR inhibitor rapamycin did not suppress EGFR-mediated LDLR expression (Fig. 1F), nor did it suppress SREBP-1 cleavage. This observation is consistent with our previous finding that EGFR signaling through PI3K/Akt promotes SREBP-1 activation in a rapamycin-insensitive fashion (14). The correlation between changes in SREBP-1 cleavage and LDLR expression suggested that SREBP-1 may play a role in linking EGFRvIII/EGFR signaling with LDLR expression, as it does in linking EGFRvIII with fatty acid synthesis (14). To test this hypothesis, SREBP-1 was knocked down in U87-EGFRvIII cells, using a short hairpin RNA (shRNA) lentivirus. This treatment led to a dose-dependent reduction in LDLR expression (Fig. 1G). To confirm that EGFR/Akt signaling regulates LDLR mediated by SREBP-1, U87/EGFR cells were stimulated with EGF, and the results showed that LDLR levels were markedly reduced after knocking down SREBP-1, using siRNA (Fig. 1H). In the liver, LDLR gene transcription is primarily under the control of SREBP-2 (34, 35), although it has also been reported to be

**Results**

**EGFRvIII Upregulates LDLR in Glioblastoma In Vivo Models**

To determine whether EGFRvIII promotes LDLR expression in vivo, we analyzed LDLR expression in tumors arising from implantation of U87MG GBM cells, with low expression of endogenous EGFR, or their isogenic counterpart, which stably express high levels of EGFRvIII. The U87MG/U87MG-EGFRvIII model system has been used extensively to study the molecular effects of EGFRvIII on GBM (9, 13, 14, 29, 30) and has been shown to faithfully recapitulate key molecular features of a patient with amplified and overexpressed EGFRvIII, including enhanced PI3K pathway activation (13, 14, 29, 30). Immunoblot analysis of EGFRvIII-expressing GBMs showed abundant LDLR expression, but expression was scarcely detectable in tumors arising from parental U87MG cells (Fig. 1A). To confirm that the elevated LDLR expression was dependent on EGFRvIII signaling, mice bearing EGFRvIII-expressing tumors were treated with the EGFR inhibitor erlotinib (150 mg/kg) for 7 days. Erlotinib treatment inhibited EGFR and Akt phosphorylation, and induced potent suppression of LDLR expression (Fig. 1B). To confirm that these findings were not unique to the U87-EGFRvIII model, we analyzed GBM39 xenografts (31). This tumor is a model of endogenous EGFRvIII expression and EGFR gene amplification that is derived from serial subcutaneous passage of a human GBM (31, 32). Consistent with the U87/EGFRvIII GBM model, LDLR expression in vivo was suppressed by erlotinib treatment (Fig. 1C). As a whole, these results show that EGFRvIII signaling can promote LDLR expression in GBMs in vivo.

Currently, the role of cholesterol metabolism in EGFR/PI3K-activated tumors and its potential therapeutic targetability are unknown.

Cholesterol metabolism in mammals is controlled through the coordinated action of SREBP and liver X receptor (LXR) transcription factors (24–26). SREBPs promote the expression of genes involved in cholesterol synthesis and enhance the uptake of extracellular cholesterol by inducing expression of the low-density lipoprotein receptor (LDLR). LXRs respond to excess cellular cholesterol by promoting ABCA1- and ABCG1-dependent cholesterol efflux and by inhibiting LDLR protein expression through induction of the E3 ubiquitin ligase inducible degrader of LDLR (IDOL) (27). We previously showed that limiting intracellular sterol availability by pharmacologically driving the LXR pathway inhibits the proliferation of rapidly dividing cell types, such as lymphocytes (28). However, the potential relevance of this pathway for cancer cell biology remains to be determined.

In our investigation, we performed integrative studies on GBM cell lines, xenograft models, and GBM clinical samples, including those from patients treated with a new EGFR tyrosine kinase inhibitor, lapatinib. Our studies reveal that GBM expression of the LDLR is driven by EGFRvIII/PI3K signaling in an SREBP-1–dependent manner and that EGFRvIII promotes enhanced dependence on LDLR uptake for tumor growth and survival. Further, we show that pharmacologic activation of LXR potently induces tumor cell death in vivo; an effect highly correlated with decreased LDLR protein expression and increased ABCA1-dependent cholesterol efflux. Taken together, these results suggest an important role for exogenous cholesterol uptake in GBM pathogenesis, pointing to an alternative pharmacologic strategy for targeting EGFRvIII-expressing GBMs and possibly other PI3K-hyperactivated cancers.

**References**

responsive to SREBP-1a in certain contexts, especially those in which SREBP-1a is highly expressed (34, 36–38). In the GBM cell system used in our work, lentiviral SREBP-2 shRNA knockdown did not result in suppression of LDLR expression (Fig. 11).

Although these results do not exclude a role for SREBP-2 in LDLR regulation in GBM cells, they suggest that EGFR/IIl EGFR signaling through PI3K/Akt promotes LDLR expression in a primarily SREBP-1-dependent manner.

Figure 1. EGFR signaling regulates cholesterol metabolism. A,atched P13K/Akt-mediated, SREBP-1-dependent pathway. A, a total of $5 \times 10^5$ U87MG and U87MG/EGFRvIII cells were implanted into the flank of immunodeficient SCID/Beige mice. After 21 days, tumors were harvested and immunoblot analysis was performed using the indicated antibodies. B, U87/EGFRvIII cells were treated for 7 days with erlotinib (150 mg/kg by oral gavage) and harvested; immunoblot analysis was performed using the indicated antibodies.

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EGFR/PI3K/Akt Signaling and Nuclear SREBP-1 Staining Correlate with Elevated LDLR Expression in GBM Patient Samples

To assess the potential clinical relevance of our observations, we performed correlation analysis of immunohistochemical staining patterns of phosphorylated EGFR (p-EGFR), p-Akt, nuclear SREBP-1, and LDLR in tumor and adjacent normal tissue from 140 GBM patients on 2 tissue microarrays (TMA). Staining of p-EGFR, p-Akt, nuclear SREBP-1, and LDLR was markedly elevated in tumor tissue of GBM patients relative to adjacent normal brain tissue \( (P < 0.001; \text{Fig. 2A}) \), with LDLR expression detected in

**Figure 2.** EGFR/PI3K/Akt signaling and nuclear SREBP-1 staining correlate with elevated LDLR expression in GBM patient samples. **A.** Analysis of p-EGFR, p-Akt, nuclear SREBP-1, and LDLR abundance (reddish brown) in 2 TMA s of 252 tumor cores and 91 matched normal tissues from 140 primary (de novo) GBM patients, using immunohistochemical analysis. Top, normal brain; middle, negative tumor (GBM A); and bottom, positive staining in a tumor (GBM B). The inset shows nuclear SREBP-1 staining indicated by the arrowheads. Images are magnified 20×; tissue is counterstained with hematoxylin. Scale bar, 20 μm. **B.** Quantitative analysis of LDLR expression in GBM versus adjacent normal tissues. **C.** Correlation analysis between LDLR expression and p-EGFR, p-Akt, or SREBP-1 in 140 GBM patients. **D.** Correlation analysis between LDLR expression and p-EGFR/p-Akt/SREBP-1 signaling pathway in 140 GBM patients. **E.** Immunohistochemical staining of p-EGFR, p-Akt, SREBP-1, and LDLR before and after 7 to 10 days of lapatinib treatment in 2 representative GBM patients. Scale bar, 20 μm.
85.7% of GBM tumor samples (Fig. 2B). LDLR staining was significantly enriched in tumors coexpressing p-EGFR, being detected in 97% of p-EGFR–positive tumors. LDLR expression was also significantly correlated with p-Akt and nuclear SREBP-1 (Fig. 2C and D). Correlation analysis cannot prove causality; therefore, we attempted to validate the causal relationship between EGFR signaling and LDLR expression identified in the GBM xenograft and cell line models (Fig. 1) by analyzing pre- and posttreatment tumor tissue that was available from 2 GBM patients treated with the EGFR/Her2 inhibitor lapatinib as part of a phase II clinical trial. We have previously shown that lapatinib inhibited EGFR/P13K signaling and SREBP-1 nuclear staining in these patients (14). Tumor tissue from 2 patients was obtained at baseline and after 7 to 10 days of lapatinib treatment. As shown in Fig. 2E, we detected decreased LDLR expression after lapatinib treatment, in association with decreased p-EGFR, p-Akt, and nuclear SREBP-1 staining (Fig. 2E). These clinical data are consistent with the model that EGFR signaling through the P13K pathway promotes LDLR expression in a SREBP-1–dependent manner.

We detected LDLR expression in some tumors that did not stain positive for p-EGFR. However, these samples showed evidence for P13K pathway activation, as determined by p-Akt staining (Fig. 2C). Therefore, other P13K pathway–activating lesions commonly found in GBM, such as the activation of other RTKs (6, 12, 39), could also promote increased LDLR expression. To test this hypothesis, we performed immunohistochemical analysis of p-PDGFR-beta and p-Met staining on the TMAs. We observed a substantial correlation between p-PDGFR-beta and p-MET, and LDLR staining (P < 0.001; Supplementary Figs. S3A–D and S4). To investigate the mechanistic basis for this finding, we tested the effect of the Met ligand HGF on SREBP-1 cleavage and LDLR expression. We observed the cell line that expresses relatively little EGFR but abundant levels of c-Met, HGF stimulated Met phosphorylation and promoted SREBP-1 cleavage and LDLR expression (Supplementary Fig. S3E). Taken together, these results indicate that EGFR signaling through Akt is associated with nuclear SREBP-1 and LDLR expression in GBM patients, and other P13K-activating RTKs can also potentially promote LDLR expression.

**GBM Cells Depend on Extracellular Cholesterol Levels for Growth**

Having shown that EGFRvIII/EGFR signaling promotes LDLR expression, we endeavored to determine whether LDL was required for GBM proliferation and survival. We measured the effect of depleting LDL from the media on GBM cell growth and survival. U87MG and U87MG-EGFRvIII GBM cells were cultured in lipoprotein-deficient serum (LPDS), and the effects on tumor proliferation and viability were measured. Sixty percent growth inhibition was detected in EGFRvIII-expressing GBM cells; only half as much was seen in parental U87 cells (Fig. 3A). Cell death was also significantly induced in LPDS (Fig. 3B). The addition of LDL to the LPDS medium returned GBM cell proliferation to baseline (Fig. 3C). In contrast, no effect of LDL addition was seen in tumor cells cultured in FBS medium (Fig. 3C). Overall, these results show that U87-GBM cells depend on LDL for optimal proliferation and survival, and suggest that EGFRvIII confers an enhanced requirement for cholesterol uptake.

**The LXR Agonist GW3965 Promotes GBM Cell Death In Vitro with Enhanced Efficacy in EGFRvIII-Expressing Tumor Cells**

Intracellular cholesterol levels can be regulated through (1) uptake of LDL through LDLR (40, 41), (2) efflux of cholesterol through ABCA1 and ABCG1 transporters (25, 42), and (3) hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase)–dependent synthesis (24, 25, 34, 43, 44). Given the ability of pharmacologic LXR activation to limit intracellular cholesterol availability (28), we hypothesized that synthetic LXR agonists might inhibit the growth and survival of GBM cells.

Indeed, treatment of U87 and U87-EGFRvIII GBM cells for 4 days with the LXR agonist GW3965 resulted in dose-dependent inhibition of growth and promotion of tumor cell death. Moreover, consistent with the enhanced dependence of EGFRvIII-bearing tumor cells on exogenous cholesterol, these cells exhibited markedly greater cell death than did those of the parental U87 cell line (Fig. 4A–D). Remarkably, tumor cell death was dose dependently rescued by the addition of LDL (Fig. 4E–G), strongly suggesting that the tumoricidal effects of GW3965 were mediated through alteration of cellular cholesterol availability.

To uncover the mechanism by which GW3965 induced tumor cell death, real-time PCR and immunoblot analyses for the LXR target genes ABCA1 and IDOL were performed. GW3965 treatment promoted dose-dependent increases in ABCA1 and IDOL, with a concomitant decrease in LDLR protein level (Fig. 5A–D; Supplementary Fig. S5). Unfortunately, no antibodies capable of detecting endogenous IDOL expression are available (27). The regulation of cholesterol efflux via ABCA1 is a 1-step process; ABCA1 is a direct transcriptional target of LXR (25). In contrast, LDLR regulation by LXR requires transcription and translation of IDOL, followed by ubiquitin-mediated degradation of LDLR (27). GW3965-mediated LDLR degradation in GBM cells took longer, and required a higher drug dose than did ABCA1 induction (Fig. 5C and D). The effects of GW3965 on ABCA1 and LDLR expression were confirmed across a panel of GBM and other cancer cell lines for which LDLR levels were linked with high levels of EGFR phosphorylation (Fig. 5E). Of interest, the dose of GW3965 required to promote cell death (Fig. 4B–D) correlated well with that required to accomplish LDLR degradation (Fig. 5C and D). Together, these results suggest that decrease of LDLR levels is required for the tumoricidal activity of GW3965.

To directly test whether LDLR degradation was required for GBM cell death in response to GW3965, we measured the effect of lentiviral LDLR shRNA knockdown, or scrambled control, on sensitivity to the drug. Low-dose GW3965 (1 or 2 μM) induced ABCA1 but did not diminish LDLR expression or cause GBM cell death (Fig. 6A–C). Lentiviral delivery of LDLR shRNA resulted in LDLR knockdown, potently promoting tumor cell death upon low-dose GW3965 treatment (Fig. 6A–C). To examine the role of IDOL-mediated LDLR degradation in promoting this apoptotic response (Fig. 6D),
Figure 3. GBM cells depend on extracellular cholesterol levels for growth. A and B, U87 and U87/EGFRvIII cells were cultured in 1% FBS or 1% LPDS medium for 3 days; live and dead cells were counted using trypan blue assay. Relative cell growth was calculated by comparing cell number with cells in 1% FBS condition (A). Dead cell percentage was calculated by comparing dead cell number with total cell amount (B). C, U87 and U87/EGFRvIII cells were cultured in 1% and 5% of FBS or LPDS medium; LDL was added to the cultured media (5 μg/mL, 1:1,000 dilution) for 3 days. Cell number was counted using a hemocytometer. D, micrographs showing morphologic differences in U87 and U87/EGFRvIII cells cultured in 5% FBS and 5% LPDS medium; addition of LDL (5 μg/mL) recovered cell phenotype and cell growth, LDL stock 5 mg/mL. Scale bar, 20 μm.
Figure 4. The LXR agonist GW3965 promotes GBM cell death in vitro with enhanced efficacy in EGFRvIII-expressing tumor cells. A. U87 and U87/EGFRvIII cells were treated with the LXR agonist GW3965 for 4 days in 1% LPDS medium; cell viability was measured using a WST-1 assay daily and normalized with day 0. Indicated cell survival percentage was calculated by normalizing with control at day 4. B and C, U87 and U87/EGFRvIII cells were treated with GW3965 for 2 days in 1% LPDS medium; cells were fixed using paraformaldehyde for 15 minutes at room temperature. Cell apoptosis was assayed by staining with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; Roche) and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). DMSO, dimethyl sulfoxide. Scale bars, 20 μm. D, apoptotic cells in B and C were quantified. E, U87/EGFRvIII cells were treated using GW3965 for 4 days in 1% LPDS medium, with or without LDL (50 μg/mL). The images represent cell morphology change after GW3965 treatment. Scale bar, 20 μm. F and G, dead cells and live cells were counted in E using trypan blue. LDL was added into media at a range of doses, as indicated. LDL stock, 5 mg/mL.
we measured the effect of adenoviral delivery of IDOL on sensitizing U87-EGFRvIII GBM cells to low-dose GW3965. Phenocopying the effect of LDLR knockdown, IDOL overexpression potently sensitized GBM cells to low-dose GW3965 (Fig. 6E and F). Neither LDLR knockdown alone nor IDOL overexpression alone was sufficient to promote GBM cell death (Fig. 6). These findings indicate that IDOL-mediated degradation of LDLR is an important component of the mechanism of GW3965-induced GBM cell death. However, the observation that targeting LDLR alone is not sufficient to elicit GBM cell death indicates that additional mechanisms, such as the promotion of ABCA1-dependent cholesterol efflux, also contribute.

**LXR Agonist Inhibits GBM Tumor Growth In Vivo**

To test the therapeutic potential of LXR agonists in the treatment of GBM, we determined the efficacy of GW3965 at blocking growth and promoting tumor cell death in vivo. U87/EGFRvIII cells were implanted s.c. in mice that were then treated with GW3965 (40 mg/kg daily by oral gavage) for 12 days. GW3965 treatment strongly induced ABCA1 expression and reduced LDLR expression (Fig. 7A). Remarkably, this activity was accompanied by a 59% inhibition of tumor growth (Fig. 7B and C) and a 25-fold increase in GBM cell apoptosis (Fig. 7D and E). These data show that an LXR agonist potently inhibits GBM growth and promotes tumor cell death in vivo.

**DISCUSSION**

Cholesterol is needed for the biogenesis and maintenance of fluidity of cell membranes (41, 44). It is also a central component of lipid rafts, specialized microdomains of the plasma membrane that serve as organizing centers for the assembly of signaling molecules (45–47). Therefore, rapidly proliferating cancer cells with highly activated signal transduction networks, such as GBM cells, are likely to have an enhanced requirement for cholesterol (48–50). However, the molecular mechanisms by which GBM cells obtain sufficient cholesterol and the potential therapeutic targetability of this process are not well understood. In our investigation, through integrated analyses in GBM cell lines, xenograft models, and GBM clinical samples, including those from patients treated with the EGFR tyrosine kinase inhibitor lapatinib, we have uncovered an EGFRvIII-activated, PI3K/SREBP-1–dependent tumor survival pathway involving LDLR. The present studies...
begin to shed light on the molecular mechanism by which an oncogene and its signal transduction effectors alter the metabolic circuitry to meet the enhanced tumor cell demand for cholesterol.

Most attempts to target cholesterol metabolism in cancer have focused on the use of the statin class of HMG-CoA reductase inhibitors that block the rate-limiting step in de novo cholesterol synthesis (51, 52). In noncancerous cells, the transcription factors SREBP and LXR maintain cholesterol homeostasis through complementary pathways of feedback inhibition and feed-forward activation. Thus, LDLR expression is suppressed by high cellular cholesterol levels through both inactivation of SREBPs and activation of the LXR-IDOL axis (27). We have shown in this article that GBM cells have devised a mechanism to subvert the normal pathways for feedback inhibition via the EGFRvIII

Figure 6. Reduction of LDLR protein levels is required for apoptotic effect of GW3965. A, U87/EGFRvIII cells were infected with LDLR shRNA lentivirus or scrambled control and selected by puromycin with 2 μg/mL for 10 days. Cells were treated with GW3965 at a range of doses, as indicated, for 48 hours. Immunoblot analysis was performed using the indicated antibodies. B and C, U87/EGFRvIII control shRNA and LDLR knockdown cells were treated with GW3965 at a range of doses, as indicated, for 3 days in 1% FBS medium. The representative images show cell morphology after GW3965 treatment (B); then cells were counted using the trypan blue assay (C). Scale bar, 20 μm. D, U87/EGFRvIII cells were infected by adenovirus Ad-LacZ or Ad-IDOL for 48 hours. Immunoblot analysis was performed using LDLR antibody. MOI, multiplicity of infection. E and F, U87/EGFRvIII cells were infected with Ad-LacZ or Ad-Idol for 8 hours, then treated with GW3965 in a range of doses, as indicated, for 3 days. Representative images after GW3965 treatment (E); tumor cells were counted using trypan blue assay (F). Scale bar, 20 μm.
and PI3K-dependent activation of SREBP-1. Twenty years ago, Rudling and colleagues (53) detected elevated LDL binding and LDLR expression in GBM relative to normal brain. However, the molecular basis for elevated LDLR expression—and its potential therapeutic implications, including the potential effect of sensitivity to statins—has not been tested. In this article, we show that constitutive EGFRvIII/PI3K-signaling through SREBP-1 results in unstrained LDLR expression (Fig. 1), thus potentially rendering tumor cells resistant to HMG-CoA reductase inhibitors (14). Consistent with this model, in the absence of extracellular cholesterol, atorvastatin significantly inhibited the growth and promoted cell death of GBM cells (Supplementary Fig. S6). These findings provide an explanation for why many tumor cells are resistant to statin treatment, and suggest alternative routes toward targeting cholesterol homeostasis in cancer.

Besides cholesterol, LDL also contains Apo B-100, fatty acids, and phospholipids (54), raising the possibility that factors in addition to cholesterol may be required by GBMs for optimal growth. Although we cannot formally exclude this possibility, we observed that overexpression of IDOL, which decreases LDLR expression (Fig. 7D), in combination with atorvastatin treatment, which inhibits endogenous cholesterol synthesis, shows remarkable antitumor synergy, although neither agent is effective alone (Supplementary Fig. S7). These data suggest that cholesterol is the critical ingredient of LDL required by GBM cells and that enhanced ability to take up exogenous cholesterol through LDLR renders statins ineffective.

Figure 7. GW3965 treatment inhibited tumor growth in vivo. A, a total of $5 \times 10^5$ U87/EGFRvIII cells were implanted into the flank of immunodeficient SCID/Beige mice ($n = 8$ per group); after tumor size reached 80 mm$^3$, GW3965 was administered at 40 mg/kg by oral gavage daily for 12 days. Tumors were harvested, and immunoblot analyses were performed using the indicated antibodies. B, tumor size was measured daily. Tumor growth was demonstrated by fold change compared with the tumor size on first day of treatment, $P < 0.05$. The experiments were repeated twice. C, representatives of U87/EGFRvIII xenograft tumors after GW3965 treatment for 12 days. D, tumor tissues were stained by TUNEL assays (Roche) to determine cell apoptosis after GW3965 treatment. Scale bar, 20 μm. E, quantification of TUNEL staining. F, pathways by which EGFR/RTK signaling mediates cholesterol metabolism and the therapeutic drug targets. The schematic shows that EGFR/PI3K/Akt signaling regulates LDLR mediated by SREBP-1; GBM cells maintain cholesterol homeostasis through uptake, biosynthesis, and efflux. It also indicates that the LXR agonist GW3965 disturbs cancer cell cholesterol homeostasis by upregulating efflux through transporter gene ABCA1 and by reducing LDL uptake through degrading LDLR mediated by IDOL.
PI3K signaling is hyperactivated as a consequence of RTK amplifications and activating mutations, PTEN loss, PI3K point mutations, and other genetic lesions, providing a core oncogenic pathway in many cancers, including up to 90% of GBMs (6, 8, 55). EGFR amplification and EGFRvIII-activating mutation are the most common oncogenes promoting PI3K signaling in GBM (1, 6). However, other RTKs that can be coexpressed in GBM, including some that may be upregulated after EGFR inhibitor therapies, like c-MET, PDGFR-alpha, and PDGFR-beta, can also engage PI3K signaling, resulting in EGFR inhibitor resistance (12). This observation prompted us to determine whether other PI3K-activating RTKs also promote LDLR expression. Consistent with this model, we detected a strong correlation between c-MET and PDGFR-beta expression and SREBP-1 and LDLR (Supplementary Fig. S3A–D). More importantly, addition of HGF can potently stimulate SREBP-1 cleavage and LDLR expression in c-MET-expressing GBM cells (Supplementary Fig. S3E), suggesting that other PI3K-activating lesions can also promote LDLR expression. These results broaden the potential spectrum of tumors that may be susceptible to anti-LDLR-mediated therapies, including LXR agonists. Furthermore, the PI3K pathway is hyperactivated not only in GBM but also in many other cancers, including breast, ovarian, endometrial, lung, prostate, renal, and lymphocyte (5, 56–58). Therefore, we hypothesize that the mechanisms discovered in our work on GBM may be relevant to many PI3K-driven cancers. Future studies will be needed to determine whether PI3K hyperactivation promotes enhanced LDLR expression and dependence on LDL in other cancers and whether this is a targetable mechanism across multiple cancer types.

mTORC1 appears to be critical for linking PI3K signaling with tumor metabolism (16, 59, 60). SREBP-1 expression and/or activity is regulated by PI3K/Akt signaling through mTORC1 in hepatocytes (61), in mouse embryonic fibroblasts (62), and in Drosophila (63). Further, mTORC1 activation of SREBP-1 has been shown to be essential for regulating lipid and sterol biogenesis (62). However, these studies have been conducted largely in noncancerous cells; the role of mTORC1 in regulating SREBP-1 and cellular metabolism in cancer remains to be elucidated.

Surprisingly, we have found that SREBP-1 activation is rapamycin insensitive, calling into question its regulation by mTOR in GBM. In preclinical models (Fig. 1F) and in GBM patients treated with rapamycin (14, 64), we have shown that SREBP-1 activation, and consequent LDLR expression, are rapamycin resistant (Fig. 1F). Two potential explanations for these results are possible. PI3K signaling to SREBP-1 may not require mTOR, perhaps owing to an alteration in the molecular circuitry linking Akt with SREBP-1 in cancer cells. Alternatively, SREBP-1 activity may be mTOR dependent, but rapamycin insensitive, because of incomplete inhibition of either mTORC1 or mTORC2 signaling. Further studies are needed to determine whether SREBP-1 is regulated by mTOR in cancer, to dissect its metabolic consequences, and to ascertain whether mTOR kinase inhibitors can block PI3K/Akt-mediated lipogenesis through SREBP-1.

The nuclear receptor LXR emerges from these studies as a potential adjuvant drug target in GBM. Although we have previously shown that forced activation of the LXR pathway with highly efficacious synthetic agonists inhibits the growth of rapidly dividing primary cells, the relevance of this effect for transformed cells has not been investigated. In this article we show that the synthetic LXR agonist GW3965 potently suppresses GBM growth and induces apoptosis in a mouse model (Fig. 7), and we demonstrate enhanced efficacy in EGFRvIII-expressing GBM cells (Fig. 4). Interestingly, we find that IDOL-mediated degradation of LDLR is necessary, but not sufficient, to induce GBM cell apoptosis (Fig. 6). Because cellular cholesterol levels depend on the integrated activities of the uptake, efflux, and synthesis pathways (44), LXR agonists may be highly beneficial because of their ability to coordinately target 2 of the 3 aspects of cholesterol regulation (27). Such drugs not only block exogenous LDL uptake but also actively promote cholesterol removal from cells and intracellular distribution out of the endoplasmic reticulum (28).

Pharmacokinetic and toxicity studies have shown that GW3965 may induce elevated hepatic triglycerides (25). Therefore, new synthetic LXR agonists are being developed that similarly activate LXR without producing the same degree of hepatic triglyceride induction. The fatty acid synthase inhibitor C75 promoted an additive antitumor growth effect when administered with GW3965, suggesting a potential role for combination therapy (Supplementary Fig. S8). Future studies will be needed to assess the efficacy and clinical utility of those compounds as potential clinical candidates as they become available for testing.

In summary, our integrated studies in GBM cell lines, mouse models, and human clinical trial samples have delineated an EGFRvIII-activated, PI3K/SREBP-1–dependent tumor survival pathway through LDLR (Fig. 7F). Our data also suggest that the LXR-IDOL-LDLR axis is a common targetable pathway in multiple tumor types (Fig. 5E; Supplementary Fig. S5). Consistent with this hypothesis, activation of LXR in different types of cancer cell lines resulted in significant cell death (Supplementary Fig. S9), raising the possibility that this axis may be a compelling drug target in multiple cancers. Further delineation of the molecular mechanisms by which PI3K signaling differentially regulates tumor cell metabolism will inform a better understanding of the links between genetic alterations and cellular metabolism in cancer, and may potentially lead to more effective, less toxic treatments.

**METHODS**

**Cell Lines**

U87 and U87-EGFRvIII, U87-EGFR, U87-EGFR-PTEN isogenic GBM cell lines; A431 epidermoid carcinoma cell line; and LN229, T98 GBM cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Cellgro) supplemented with 10% FBS (Omega Scientific) in a humidified atmosphere of 5% CO₂, 95% air at 37°C. U87-EGFRvIII cells were a kind gift from Dr. Webster Cavenee. U87-EGFR cells were generated by retrovirus-mediated transduction of wild-type EGFR into U87 cells followed by selection of stable clones. U87-EGFR-PTEN cells were generated by plasmid-mediated transfection of PTEN into U87-EGFR cells followed by selection for stable clones. These cell lines have previously been reported (9, 29). The H1975 non–small-cell lung carcinoma cell line was cultured in RPMI 1640 with 10% FBS, and the HeLa cervical cancer cell line was cultured in DMEM with 10% FBS. Both are from American Type Culture Collection.
Antibodies and Reagents

Antibodies were used against the following: LDLR, ABCA1, ABCG1 (Abcam); p-Akt Ser473, Akt, p-S6 Ser235/236, p-Met Tyr1234, p-PDGFR Tyr1021 (Cell Signaling); β-actin (Sigma-Aldrich); SREBP-1 and SREBP-2 (BD Pharmingen); p-Met Tyr1349, p-PDGFR Tyr579 (Abgent); EGF/R/EGFRvIII cocktail antibody (Upstate); and p-EGFR Tyr1086 (Invitrogen). Reagents used were as follows: erlotinib (ChemieTr, LY294002, rapamycin, Polybrene, puromycin (Sigma-Aldrich); Akt-1/2 (Calbiochem); and atovastatin (Toronto Research Chemicals, Canada). GW3965 was gifted from GlaxoSmithKline. Also used were scramble shRNA, SREBP-1 shRNA, SREBP-1 siRNA, and SREBP-2 shRNA lentivirus (Santa Cruz); scramble shRNA, LDLR shRNA lentivirus (Sigma-Aldrich); and human LPDS and LDL (Intracel).

Cell Proliferation and Death Assays

The details are described in the Supplementary Material.

shRNA Assay

The details are described in the Supplementary Material.

Western Blotting

Western blotting is shown in the Supplementary Material and done as previously described (65).

Immunohistochemical Assay

Paraffin-embedded tissue blocks were sectioned using the University of California, Los Angeles, Department of Pathology and Histology and Tissue Array Core Facility. Immunohistochemical staining was performed as previously described and details are provided in the Supplementary Material (9, 64).

Tissue Microarrays

TMAs were used to analyze p-EGFR Tyr1086, p-Akt Ser473, nuclear SREBP-1, LDLR, p-MET, and p-PDGFR Tyr579 immunohistochemical staining in 140 GBM patient samples. TMAs enable tumor tissue samples from hundreds of patients to be analyzed on the same histologic slide. The details are described in the Supplementary Material and the procedure was done as previously described (14, 55).

Phase II Lapatinib Clinical Trial

The details are addressed in reference 14.

In Vitro Staining with Terminal Deoxynucleotidyl Transferase–Mediated DUTP Nick End Labeling

The details are addressed in the Supplementary Material and done as previously described (14).

Real-Time PCR

A total of 6 × 10^4 cells were seeded into 6-well plates in 5% FBS for 24 hours, then changed to 1% LPDS medium and treated with GW3965 in time course manner. Cells were washed once using PBS; then total RNA was extracted using TRizol reagent according to its protocol (Invitrogen). Next, 800 ng RNA was complementarily synthesized to cDNA and amplified using real-time PCR (Bio-Rad), and its values were normalized against the internal control gene 36B4 (RPLPO) for each replicate. The primers used were as follows: ABCA1 forward: 5′-AAAAGTGGTGTGGGGCCCTTTG-3′, reverse: 5′-GTTTCCGACCGTGGGATCTT-3′; IDOL forward: 5′-CGAGGACTCAGCTCAACA-3′, reverse: 5′-TGACGGTACAATGTAATGCTC-3′; 36B4 forward: 5′-ATGCGACGATCTACAAC-3′, reverse: 5′-TCGTTGGTACCGCGTGGTGA-3′.

Xenograft Model

Isogenic human malignant glioma cells (U87, U87-EGFRvIII) and human primary GBM model GBM39 (31, 32) were implanted into immunodeficient SCID/Beige mice for s.c. xenograft studies. SCID/Beige mice were bred and kept under defined-flora pathogen-free conditions at the American Association for Laboratory Animal Care-approved Animal Facility of the Division of Experimental Radiation Oncology, University of California, Los Angeles. The details are described in the Supplementary Material.

Immunohistochemistry and Image Analysis–Based Scoring

Tissue sections were cut from blocks of formalin-fixed paraffin tumor tissue from TMA or GBM patients treated with lapatinib. Tumor specimens were obtained according to a protocol approved by the Institutional Review Board of University of California, Los Angeles. The first set of paired pre- and posttreatment tumor tissues for the lapatinib trial was examined. The details are described in the Supplementary Material, and the process was done as previously described (14, 64).

Statistical Analysis

Results are shown as mean ± SEM. Fisher’s exact test was used to assess correlations between various molecular markers. Other comparisons in cell-growth assays, tumor volumes, tumor metabolism, and cell death were performed using the 2-tailed t test as well as by ANOVA, as appropriate. P < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

P. Tontonoz is an investigator at the Howard Hughes Medical Institute. M.P. Mehta has served as a consultant to Adnexus, Bayer, Genentech, Merck, Schering Plough, and Tomotherapy; he serves on the Board of Directors of Pharmacys, and as an advisor to Stemina, and is on the Data Safety Monitoring Board for Apogenix. He holds stock options in Colby, Procuritis, Pharmacys, and Tomotherapy. No other potential conflicts of interest were disclosed.

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REFERENCES

EGFR Signaling Regulates Cholesterol Metabolism


Correction: An LXR Agonist Promotes Glioblastoma Cell Death through Inhibition of an EGFR/AKT/SREBP-1/LDLR–Dependent Pathway

In this article (Cancer Discovery 2011;1:442–56), which was published in the October 2011 issue of Cancer Discovery (1), the following errors appear in the Results section. These changes do not affect any of the data presented in the manuscript or the conclusions. The authors regret these errors. The corrected passages are provided below:

Results, page 444, second column, 2nd paragraph:
Erlotinib (10 μM), LY294002 (20 μM), and Akti 1/2 (5 μM) treatment, targeted to block EGFR, PI3K, and Akt signaling respectively, each produced potent suppression of EGF-mediated SREBP-1 cleavage and LDLR expression (Fig. 1F).

Results, page 445, Figure 1E legend:
The following text was deleted: P, the precursor of SREBP-1; N, the N-terminus of SREBP-1, which is the active form.

The correct text is as follows:
U87/EGFR cells were placed in serum-free medium for 24 hours and stimulated with EGF (20 ng/mL) for the indicated times; then immunoblot analysis was performed using the indicated antibodies.

Results, page 445, Figure 1I legend:
shRNA lentiviral knockdown of SREBP-2 had no effect on LDLR levels. Immunoblot analysis was performed using the indicated antibodies. P, the precursor of SREBP-2; C, the C-terminus of SREBP-2.

In addition, the Western blot in Fig. 1I was mislabeled due to a production error. The corrected panel appears on the next page.
Figure 1. EGFRvIII/EGFR signaling promotes LDLR expression through a PI3K/Akt-mediated, SREBP-1-dependent pathway. A, a total of $5 \times 10^5$ U87MG and U87MG/EGFRvIII cells were implanted into the flank of immunodeficient SCID/Beige mice. After 21 days, tumors were harvested and immunoblot analysis was performed. M, the mature form of LDLR, which is glycosylated; P, the precursor of LDLR, nonglycosylated. B, U87/EGFRvIII tumors were treated for 7 days with erlotinib (150 mg/kg by oral gavage) and harvested; immunoblot analysis was performed using the indicated antibodies. C, GBM39 cells, a human serially passaged model of endogenous EGFRvIII expression, were implanted in the flanks of SCID mice and treated for 7 days with erlotinib (150 mg/kg by oral gavage). Immunohistochemical analysis of p-EGFR and LDLR was performed using the indicated antibodies. D, Western blot was performed to analyze GBM39 tumor lysates, using indicated antibodies. E, U87/EGFR cells were placed in serum-free medium for 24 hours and stimulated with EGF (20 ng/mL) for the indicated times; then immunoblot analysis was performed using the indicated antibodies. F, effect of the EGF inhibitor erlotinib (10 μM), the PI3K inhibitor LY294002 (20 μM), the Akt inhibitor Akti-1/2 (5 μM), or the mTORC1 inhibitor rapamycin (1 nM) for 12 hours on SREBP-1 cleavage and LDLR protein levels in U87/EGFR cells. Cells were pretreated with inhibitors for 30 minutes before stimulation with EGF, 20 ng/mL; immunoblot analysis was performed using the indicated antibodies. G, U87/EGFRvIII cells were infected using SREBP-1 shRNA lentivirus for 48 hours at the indicated doses; immunoblot analysis was performed using the indicated antibodies. H, U87/EGFR cells were transfected using SREBP-1 siRNA (10 nM) for 24 hours; then serum-free overnight, cells were stimulated with EGF (20 ng/mL) for 16 hours. Immunoblot analysis was performed using the indicated antibodies. I, shRNA lentiviral knockdown of SREBP-2 had no effect on LDLR levels. Immunoblot analysis was performed using the indicated antibodies. P, the precursor of SREBP-2; C, the C-terminus of SREBP-2.

REFERENCE


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