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

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Abstract

Glioblastoma (GBM) is the most common malignant primary brain tumor of adults and one of the most lethal of all cancers. EGFR mutations (EGFRvIII) and PI3K hyperactivation are common in GBM, promoting tumor growth and survival, including through 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. Here, studies in GBM cell lines, xenograft models and GBM clinical samples, including from patients treated with the EGFR tyrosine kinase inhibitor lapatinib, uncovered an EGFRvIII-activated, PI3K/SREBP-1-dependent tumor survival pathway through the LDL receptor. Targeting LDLR with the Liver X Receptor (LXR) agonist GW3965 caused IDOL (Inducible Degrader Of LDLR)-mediated LDLR degradation and increased expression of the ABCA1 cholesterol efflux transporter, potently

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promoting tumor cell death in an in vivo GBM model. These results demonstrate that EGFRvIII can promote tumor survival through PI3K-SREBP-1 dependent up-regulation of LDLR, and suggest a role for LXR agonists in the treatment of GBM patients.

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 median patient survival of 12–15 months from initial diagnosis, despite aggressive therapy (3). Therefore, new treatment strategies are needed (4). The 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 EGFR amplification and activating mutation (EGFRvIII), and 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 (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, including GBM (13, 14, 17, 20). We recently showed that mutant EGFRvIII expressing GBMs promote lipogenesis through PI3K-dependent activation of the master transcriptional regulator SREBP-1, and we demonstrated 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. 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 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 LDL 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 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.

Here, we performed integrative studies in GBM cell lines, xenograft models and GBM clinical samples, including from patients treated with a new EGFR tyrosine kinase inhibitor lapatinib. Our studies demonstrate 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 LDL 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.

Results

EGFRvIII upregulates LDLR in GBM 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 that 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 (Figure 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 (Figure 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 (Figure 1C). Taken together, these results demonstrate that 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 demonstrated that stimulation of wild-type EGFR activates many of the same pathways as EGFRvIII, including PI3K signaling (9, 13, 14, 29). To establish the kinetics of EGFR-mediated LDLR up-regulation, and to assess whether it is a function of EGFR signal strength, we stimulated U87MG cells stably over-expressing 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 Figure 1A), resulting in increased LDLR protein expression detectable two hours after stimulation (Figure 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 supplemental Figure 1B, 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 EGFR, or a kinase-dead version of EGFR, under the control of a doxycycline-regulatable promoter. Doxycycline treatment resulted in a dose-dependent increase in EGFR expression, phosphorylation and LDLR expression. These changes were not seen in LN229 cells expressing kinase-dead EGFR (Supplemental Figure 2). These results demonstrate that signal flux through EGFR can promote LDLR expression in GBM cells.
We used a pharmacological approach to determine whether PI3K signaling downstream of EGFRvIII/EGFR was required for regulation of LDLR expression. Erlotinib (10 uM), LY294002 (10 uM) and Akti 1/2 (5 uM) treatment, targeted to block EGFR, PI3K and Akt signaling respectively, each produced potent suppression of EGF-mediated SREBP-1 cleavage and LDLR expression (Figure 1F). In contrast, the allosteric mTOR inhibitor rapamycin did not suppress EGF-mediated LDLR expression (Figure 1F), nor did it suppress SREBP-1 cleavage. This 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 shRNA lentivirus. This treatment led to a dose-dependent reduction in LDLR expression (Figure 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 (Figure 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 responsive to SREBP-1a in certain contexts, especially those where SREBP-1a is highly expressed (34, 36–38). In the GBM cell system used here, lentiviral SREBP-2 shRNA knockdown did not result in suppression of LDLR expression (Figure 1I). Although these results do not exclude a role for SREBP-2 in LDLR regulation in GBM cells, they suggest that EGFRvIII/EGFR signaling through PI3K/Akt promotes LDLR expression in a primarily SREBP-1-dependent manner.

**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 p-EGFR, p-Akt, nuclear SREBP-1 and LDLR in tumor and adjacent normal tissue from 140 GBM patients on two tissue microarrays (TMAs). p-EGFR, p-Akt, nuclear SREBP-1 and LDLR staining were markedly elevated in tumor tissue of GBM patients relative to adjacent normal brain tissue (p<0.001) (Figure 2A), with LDLR expression detected in 85.7% of GBM tumor samples (Figure 2B). LDLR staining was significantly enriched in tumors co-expressing p-EGFR, being detected in 97% of p-EGFR positive tumors. LDLR expression was also significantly correlated with p-Akt and nuclear SREBP-1 staining (p<0.001) (Figure 2, C 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 (Figures 1) by analyzing pre- and post-treatment tumor tissue that was available from two 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/PI3K signaling and SREBP-1 nuclear staining in these patients (14). Tumor tissue from two patients was obtained at baseline and after 7–10 days of lapatinib treatment. As shown in Figure 2E, we detected decreased LDLR expression after lapatinib treatment, in association with decreased p-EGFR, p-Akt and nuclear SREBP-1 staining (Figure 2E). These clinical data are consistent with the model that EGFR signaling through the PI3K 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 PI3K pathway activation, as determined by p-Akt staining (p<0.001) (Figure 2C). Therefore, other PI3K 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) (Supplemental Figure 3, A–D; Supplemental Figure 4). To investigate the mechanistic basis for this finding, we tested the effect of the Met ligand HGF on SREBP-1 cleavage and LDLR expression. In U251 GBM cells, a cell line that expresses relatively little EGFR but expresses abundant levels of c-Met, HGF stimulated Met phosphorylation and promoted SREBP-1 cleavage and LDLR expression (Supplemental Figure 3E). Taken together, these results demonstrate that EGFR signaling through Akt is associated with nuclear SREBP-1 and LDLR expression in GBM patients, and other PI3K-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 (Figure 3B). The addition of LDL to the LPDS medium returned GBM cell proliferation to baseline (Figure 3, C and D). In contrast, no effect of LDL addition was seen in tumor cells cultured in FBS medium (Figure 3C). Taken together, these results demonstrate 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 or ABCG1 transporters (25, 42) and 3) 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 compared to the parental U87 cell line (Figure 4, A–D). Remarkably, tumor cell death was dose-dependently rescued by addition of LDL (Figure 4, E–G), strongly suggesting that the tumoricidal effects of GW3965 were mediated through altering 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 (Figure 5, A–D; Supplemental Figure 5). Unfortunately, there are no antibodies available capable of detecting endogenous IDOL expression (27). The regulation of cholesterol efflux via ABCA1 is a one 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 (Figure 5, C 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 (Figure 5E). Interestingly, the dose of GW3965 required to promote cell death (Figure 4, B–D) correlated well with that required to accomplish LDLR degradation (Figure 5, C and D). Taken 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 uM) induced ABCA1, but did not diminish LDLR expression or cause GBM cell death (Figure 6, A–C). Lentiviral delivery of LDLR shRNA resulted in LDLR knockdown, potently promoting tumor cell death upon low dose GW3965 treatment (Figure 6, A–C). To examine the role of IDOL-mediated LDLR degradation in promoting this apoptotic response (Figure 6D), 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 (Figure 6, E and F). Neither LDLR knockdown alone, nor IDOL overexpression alone, were sufficient to promote GBM cell death (Figure 6). Taken together, these results demonstrate 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 as treatment for GBM, we determined the efficacy of GW3965 at blocking growth and promoting tumor cell death in vivo. U87/EGFRvIII cells were implanted subcutaneously 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 (Figure 7A). Remarkably, this was accompanied by 59% inhibition of tumor growth (Figure 7, B and C), and a 25-fold increase in GBM cell apoptosis (Figure 7, D and E). These data demonstrate 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. Here, through integrated analyses in GBM cell lines, xenograft models and GBM clinical samples, including 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 non-cancerous 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 here that GBM cells have devised a mechanism to subvert the normal pathways for feedback inhibition via the EGFRvIII and PI3K-dependent activation of SREBP-1. Twenty years ago, Rudling and colleagues detected elevated LDL binding and LDLR expression in GBM relative to normal brain (53). 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. Here, we show that constitutive EGFRvIII/PI3K-signaling through SREBP-1 results in unrestrained LDLR expression (Figure 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 (Supplemental Figure 6). These findings provide an explanation for why many tumor cells are resistant to statin treatment, and suggest alternative routes towards targeting cholesterol homeostasis in cancer.

In addition to 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 (Figure, 7D), and in combination with atorvastatin treatment, which inhibits endogenous cholesterol synthesis, show remarkable anti-tumor synergy, although neither agent is effective alone (Supplemental Figure 7). These data suggest that cholesterol is the critical ingredient of LDL required by GBM cells, and that enhanced ability to take up exogenous cholesterol though LDLR renders statins ineffective.

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 co-expressed 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 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 expression (Supplemental Figure 3, A–D). More importantly, addition of HGF can potently stimulate SREBP-1 cleavage and LDLR expression in c-MET-expressing GBM cells (Supplemental Figure 3E), 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 here in 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 are regulated by PI3K/Akt signaling through mTORC1 in hepatocytes (61), 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 non-cancerous 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 pre-clinical models (Figure 1F) and in GBM patients treated with rapamycin (14, 64), we have shown that SREBP-1 activation, and consequent LDLR expression, are rapamycin resistant (Figure 1F). There are two potential explanations for these results. PI3K signaling to SREBP-1 may not require mTOR, perhaps due 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 due to 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 determine 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. Here we show that the synthetic LXR agonist GW3965 potently suppresses GBM growth and induces apoptosis in a mouse model (Figure 7), and we demonstrate enhanced efficacy in EGFRvIII-expressing GBM cells (Figure 4). Interestingly, we find that IDOL-mediated degradation of LDLR is necessary, but not sufficient, to induce GBM cell apoptosis (Figure 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 two of the three aspects of cholesterol regulation (27). Such drugs not only block exogenous LDL uptake, they also actively promote cholesterol removal from cells and intracellular distribution out of the endoplasmic reticulum (ER) (28).

Pharmacokinetic and toxicity studies have demonstrated 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 anti-tumor growth effect when administered with GW3965, suggesting a potential role for combination therapy (Supplemental Figure 8). 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 (Figure 7F). Our data also suggest that LXR-IDOL-LDLR axis is a common targetable pathway in multiple tumor types (Figure 5E; Supplemental Figure 5). Consistent with this hypothesis, activation of LXR in different types of cancer cell lines resulted in significant cell death (Supplemental Figure 9), 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.
Materials and Methods

Cell lines

U87 and U87-EGFRvIII, U87-EGFR, U87-EGFR-PTEN isogenic glioblastoma cell lines, A431 epidermoid carcinoma cell line, and LN229, T98 glioblastoma cell lines were cultured in DMEM (Cellgro) supplemented with 10% FBS (Omega Scientific) in a humidified atmosphere of 5% CO\textsubscript{2}, 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). H1975 non-small cell lung carcinoma cell line was cultured in RPMI1640 with 10% FBS, and HeLa cervical cancer cell line was cultured in DMEM with 10% FBS. Both are from ATCC.

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); SREBP-1 and SREBP-2 (BD Pharmingen); p-Met Tyr1349, p-PDGFR Tyr579 (Abgent); EGFR/EGFRvIII cocktail antibody (Upstate); p-EGFR Tyr1086 (Invitrogen). Reagents used are Erlotinib (ChemieTex), LY294002, Rapamycin, Polybrene, Puromycin (Sigma); p-Met Tyr1349, p-PDGFR Tyr579 (Abgent); EGFR/EGFRvIII cocktail antibody (Upstate); p-EGFR Tyr1086 (Invitrogen). Reagents used are Erlotinib (ChemieTex), LY294002, Rapamycin, Polybrene, Puromycin (Sigma); Akti-1/2 (Calbiochem); Atorvastatin (Toronto Research Chemicals, Canada). GW3965 was gifted from GlaxoSmithKline. Scramble shRNA, SREBP-1 shRNA, SREBP-1 siRNA and SREBP-2 shRNA lentivirus (Santa Cruz); Scramble shRNA, LDLR shRNA lentivirus (Sigma). Human lipoprotein deficient serum (LPDS) and low density lipoprotein (LDL) (Intracell).

Cell Proliferation and Death Assays

The details are described in supplemental materials.

ShRNA assay

The details are described in supplemental materials.

Western Blotting

Western blotting is shown in supplemental materials and done as previously described (65).

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 details were shown in supplemental materials (9, 64).

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. The details are described in supplemental materials and done as previously described (14, 55).

Phase II Lapatinib Clinical Trial

The details are addressed in Guo et al. Science Signaling 2009 (14).
**TUNEL Staining**

*In vitro* TUNEL staining

The details are addressed in supplemental materials and done as previously described (14).

**Real time PCR**

6 × 10^3 cells were seeded into 6 well plates in 5% FBS for 24 hrs, 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 following its protocol (Invitrogen). 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 (RPLP0) for each replicate. The primers used were: ABCA1 forward 5′-AACAGTTTTGCGCCCTTTTG-3′, reverse 5′-AGTTCCAGGCTGGGTACTTT-3′. Idol forward 5′-CGAGGACTGCCTCAACCA-3′, reverse 5′-TGCAGTCCAAAATAGTCAACTTCT-3′. 36B4 forward 5′-AATGGCAGCATCTACAAC-CC-3′, reverse 5′-TCGTTTGACCCGTTGATGA-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 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. The details are described in supplemental materials.

**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 was examined. The details are described in supplemental materials and done as previously described (14, 64).

**Statistical Analysis**

Results are shown as mean ± SEM. Fishers 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 two-tailed t test as well as by ANOVA as appropriate. *P*<0.05 was considered as statistically significant.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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References


Cancer Discov. Author manuscript; available in PMC 2012 March 15.
60. Yecies JL, Manning BD. mTOR links oncogenic signaling to tumor cell metabolism. J Mol Med. 2011
Significance
This study demonstrates 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 demonstrate that an LXR agonist causes IDOL-mediated LDLR degradation and increases expression of the ABCA1 cholesterol efflux transporter, potently promoting GBM cell death \textit{in vivo}. These results suggest a role for LXR agonists in the treatment of GBM patients.
Figure 1. EGFRvIII EGFRvIII/EGFR signaling promotes LDLR expression through a PI3K/Akt-mediated, SREBP-1 dependent pathway

A) $5 \times 10^5$ U87MG and U87MG/EGFRvIII cells were implanted into the flank of immunodeficient SCID/Beige mice. After 21 days, tumors were harvested and immunoblot analysis for p-EGFR, EGFR, p-Akt and LDLR was performed. M is mature form of LDLR, which is glycosylated. P is precursor of LDLR, non-glycosylated. 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 (IHC) of p-EGFR and LDLR was performed. Scale bar = 20 um. 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, stimulated with EGF (20 ng/ml) for the indicated times, and immunoblot analysis was performed using the indicated antibodies. P is precursor of SREBP-1; N is N-terminus of SREBP-1, which is
active form. F) Effect of the EGFR inhibitor erlotinib (10 uM), the PI3K inhibitor LY294002 (20 uM), the Akt inhibitor Akti-1/2 (5 uM), 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 EGF 20 ng/ml stimulation; immunoblot analysis was performed using the indicated antibodies. G) U87/EGFRvIII cells were infected using SREBP-1 shRNA lentivirus for 48 hrs 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 is precursor of SREBP-1; C is C-terminus of SREBP-2.
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 two tissue microarrays (TMA) of 252 tumor cores and 91 matched normal tissues from 140 primary (de novo) GBM patients using immunohistochemical analysis (IHC). Top row is normal brain, the middle row is a negative tumor (GBM A) and the bottom row shows positive staining in a tumor (GBM B). Inset shows nuclear SREBP-1 staining indicated by green arrow. Images are magnified 20×; tissue is counterstained with hematoxylin. Scale bar = 20 um.

B) Quantitative analysis of LDLR expression in GBM vs. 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 7–10 days of lapatinib treatment in two representative GBM patients. Scale bar = 20 um.
Figure 3. GBM cells depend on extracellular cholesterol levels for growth

A, B) U87 and U87/EGFRvIII cells were cultured in 1% FBS or 1% lipoprotein depleted serum (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 cells number with total cell amount (B).

C) U87 and U87/EGFRvIII cells were cultured in 1% and 5% of FBS or LPDS medium; low density lipoprotein was added to the cultured media (5 ug/ml, 1:1,000 dilution) for 3 days. Cell number was counted using a hemocytometer.

D) Micrographs showing morphological differences in U87 and U87/EGFRvIII cells cultured in 5% FBS and 5% LPDS medium; addition of LDL (5 ug/ml) recovered cell phenotype and cell growth. LDL stock 5 mg/ml. Scale bar = 20 um.
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 LXR agonist GW3965 for 4 days in 1% LDPS medium; cell viability was measured using WST-1 assay daily and normalized with day 0. Indicated cell survival percentage was calculated by normalizing with control at day 4. B, 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 TUNEL staining (Roche) and counterstained with 4’,6-diamidino-2-phenylindole (DAPI). DMSO, dimethy sulfoxide. Scale bar, 20 um. 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 ug/ml). The images represent cell morphology change after GW3965 treatment. Scale bar = 20 um. F, G) Dead cells and live cells were counted in panel E using trypan blue. LDL was added into media at a range of doses as indicated. LDL stock 5 mg/ml.
Figure 5. The LXR agonist GW3965 up-regulates expression of the cholesterol transporter gene ABCA1 and the E3 ubiquitin ligase IDOL and reduces LDLR levels

A, B) U87/EGFRvIII cells were treated with GW3965 5 μM for the indicated times. Total cellular RNA was extracted, and ABCA1 and IDOL gene expression was quantified using real-time PCR.

C) U87/EGFRvIII cells were treated with GW3965 for 24 hrs at a range of doses as indicated. Immunoblot analyses were performed using the indicated antibodies.

D) Cells were treated with GW3965 at 5 μM for the indicated times. Immunoblot analyses were performed using the indicated antibodies.

E) Multiple cancer cell lines were treated with GW3965 at 5 μM for the indicated times. Immunoblot analysis was performed using the indicated antibodies.
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 hrs. Immunoblot analysis was performed using the indicated antibodies. B, 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 trypan blue assay (C). Scale bar = 20 um. D) U87/EGFRvIII cells were infected by adenovirus Ad-LacZ or Ad-Idol for 48 hrs. Immunoblot analysis was performed using LDLR antibody. E, F) U87/EGFRvIII cells were infected with Ad-LacZ or Ad-Idol for 8 hrs, then treated with GW3965 in a range of dose as indicated for 3 days. Representative images after GW3965 treatment (E); tumor cells were counted using trypan blue assay (F). Scale bar = 20 um.
Figure 7. GW3965 treatment inhibited tumor growth in vivo

A) 5 × 10^5 U87/EGFRvIII cells were implanted into the flank of immunodeficient SCID/Beige mice (n=8/group); after tumor size reached 80 mm^3, GW3965 was administrated at 40 mg/kg by oral gavage daily for 12 days. Tumors were harvested and immunoblot analyses was 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 um.

E) Quantification of TUNEL staining.

F) Model demonstrating the pathways by which EGFR/RTK signaling mediates cholesterol metabolism and the therapeutic drug targets. The scheme 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 LXR agonist GW3965 disturbs cancer cell cholesterol homeostasis by up-regulating efflux through transporter gene ABCA1, and by reducing LDL uptake through degrading LDLR mediated by IDOL.