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Lonafarnib (SCH66336) improves the activity of temozolomide and radiation for orthotopic malignant gliomas

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Abstract

Malignant gliomas are highly lethal tumors resistant to current therapies. The standard treatment modality for these tumors, surgical resection followed by radiation therapy and concurrent temozolomide, has demonstrated activity, but development of resistance and disease progression is common. Although oncogenic Ras mutations are uncommon in gliomas, Ras has been found to be constitutively activated through the action of upstream signaling pathways, suggesting that farnesyltransferase inhibitors may show activity against these tumors. We now report the *in vitro* and orthotopic *in vivo* results of combination therapy using radiation, temozolomide and lonafarnib (SCH66336), an oral farnesyl transferase inhibitor, in a murine model of glioblastoma. We examined the viability, proliferation, farnesylation of H-Ras, and activation of downstream signaling of combination-treated U87 cells *in vitro*. Lonafarnib alone or in combination with radiation and temozolomide had limited tumor cell cytotoxicity *in vitro* although it did demonstrate significant inhibition in tumor cell proliferation. *In vivo*, lonafarnib alone had a modest ability to inhibit orthotopic U87 tumors, radiation and temozolomide demonstrated better inhibition, while significant anti-tumor activity was found with concurrent lonafarnib, radiation, and temozolomide, with the majority of animals demonstrating a decrease in tumor volume. The use of tumor neurospheres derived from freshly resected adult human glioblastoma tissue was relatively resistant to both temozolomide and radiation therapy. Lonafarnib had a significant inhibitory activity against these neurospheres and could potentate the activity of temozolomide and radiation. These data support the continued research of high grade glioma treatment combinations of farnesyl transferase inhibitors, temozolomide, and radiation therapy.

Keywords

Ras; farnesyltransferase inhibitor; radiation therapy; orthotopic glioma; temozolomide; stem cell

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Introduction

Glioma is the most common type of primary brain tumor diagnosed in the United States. The standard of care for this disease includes a multi-modal approach usually involving surgical resection, radiation therapy, and chemotherapy [1] with temozolomide [2]. Despite this aggressive treatment regimen, glioblastoma (GBM) remains highly lethal, with a median survival time of approximately 1 year [3]. With its extensive infiltrative and aggressive nature, GBM is highly refractory to the current available therapies.

At the molecular and genetic levels, epidermal growth factor receptor (EGFR) activation, loss of or mutation in p53, aberrant telomerase activity, and loss of phosphatase and tensin homolog (PTEN) are commonly observed in glioma [4–8]. Receptor tyrosine kinases (RTKs) such as the EGFR can function, in part, through activation of the Ras/Raf/Mitogen-Activated protein kinase (MAPK) signaling cascade. Thus, it is not surprising that EGFR upregulation of Ras signaling is one of the major intracellular pathways implicated in glioma growth and development [9–12].

Although activating Ras mutations are quite common in many types of cancer, such mutations are very rare in gliomas. Thus, the elevation of Ras activity characteristic of this disease is predominantly wild type. This aspect of biology suggests gliomas may be sensitive to the effects of a farnesyltransferase inhibitor (FTI). FTIs block the farnesylation of Ras, and render it unable to associate with the plasma membrane. Unfarnesylated Ras remains cytosolic and can not participate in intracellular signaling. Tipifarnib (R115777) has completed phase I testing both alone [13] and in combination with radiation therapy [14] and has been well tolerated. Lonafarnib (SCH66336, Sarasar®, Schering- Plough) is another potent orally active tricyclic FTI with less myelosuppressive side effects, now in clinical trials [15, 16]. Lonafarnib, which competes with the CAAX peptide substrate motif, blocks farnesyltransferase activity *in vitro* with an IC50 value of 1–2 nM.

Recent evidence indicates FTIs may be capable of exploiting some of the molecular characteristics of glioma. For example, it has been reported that tumors with endogenous Ras activation are more resistant to radiation therapy (XRT) than those in which Ras activity has been blocked [17–20], and the use of FTIs to inhibit activated Ras in human tumor cells resulted in XRT sensitization *in vitro* [21]. Moreover, cells with amplification of EGFR, which is common in GBM, have been shown to be more sensitive to lonafarnib *in vitro* [22].

Given this evidence, we tested whether the addition of an FTI given concurrently with the standard of care would potentiate the anti-tumor effects in an orthotopic model of glioma. In this study, we looked for preclinical evidence of synergy or additive effects of combination therapy including XRT, temozolomide (Tem), and lonafarnib. We found the addition of the FTI lonafarnib to a regimen of concomitant XRT and Tem enhanced growth inhibition *in*

vitro and *in vivo*. Our data suggest the addition of a FTI to the current standard of care for gliomas should be evaluated for its ability to improve the outcome of glioma patients.

Materials & Methods

Cell Culture & Luciferase Expression

U87 human glioma cells (American Type Culture Collection, Manassas, VA) expressing MGMT as determined by immunohistochemistry were cultured in DMEM with 10% FCS (Invitrogen/GIBCO, Carlsbad, CA). The coding sequences for luciferase and neomycin phosphotransferase were fused and introduced into a pMMP retrovirus (Richard Mulligan, Children's Hospital, Boston), generating pMMP-LucNeo. pMMP-LucNeo was packaged in 293T cells by cotransfection with plasmids encoding helper functions (from pMD murine leukemia virus) and vesicular stomatitis virus (VSV)-G envelope protein (pMD-G; Richard Mulligan). Pseudotyped VSV-G was applied to cells with 8 ug/ml polybrene (Sigma, St. Louis, MO). LucNeo-expressing U87 cells were selected in growth media supplemented with 1 mg/ml G418 (Invitrogen/GIBCO, Carlsbad, CA).

In vivo drug prep, dosing, & XRT

lonafarnib (SCH66336, Sarasar®) and Temozolomide (Temodar®, Tem) were kindly provided by Schering-Plough (Kenilworth, NJ). Drugs were reconstituted in 4% DMSO in 20% (2-hydroxypropyl)-beta-cyclodextrin in PBS. Lonafarnib was given once daily at 80mg/kg with twice weekly weightings to ensure accurate dosing. Tem was given by gavage at 5mg/kg 90 min prior to XRT. For irradiation, anesthetized mice were placed in a lead shielding apparatus which limited radiation exposure to the head only. Treatment (2.5Gy/day for two days) was delivered using a Gammacell 40 (MDS Nordion, Ottawa, Canada) irradiator delivering 100 rads/min. For *in vivo* combination experiments, suboptimal doses of XRT/Tem were selected to permit identification of synergistic effects of lonafarnib.

In vitro Drug Stocks, Treatments, & XRT

Drugs were reconstituted in DMSO and stock solutions (10mM Lonafarnib, 25mM Tem) were stored at -20°C. Drugs were diluted in culture media and fresh media was prepared and applied to cells every 24 h. For XRT, culture plates were irradiated using a GammaCell 1000 (MDS Nordion, Ottawa, Canada) at a rate of 80 rads/min.

Non-Radioactive MTS Cytotoxicity Assay

CellTiter96 Aqueous Assay kit was purchased from Promega (Madison, WI). Assays were performed under manufacturer's instructions with 5000 cells/well in a 96-well tissue culture plate. Plates were irradiated 24 h after drug exposure and assayed 96 h after XRT, with fresh drug treatments applied each day. For quantification, dye was added directly to each well, plates were washed as per the manufactures recommendation and cell viability determined by optical density. Significance was analyzed using the Student's T-test.

Proliferation Assay

12-well plates were seeded with 100,000 cells/well. Drug treatments were initiated 24 h after plating, and media was replaced every 24 h for a total of 96 h of drug exposure. Plates were irradiated after 24 h of drug exposure. Cells from triplicate sets of treatments were trypsonized and counted 48 h after irradiation using a Z1 series coulter counter (Beckman Coulter, Fullerton, CA), and compared to cell numbers from wells counted on Day 1 (the day drug treatment was initiated). Proliferation after drug treatments were normalized to the control wells and expressed as % of the control treatment. Significance was analyzed using the Student's T-test.

Downstream Pathway Analysis

2.5×10^6 cells per 100mm³ dish were seeded, and drug treatments initiated 24 h after plating. Plates were irradiated after 24 h of drug exposure, and cells were lysed after 48 h of drug exposure (24 h after XRT). Total protein was extracted with ice-cold T-Per (Pierce, Rockford, IL) supplemented with protease and phosphatase inhibitors, and quantitated using the BCA protein assay kit (Pierce, Rockford, IL). 500ug of total protein was used to probe different Human Phospho-RTK Human Phospho-MAPK Arrays (R&D Systems, Minneapolis, MN). Arrays were washed and developed according to manufacturer's instructions, and exposed to film. Films were scanned using a flatbed scanner, and dots were quantitated using ImageJ (NIH, Bethesda, MD). Relative changes between treatment groups were expressed as % of control, with significance assessed by Student's T-test.

Western Blotting of H-Ras

2.5×10^6 cells per 100mm³ dish were seeded, and drug treatments initiated 24 h after plating. Plates were irradiated after 24 h of drug exposure, and cells were lysed after 48 h of drug exposure (24 h after XRT). Total protein was extracted with ice-cold T-Per (Pierce, Rockford, IL) supplemented with protease and phosphatase inhibitors, and quantitated using the BCA protein assay kit (Pierce, Rockford, IL). Samples (20 µg total protein) were run on 4–15% Tris HCl SDS-PAGE Criterion gels (Biorad, Hercules, CA) and probed for H-Ras (1:500 Santa Cruz Biotech, Santa Cruz, CA) and α -tubulin (1:5000, Sigma, St. Louis, MO) as an internal loading control. Blots were exposed to film, and films were scanned using a flat bed scanner. Bands were quantitated using ImageJ (NIH, Bethesda, MD), and graphed using Excel (Microsoft, Redmond, WA). H-Ras was normalized to the loading control and expressed as a % of the control treatment. Significance was assessed using the Student's T-test.

Tumor Cell Line Xenografts

Tumor cell lines were harvested in mid-logarithmic growth phase and resuspended in PBS. Homozygous NCR nude mice (Taconic Farms, Hudson, NY) were anesthetized with Ketamine/Xylazine before exposure of the cranium and removal of the periosteum with a size 34 inverted cone burr (Roboz, Gaithersburg, MD). Mice were fixed in a stereotactic frame (Stoelting, Wood Dale, IL), and 5×10^4 cells in 10 µl of PBS were injected through a 27-gauge needle over 5 min at 2 mm lateral and posterior to the bregma and 3 mm below the

dura. The incision was closed with staples. Animals were observed daily for signs of distress or development of neurologic symptoms at which time the mice were sacrificed.

***In Vivo* Imaging**

Mice were anesthetized with Ketamine/Xylazine, injected with D-luciferin at 50 mg/kg i.p. (Promega, Madison, WI), and imaged with the Xenogen IVIS 100 Imaging System for 10–120 s, bin size 2 as previously published [23, 24]. To quantify bioluminescence, identical circular regions of interest were drawn to encircle the entire head of each animal, and the integrated flux of photons (photons per second) within each region of interest was determined by using the Xenogen LIVING IMAGES software package. Data were normalized to bioluminescence at the initiation of treatment for each animal. Statistical significance was assessed using the Student's T-test.

Glioma Neurosphere Assay

Collection and use of fresh and discarded human tumor tissue was approved by the Brigham and Women's Hospital Institutional Review Board. After frozen section diagnosis of malignant glioma by the attending neuropathologist, tumor material was grossly dissected from the tissue sample. Portions of the tumors were collected in chilled media for the studies described here and other portions were allocated for paraffin embedding for histological diagnosis and for genotyping. Expansion of tumor material and propagation was accomplished by subcutaneous implantation in Icr SCID mice (cells were never grown on plastic). When tumors reached ~1 cm, tumors were disaggregated, cells were counted and then grown in serum-free media with EGF, FGF and LIF as described previously to form tumorspheres [25, 26]. Drugs (SCH 5uM, TMZ 100uM) were added immediately after plating cells into 24 well plates and radiation given at 24hrs after plating and tumor neurospheres were counted in triplicate 10 days after plating.

Results

Enhanced cytotoxic and anti-proliferative effects of lonafarnib (SCH66336)/XRT/Tem combination in U87 glioma cells *in vitro*

The effect of lonafarnib, and different combinations of lonafarnib, XRT, and Tem on U87 cytotoxicity *in vitro* were examined over a wide range of doses using the standard MTS assay (Promega, Madison, WI). Doses of XRT and Tem were designed to provide a small but significant decrease in proliferation alone (91% and 76%, respectively) and a moderate effect in combination (71%) to allow easy identification of synergistic action with lonafarnib (Fig. 1A). Lonafarnib alone or SCH/Tem did not induce dramatic U87 cell cytotoxicity *in vitro* at physiologically achievable doses (<10µM, Fig. 1A). Lonafarnib/XRT/Tem was significantly more effective than lonafarnib alone (65% vs 85%, respectively, $p < 0.001$), but cell viability still remained high (Fig. 1A). Although we did find an increase in U87 cytotoxicity, it was difficult to determine the added benefit of the combination treatment with this method. To further analyze the effects of the combination we employed a traditional proliferation assay. Lonafarnib alone, at the lowest dose (5µM) was significantly more effective in blocking U87 proliferation than XRT/Tem (54% proliferation vs 83%, respectively, $p < 0.01$, Fig. 1B) although increasing doses of lonafarnib did not further

increase the effect. Combination of lonafarnib/XRT/Tem enhanced inhibition of proliferation and was more effective than either lonafarnib alone or XRT/Tem (24% proliferation with lonafarnib/XRT/Tem vs 54% with 5uM lonafarnib, and 83% with XRT/Tem, $p<0.001$). This assay clearly showed the impact of adding lonafarnib to the standard of XRT/Tem. *In vitro* dosages of 10uM lonafarnib and 100uM Tem, which are physiologically achievable *in vivo*, were chosen for subsequent experiments based on cytotoxicity and proliferation data.

Farnesylation of H-Ras is inhibited by lonafarnib (SCH66336) alone and in combination with XRT and Tem, but not XRT, Tem, or XRT/Tem *in vitro*

In order to assess the immediate downstream effects of lonafarnib and ascertain whether the *in vitro* levels were sufficient to inhibit farnesylation of H-Ras, we examined the level of farnesylation of H-Ras in response to combinations of lonafarnib (10uM), XRT (6Gy), and Tem (100uM). Cells were treated for 24h and then given a single pulse of XRT and a media change (drug containing). Cells were harvested for Western blot 24h after the pulse of XRT. Two species of H-Ras were found, the upper band, unprocessed inactive Ras, and the faster migrating lower band which is farnesylated and active [27] (Fig. 2A). Treatment of U87 cells with XRT, Tem, or XRT/Tem were identical to control treated samples. Treatment with any combination including lonafarnib had a dramatic, near complete suppression of H-Ras farnesylation (lower band). When normalized for loading with α -tubulin, levels of H-Ras were found to be very consistent in all groups not exposed to lonafarnib (90–100% relative density, Fig. 2B). Interestingly, all treatment groups containing lonafarnib showed a significantly higher level of unfarnesylated H-Ras (116–137%) compared to control treatment (Fig. 2B).

Effect of lonafarnib (SCH66336)/XRT/Tem combination treatment on signaling molecules *in vitro*

Next we characterized the activation state of some of the common signaling pathways after *in vitro* treatment of U87 cells with lonafarnib alone, lonafarnib/XRT, and lonafarnib/XRT/Tem. We examined both serine/threonine molecules associated with the MAPK pathways and RTK signaling components. Activation of the EGFR (phosphoEGFR, or pEGFR) was reduced by 44% with lonafarnib alone ($p<0.01$), but this attenuation was lost with lonafarnib/XRT. Interestingly, the combination of lonafarnib /XRT/Tem resulted in a significant increase (126%, $p<0.01$) of EGFR activation (Fig. 3A,B).

An effect of combination treatment was found on the hepatocyte growth factor receptor (HGFR, c-Met) phosphorylation. Lonafarnib alone caused a modest decrease of ~9% in HGFR activation ($p<0.05$, Fig. 3B), while significant inhibition of HGFR activation was found with lonafarnib /XRT (51%, $p<0.001$), and with the combination of lonafarnib /XRT/Tem (53%, $p<0.01$). Another interesting finding was the effect of lonafarnib on the activation of the RTK EphA7 (Fig. 3B). Phosphorylation of this receptor, which is activated by a GPI-linked ephrin ligand, was significantly decreased by 63% in lonafarnib alone and lonafarnib /XRT ($p<0.05$) and 58% by lonafarnib /XRT/Tem ($p<0.05$).

Downstream elements commonly associated with Ras, ERK and p38 δ showed changes in response to treatment (Fig. 3B). Activation of ERK1 was decreased to 82% with lonafarnib

alone, although was not found to be statistically significant ($p=0.08$), while the combination of lonafarnib /XRT caused a significant increase of phosphorylation (120%, $p<0.05$). Treatment of U87 cells with lonafarnib /XRT/Tem resulted in significant reduction of ERK1 activation (81%, $p<0.05$, Fig. 3B). p38 δ showed a modest, but significant increase (113%, $p<0.05$) in response to lonafarnib alone, and this increase was attenuated to control levels by the combination of lonafarnib /XRT/Tem (Fig. 3B and Supplemental Fig. 1).

Signaling through the PI3K/AKT pathway functions independently of Ras, and we found no significant effect on AKT with lonafarnib alone or in combination with XRT and Tem on U87 glioma cells *in vitro* (data not shown). We found little change in the activation of RSK, JNK1, or GSK-3 α/β with lonafarnib alone, or with combination treatment (data not shown).

Addition of lonafarnib (SCH66336) potentiates the anti-tumor affect of XRT/Tem on orthotopic U87 glioma *in vivo*

Given the encouraging *in vitro* data generated with the combination of lonafarnib /XRT/Tem, we next tested this drug regimen *in vivo*. Using an orthotopic murine model of U87 glioma we assessed the ability of lonafarnib alone and lonafarnib /XRT/Tem to block the growth of intracranial tumors. Lonafarnib alone (80 mg/kg by oral gavage, once daily) had limited ability to inhibit orthotopic U87 tumors compared to vehicle treated control animals (T/C of 0.67, Fig. 4A,B). The combination of XRT/Tem (2.5Gy/day for 2 days; 5 mg/kg by oral gavage 90 min prior to XRT) was designed to produce modest tumor growth inhibition *in vivo* (T/C of 0.42). Concurrent lonafarnib/XRT/Tem (lonafarnib 80 mg/kg by oral gavage, once daily, XRT 2.5Gy/day for 2 days, and Tem 5 mg/kg by oral gavage 90 min prior to XRT) provided the strongest growth reduction (T/C of 0.02) and was significantly more effective than XRT/Tem ($p<0.04$), with the majority of animals demonstrating a decrease in tumor volume ($p<0.05$, Fig. 4A,B) after two weeks and persisting after 4 weeks ($p<0.05$, Fig. 4B).

Addition of lonafarnib (SCH66336) potentiates the anti-tumor affect of XRT/Tem on human glioblastoma neurospheres *in vitro*

The utility of established tumor cell lines grown in serum to predict treatment response *in vitro* or *in vivo* remains controversial. To assess the activity of the combination of lonafarnib/XRT/Tem in a more clinically relevant model [28], we treated glioblastoma neurospheres derived from surgical specimens of adult glioblastoma. Lonafarnib alone demonstrated significant inhibition of neurosphere proliferation (lane 2) compared to temozolomide (lane 3), radiation (600 rad) (lane 5) and radiation + temozolomide (lane 7). The addition of lonafarnib to either temozolomide (lane 4), radiation (lane 6) or to radiation and temozolomide (lane 8) demonstrate almost complete inhibition of proliferation (Fig. 5). Thus, while radiation and/or temozolomide had only limited proliferation inhibition on this population, lonafarnib demonstrated significant activity alone compared to controls (lanes 1 versus 2, $p<0.0001$, Fig. 5), and significantly improved the results when combined with radiation and temozolomide (lanes 7 versus 8, $p<0.001$, Fig. 5).

Discussion

Glioma is the most common primary brain tumor diagnosed in the United States. The standard of care for a newly diagnosed adult high-grade glioma, GBM, is XRT and Temozolomide treatment. While active, this therapy is not curative in the vast majority of patients. Current clinical strategies are to augment the effectiveness of the current standard therapy by adding in novel chemotherapeutic and biological agents.

Excessive growth factor stimulation of glioma cells results in aberrant constitutive activity of Ras. The overabundance of Ras activity promotes glioma cell growth and proliferation, and is a target of novel anti-cancer drug design. FTIs block the activation of Ras by inhibiting its farnesylation, rendering it unable to associate with the plasma membrane which is required for its activity (Fig. 6). FTIs have been shown to block *in vitro* anchorage-dependent and anchorage-independent growth and proliferation of a variety of cancer cell lines [29–31]. In addition to blocking the activation of Ras, treatment of cancer cells *in vitro* with FTI has been shown to reverse radiation resistance and increase the sensitivity of the cells to XRT [17–21]. FTIs have little to no effect on normal cells *in vitro* and *in vivo* [32] and show little systemic toxicity in animal models [33, 34], supporting FTIs as promising agents for the treatment of cancer.

Lonafarnib has been tested pre-radiation for adults with glioblastoma without significant activity [35] while it has shown promise in combination with the cytotoxic drug Paclitaxel in Non Small Cell Lung Cancer (NSCLC) [36]. This reinforces the development of combination therapies, which may provide additional efficacy. Our goal was to assess, *in vitro* and *in vivo*, if lonafarnib/ XRT/Tem showed evidence of additive or synergistic activity in a preclinical model of GBM. We tested the effectiveness of the FTI lonafarnib in combination with XRT and Tem *in vitro* and an *in vivo* animal model of orthotopic U87 GBM as well as in adult glioblastoma derived tumorspheres. As predicted by previously published studies, lonafarnib alone had limited single agent activity *in vitro* and *in vivo*. We found lonafarnib did not induce dramatic cell death as measured by MTS cytotoxicity assay. In contrast, lonafarnib inhibited glioma cell proliferation, and showed marked increase in activity when combined with XRT and Tem. *In vivo* activity of lonafarnib may be related to a combination of the cytotoxic and antiproliferative effects.

Doses of lonafarnib used in our *in vitro* studies were sufficient to block activation of H-Ras in the U87 glioma cells, and are physiologically achievable *in vivo*. In addition to preventing the prenylation of H-Ras, overall levels of H-Ras protein were elevated in treatments including lonafarnib. This suggests accumulation of unprocessed H-Ras seen in other cell lines after treatment with an FTI [27, 37], is also found in the U87 cells.

To identify compensatory mechanisms a cell may upregulate after treatment with lonafarnib/XRT/Tem, we examined several RTK and MAPK signaling molecules after combination treatment. We found activation of EGFR was strongly inhibited in U87 cells *in vitro* by lonafarnib alone, but this effect was lost with lonafarnib/XRT, and activation was induced by lonafarnib/XRT/Tem. The response of the cell to damage by XRT/Tem resulting in increased activation of EGFR, perhaps as a survival mechanism as the cell tries to

overcome the effects of XRT/Tem are of interest. We are investigating the possible significance of adding an EGFR inhibitor to the lonafarnib/XRT/Tem combination.

Ras is a major signaling mediator of EGFR and it has been reported that inhibition of Ras can feedback and block EGFR activation. We found EGFR activation was reduced after treatment with lonafarnib and dramatic synergy was found with lonafarnib/XRT/Tem combination treatment. Previous studies have reported near complete inhibition of EGFR expression and activation of EGFR in U-373 and T98G glioma cell lines after *in vitro* treatment with a FTI [38]. Our data support this bidirectional signaling in U87 glioma cells *in vitro*.

Although the Ras pathway is mostly known for its role in growth and transformation, it also has been shown to affect axon guidance and cell adhesion. The ephrin-Ras relationship has been shown to function in these processes. Eph receptors (EphR) are among the few RTKs that negatively regulate H-Ras and the downstream MAPK pathway in a wide range of cell lines *in vitro*. EphR negatively regulates the Ras/MAPK/ERK pathway through p120RasGAP, which inhibits the positive signals of Grb2/Sos [39–41]. We found that activation of EphA7 was dramatically inhibited by lonafarnib, alone and in combination with XRT/Tem. This unexpected finding was quite striking, and indicates the EphR may play a more significant role in cellular proliferation than previously thought. EphR activation can block the activation of the Ras/MAPK/ERK pathway by platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and angiopoietin-1 (Ang-1), suggesting cross-talk between downstream signals of Eph receptors and other RTKs [41–44]. Separation of the direct anti-tumor and anti-angiogenic effects of FTIs [45] is difficult due to the significant overlap of signaling via the Ras pathway in both tumor and endothelial cells. Similarly, radiation therapy and temozolomide target dividing endothelial cells in the same way that they target dividing tumor cells. The ability to potentially target both populations simultaneously however may be of therapeutic benefit *in vivo* and account for the significant inhibition of tumor growth in the orthotopic models. Our data suggests that in glioma cells, the EphR-Ras relationship is bidirectional, and that inhibition of H-Ras by a FTI potentially blocks the activation of EphA7.

Our data show that glioma cells counterbalance the inhibition of H-Ras by lonafarnib with compensatory pathway activation *in vitro*. Activation of p38 δ was increased after treatment with lonafarnib alone, but the increase was abrogated when XRT and Tem were added. Although Ras is a main signaling input for the MAPK/ERK signaling cascade, we found little downregulation of ERK1 activation with lonafarnib alone. Alternative prenylation of K-Ras and N-Ras by geranylgeranyl transferase-I still occurs in the presence of an FTI [46, 47], which may account for the continued activity of MAPK/ERK signaling and thus possible resistance to FTIs. This also could suggest other signaling mechanisms that function through the MAPK/ERK cascade are sufficient to fully activate the pathway *in vitro*. Activation of ERK1 was significantly upregulated with lonafarnib /XRT and significantly less in lonafarnib/XRT/Tem treated cells. Our data suggests XRT causes a rise in MAPK/ERK signaling, as others have reported [48], which is blocked by the addition of Tem.

Our results indicate that there is added *in vitro* efficacy with the combination of lonafarnib/XRT/Tem. Although we did not find lonafarnib alone or in combination with XRT/Tem to be highly cytotoxic, it was found to be potentially cytostatic. Because XRT/Tem can induce significant inhibition of glioma growth *in vivo*, we reduced the amount of XRT/Tem given to animals in our preclinical model of GBM in order to be able to evaluate the potential effects of lonafarnib. Given that we are using only two doses of XRT/Tem, the *in vivo* response to lonafarnib/XRT/Tem in humans in the clinic may be amplified with the extended course of XRT/Tem typically prescribed for GBM. The combination of lonafarnib/XRT/Tem *in vivo* not only prevented progression of tumor growth, but also caused a significant decrease in tumor size. This indicates the combination of lonafarnib/XRT/Tem may manifest differently *in vivo* from what is found *in vitro* and highlights the role tumor microenvironment plays in a tumor's response to biologic agents. Our goal is to improve response of GBM to standard modalities, and the addition of a drug that potentially inhibits cell proliferation could maximize the effects of standard therapy.

Current strategies for the treatment of malignant gliomas have resulted in prolongation of survival but most patients eventually succumb to disease. This is thought in part to be related to the presence of a rare population of glioma stem cells that are highly radiation and chemotherapy resistant [49, 50]. Developing new strategies that specifically inhibit these cells may be critical in improving the outcome of this disease [51]. Our results demonstrating a significant inhibitory action of lonafarnib on glioma stem cell derived neurospheres and the complete suppression of proliferation when combined with radiation and temozolomide provide additional rationale for further testing of this combination.

We hope to now translate the combination of lonafarnib/XRT/Tem to the clinical setting. To test this combination, we suggest a clinical trial in both adult and pediatric patients with newly diagnosed gliomas where lonafarnib is added concurrently to the standard of care XRT/Tem schedule. Our results support the continued investigation of combining novel molecular therapeutic agents with XRT/Tem with the goal of offering progress in the treatment of highly aggressive brain tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a

SCH		XRT/Tem		SCH/XRT/Tem	
Treatment	% Viability $\pm$ S.E.M.	Treatment	% Viability $\pm$ S.E.M.	Treatment	% Viability $\pm$ S.E.M.
SCH 0 μ M	100% $\pm$ 3.34	Tem 100 μ M	91% $\pm$ 5.58	SCH 10 μ M/ Tem	84% $\pm$ 2.19
SCH 5 μ M	84% $\pm$ 2.79	XRT 6 Gy	76% $\pm$ 3.46	SCH 10 μ M/ XRT	72% $\pm$ 2.28
SCH 10 μ M	85% $\pm$ 2.70	XRT/ Tem	71% $\pm$ 2.13	SCH 10 μ M/ XRT/ TEM	65% $\pm$ 2.29

Control vs. 10 μ M ACH $p < 0.05$; 10 μ M SCH vs. SCH/XRT/ Tem $p < 0.01$ **Fig. 1.**

Combination treatment has an effect on U87 cell cytotoxicity and proliferation *in vitro*. (A) Lonafarnib alone or SCH/Tem (10 μ M/100 μ M) induced some U87 cell cytotoxicity compared to vehicle treated control while SCH/XRT/Tem was notably more effective than lonafarnib alone, but cell viability still remained high. (B) Combination treatment has a strong synergistic effect on U87 cell proliferation. The combination of SCH/XRT/Tem displayed significantly more inhibition than lonafarnib alone (5 μ M 0Gy vs 6Gy $p < 0.001$, 10 μ M 0Gy vs 6Gy $p < 0.001$, 15 μ M 0Gy vs 6Gy $p < 0.001$). The number of cells per well of quadruplicate treatments was normalized to control and plotted as the mean $\pm$ s.e.m. Statistical significance was determined by Student's t-test (** $p < 0.01$, *** $p < 0.001$). Results are representative of three independent experiments.

b

Treatment	% Relative H-Ras
Control	100 ± 4.5
XRT (6 Gy)	97.77 ± 4.6
Tem (100 µM)	98.03 ± 1.2
SCH66336 (10 µM)	136.94 ± 4.8
SCH/XRT	135.10 ± 6.6
XRT/Tem	92.55 ± 4.3
SCH/Tem	116.84 ± 1.3
SCH/XRT/Tem	125.19 ± 2.4

Fig. 2.

Lonafarnib (SCH66336) blocks farnesylation and increases overall levels of H- Ras *in vitro*.

(A) Lonafarnib alone strongly blocked the farnesylation of H-Ras protein, with the farnesylated form barely visible by immunoblot (lower band, active H- Ras). (B) Overall levels of H-Ras protein were also altered in any treatment containing lonafarnib. Levels of H-Ras were normalized to α -tubulin and plotted as the mean of three replicates $\pm$ s.e.m. Statistical significance of increased unfarnesylated H-Ras was determined by Student's t-test (* $p < 0.05$, ** $p < 0.01$). Results representative of three independent experiments.

Fig. 3.

Combination treatment influences activation of MAPK and RTK pathways (A)

Representative Proteome Profiler (R&D Systems, Minneapolis MN) dot blot is shown for

EGF receptor (EGFR). (B) Phosphorylation of EGFR was significantly attenuated by

lonafarnib alone but reversed with the addition of XRT and Tem. Activation of pEphA7 was also highly suppressed with lonafarnib alone while ERK1 activation was slightly reduced by lonafarnib. Levels of p38δ were induced by SCH alone but not by SCH/XRT or SCH/XRT/Tem.

The optical density of duplicate dots were normalized to control and plotted as the mean $\pm$ s.e.m. Statistical significance was determined by Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Fig. 4. Combination of SCH/XRT/Tem displays additional anti-tumor activity over XRT/Tem in *in vivo* orthotopic U87 gliomas (A) Lomafarnib alone was not effective in blocking intracranial tumor growth (n=6) compared to vehicle treated control animals (n=5). XRT/Tem treatment was designed to produce modest tumor growth inhibition so the effect of combination treatment could be assessed. (B) Combination of SCH/XRT/Tem displays additional anti-tumor activity over XRT/Tem in *in vivo* orthotopic U87 gliomas. Combination of SCH/XRT/Tem inhibited intracranial tumor growth. Relative tumor burden is plotted on the Y axis as the tumor volume relative to the day treatment is initiated. Statistical significance was determined by Student's t-test (* $p<0.05$) and are the average of the means of 4 different experiments.

Fig. 5. SCH66336 alone and the combination of SCH/XRT/Tem significantly inhibits glioblastoma neurosphere proliferation *in vitro*. Results are presented as normalized number of neurospheres and error bars with 95% confidence intervals.

Fig. 6. Schematic representation of the ras signaling pathway. Lonafarnib (SCH66336) blocks the farnesylation of Ras-GDP forcing it to remain in the cytoplasm where it cannot activate the Ras signal transduction pathway. Synergistic inhibition of tumor growth by lonafarnib after treatment with radiation and temozolomide suggests that resistance to DNA damage is mediated by Ras-GTP or other farnesylation dependent pathways. FT = Farnesyl Group; FT = Farnesyltransferase; SCH = Lonafarnib (SCH66336); RTK = Receptor tyrosine kinase; XRT = Radiation therapy; TMZ = Temozolomide.