Blockade of TGF-beta signaling by the TGFβRI kinase Inhibitor

LY2109761 enhances radiation response and prolongs survival in glioblastoma

Mengxian Zhang,1, 2, 3 Susanne Kleber,4 Manuel Röhrich, 2, 3 Carmen Timke2, 3
Na Han,1 Jochen Tuettenberg,4 Ana Martin-Villalba,5 Juergen Debus,3 Peter Peschke,2 Ute Wirkner,2 Michael Lahn,6 Peter E. Huber2, 3, 7

1 Department of Oncology, Tongji Hospital, Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430030, China
2 Department of Radiation Oncology, German Cancer Research Center (DKFZ), 280 INF, Heidelberg 69120, Germany
3 Department of Radiation Oncology, University Hospital Center, 400 INF, Heidelberg 69120, Germany
4 SHG-Klinik Idar-Oberstein Klinik für Neurochirurgie, 2 Dr.-Ottmar-Kohler-Straße, Idar-Oberstein 55743, Germany
5 Department of Neurobiology of Brain Tumors, German Cancer Research Center (DKFZ), 280 INF, Heidelberg 69120, Germany
6 Oncology Early Clinical Investigation, Lilly Research Laboratories, 46285 Indianapolis, USA

7 Correspondence to:
Peter E. Huber M.D. Ph.D.
Department of Radiation Oncology
German Cancer Research Center (DKFZ)/University of Heidelberg Medical Center
280 Im Neuenheimer Feld
Heidelberg 69120
Germany
Phone: +49 6221 42 2515
Fax: +49 6221 42 2514
Email: p.huber@dkfz.de

Key words:
Glioblastoma, TGF-β, radiotherapy, cancer stem-like cell, orthotopic model, expression microarray, LY2109761

Grant support: This work was supported in part by grants from DFG National Priority Research Program the Tumor-Vessel Interface (SPP1190), National Aeronautics and Space Administration Specialized Center of Research NNJ04HJ12G, Kompetenzverbund Strahlenforschung (KVSF, 03NUK004A,C) of Bundesministerien fuer Bildung, Forschung und Umwelt (BMBF/BMU).
Abstract

Glioblastoma multiform (GBM) is a highly aggressive primary brain tumor that tends to be resistant to the ionizing radiotherapy used to treat it. Because transforming growth factor (TGF)-β is a modifier of radiation responses, we performed a preclinical study of the antitumor effects of the TGF-β receptor (TβR) I kinase inhibitor LY2109761 in combination with radiotherapy. LY2109761 reduced clonogenicity and increased radiosensitivity in GBM cell lines and cancer stem-like cells (CSCs), augmenting the tumor growth delay produced by fractionated radiotherapy in a supra-additive manner in vivo. In an orthotopic intracranial model, LY2109761 significantly reduced tumor growth, prolonged survival and extended the prolongation of survival induced by radiation treatment. Histological analyses showed that LY2109761 inhibited tumor invasion promoted by radiation, reduced tumor microvessel density and attenuated mesenchymal transition.

Microarray-based gene expression analysis revealed signaling effects of the combinatorial treatments that supported an interpretation of their basis. Together, these results show that a selective inhibitor of the TβR-I kinase can potentiate radiation responses in glioblastoma by coordinately increasing apoptosis and CSLC targeting while blocking DNA damage repair, invasion, mesenchymal transition and angiogenesis. Our findings offer a sound rationale for positioning TGF-β receptor kinase inhibitors as radiosensitizers to improve the treatment of glioblastoma.
LY2109761 and radiation in an orthotopic glioblastoma model  

Zhang et al.

**Introduction**

GBM continues to be the most frequent and most malignant human brain tumor, which is highly resistant to current conventional treatments and has one of the worst survival rates among all human cancers. New strategies to treat this deadly disease are desperately needed. Emerging evidence suggests that many cancers, including hematopoietic and solid tumors, may be driven by a small subpopulation of cancer stem-like cells (CSLCs, or cancer stem cells, CSCs, or tumor-initiating cells, TICs). GBM are among the first solid cancers in which CSLCs were identified (1, 2). CSLCs markers such as CD133 and side population have been used to prospectively isolate a small fraction of cells in human brain tumors with increased potential to generate tumor neurospheres and xenografts (3, 4). Recent reports suggest that the expression of the CD133 antigen in gliomas and other brain tumors could serve as a prognostic indicator for tumor recurrence, malignant progression, treatment resistance and patient survival (5). Several studies indicate that conventional radiation therapies appear to predominantly target the better-differentiated CD133- population, while leaving many CD133+ CSLCs alive (6). Thus targeting CSLCs using inhibitors of transforming growth factor-β (TGF-β) could be a promising attempt to improve glioblastoma treatment (7).

TGF-β is a family of polypeptides that regulates a wide variety of biological functions including cell proliferation, migration, survival, angiogenesis, immunosurveillance and embryonic stem cells maintenance and differentiation. The multifunctional effects of TGF-β are elicited through dimerization of the type I (TβRI) and type II (TβRII) serine/threonine kinase receptors. Upon TGF-β binding, the receptor complex phosphorylates the transcription factors SMAD2 and SMAD3, which then binds to SMAD4 and translocates to the nucleus, where they regulate transcription of various target genes (8). The overexpression of TGF-β ligands has been reported in various malignant entities including malignant gliomas (9-11). In malignant glioma patients, elevated levels of TGF-β has been reported to be associated with high tumor grade, advanced tumor stages and poor patient outcome (9, 12). In addition to be implicated in invasion and intratumoral angiogenesis in glioblastoma, TGF-β signaling has also been reported to play a pivotal role in maintenance of stemness of glioma CSLCs (13,
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

14). These multiple roles of TGF-β in glioma initiation and progression have promoted the development of therapeutic agents based on the inhibition of the TGF-β pathway (13, 15). LY2109761, a novel TβRI kinase inhibitor, has shown a SMAD2-selective inhibitory profile with anti-tumor activity in various tumor models, such as colorectal cancer (16), pancreatic cancer (17), and hepatocellular carcinoma (18).

Since radiotherapy is a mainstay treatment modality in glioblastoma, but may promote radioresistance in glioma stem cells (6), a combination with an inhibitor of TGF-β, which targets in particular CSLCs (7), seems promising. We investigated here the combination effects of radiation and LY2109761 in established human glioblastoma cell lines and in GBM CSLCs in vitro and in vivo in a subcutaneous and an orthotopic tumor model. Our data indicate that LY2109761 is an effective treatment approach alone and augments the radiation treatment response in particular in GBM CSLCs.

METHODS AND MATERIALS

Cell cultures and treatment conditions

The human GBM cell lines U87MG and T98 were obtained from the American Type Culture Collection (ATCC, Bethesda, MD). CD133+ Glioblastoma CSLCs (NMA-23) were maintained in their undifferentiated state using Neurobasal Media supplemented with epidermal growth factor and fibroblastic growth factor, sodium pyruvate, glutamine, B27, non-essential amino acids and penicillin/ streptomycin (Gibco, Grand Island, NY). NMA-23 cells were isolated from human glioblastoma surgical sample using fluorescence-activated cell sorting (FACS) method with a PE-labeled CD133 antibody (Miltenyi Biotec, Auburn, CA) as previously described (19). The collection of human biopsy tissue was approved by the regional ethical committee. LY2109761 was kindly provided by Eli Lilly (Indianapolis, IN, USA), constituted in DMSO (10 mmol/L) and stored at -20°C. Cell exposures with LY2109761 were performed 2 hour prior to irradiation with 6 MV X-rays (Mevatron, Siemens, Erlangen, Germany) at a dose rate of 2.5 Gy/min.
Small interfering RNA treatment

Validated Stealth RNAi (Invitrogen, Carlsbad, CA) specific to TβRI (TGBFR1) was transfected into U87MG and NMA-23 by using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Stealth RNAi negative control with low GC content (Invitrogen, Carlsbad, CA) was used as negative control. The expression of TGBFR1 was measured by quantitative real-time PCR (qRT-PCR) and phosphorylation of SMAD2 was examined using Western blot.

Clonogenic assay

Increasing numbers of cells (pretreated with LY2109761 for 2 h or transfected with siRNA targeting TGBFR1 or nonsense siRNA for 48 h) were plated in 25 cm² flasks, irradiated and returned to the incubator for 10–14 days. Colonies formed were stained with crystal violet (Sigma, Germany) and those with at least 50 cells were counted by microscopic inspection. The linear quadratic (LQ) equation was fitted to data sets to generate survival curves and dose enhancement factor for drugs was calculated at 10% surviving fraction (DEF0.1).

Neurosphere formation, limiting dilution and proliferation assay

For the neurosphere formation assay, 1,000 NMA-23 cells (pretreated with LY2109761 for 2 h or transfected with siRNA targeting TGBFR1 or nonsense siRNA for 48 h) were seeded on 25 cm² flasks and irradiated. After 7 days incubation, numbers of neurospheres consisting of at least 50 cells were counted under a microscope. For the limiting dilution assay, increasing numbers of NMA-23 cells were plated in 96-well plates in 200 μl serum-free medium and treated with radiation or LY2109761 or their combination. After 7 days culture, the percentage of wells not containing neurospheres for each cell-plating density was calculated and plotted against the number of cells plated per well. For the proliferation assay, 50,000 NMA-23 cells were seeded on 25 cm² flasks over night and then treated as described, incubated for another 72 h, when the numbers of living cells were counted.
**Immunofluorescent staining for γH2AX**

Cells were grown and treated in chamber slides. At specified time points, cells were fixed with 3% paraformaldehyde for 10 min and then permeabilized with 0.5% Triton-X-100 for 30 min on ice. Antibody (AlexaFluor488 anti-H2AX phosphorylated (Ser139), Biolegend, San Diego, CA) was added at a dilution of 1:100 in 3% BSA and incubated overnight at 4°C. Cells were washed and nuclei were counterstained with 1 µg/ml 4,6-diamidino-2-phenylindole. Foci were counted by the automated image-analysis system Metacyte with a Zeiss Axioplan-2 imaging epi-fluorescence microscope equipped with ISIS software (MetaSystems, Altussheim, Germany). For each treatment condition, γH2AX foci were analyzed in at least 400 cells and median number of foci was determined.

**Apoptosis and flow cytometry**

24 h after treatments, U87MG cells and NMA-23 cells were prepared for FACS analysis (FACScan, Becton Dickinson). Cells were fixed in 70% ethanol, centrifuged, washed in PBS and the supernatant was removed. Cells were resuspended in the staining solution of PBS, RNAse (200µg/ml) and propidium iodide (10µg/ml) and were analyzed for cells with sub-G1 DNA content. To measure caspase-3 activity, cells were treated according to the manufacturer’s suggestions (BD Pharmingen), incubated for 20 min on ice, pelleted, washed and resuspended in washing buffer plus PE conjugated monoclonal active caspase-3 (BD Pharmingen) and analyzed by flow cytometry and Flowjo software (Tree Star, Inc. Ashland, OR).

**Western blot analysis**

6 h after treatment as indicated, cells were washed two times with PBS and lysed using Qproteome Mammalian Protein Prep Kit (QIAGEN GmbH, Hilden, Germany) supplemented with a complete protease and phosphate inhibitor cocktail (Sigma, Munich, Germany). Protein concentration was measured using a Bradford assay (Pierce Chemical Co., Rockford, IL), and samples were immunoblotted with antibodies against Phospho (P)-SMAD2 and total
SMAD2 (Cell signalling) with an anti β-actin monoclonal antibody (Sigma) as internal loading control.

**Microarray analysis**

U87MG cells were solubilized and homogenized in TRIzol (Invitrogen, Carlsbad, CA) 6 h after radiation +/- LY2109761 treatment (4 Gy, 10 µmol/L). For LY2109761 treatment cells were exposed to LY2109761 for two hours prior to irradiation as in the other in vitro assays. Total RNA was isolated according to the manufacturer’s instruction, purity and integrity of the RNA was assessed with Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA), and 200 ng of total RNA was used for amplification and labeling using the Agilent’s Low Input Quick-Amp Labeling Kit (#5190-0442) following the detailed kit protocol. Cyanine 3 labeled cRNA was purified using QIAGEN’s RNeasy mini spin columns. Quantity and cyanine incorporation of labeled cRNAs was determined using a Nanodrop ND.1000 Spectrophotometer (Peqlab Biotechnologies GmbH). The labeled probes were then hybridized to an Agilent 4x44 K Whole Human Genome Microarray (G4112F) containing 45,015 features representing 41,000 unique probes. After washing, microarrays were scanned using an Agilent Array scanner (Agilent Technologies). Data were extracted with Agilent feature extraction software (Agilent version 9.1) and statistically analyzed with SUMO software as previously described (20). Ingenuity pathway analysis was used for functional analysis (IPA, Ingenuity Systems, Inc., Redwood City, CA) (21). Microarray data were deposited in “ArrayExpress” (Accession No.: E-TABM-1148).

**Quantitative real-time PCR (qRT-PCR)**

To quantify mRNA expression, qRT-PCR was performed using QuantiTect Primer assay (QIAGEN GmbH, Hilden, Germany) and QuantiTect SYBR Green RT-PCR Kit (#204243, QIAGEN) on a LightCycler 480 instrument (Roche Diagnostics). The relative expression of the target genes were calculated by normalizing the \(C_p\) (crossing point) values with those of housekeeping gene GAPDH.

**Tumor growth in the s.c. U87MG model in Balb mice**
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

All animal experiments were approved by in-house and governmental animal protection committees. 5 x 10⁶ U87MG cells were injected subcutaneously (s.c.) into the right hind limb of 6- to 8 week-old BALB/c athymic nude mice (Charles River Laboratories, Sulzfeld, Germany). Animals were randomized into four groups (control, LY2109761, radiation, radiation plus LY2109761, n=10 each). Treatments started when tumors were established and reached a volume of approximately 150 mm³. Tumors were irradiated with fractionated radiotherapy (5 x 2 Gy, day 0 – 4 for 5 consecutive days) using a 6 MV LINAC (Siemens, Germany). LY2109761 was administered orally at 50 mg/kg twice daily (days 1-5 of each week) until the end of observation. Tumor volume for the s.c. experiment was determined 3 times weekly by direct measurement with calipers (volume = length x width x width x 0.5).

**Tumor growth and animal survival in the orthotopic CSLC model in SCID mice**

Beige SCID mice (8-wk old, 20g; Charles River Laboratories, Sulzfeld, Germany) were anesthetized and stereotactically inoculated with NMA-23 cells (10⁴ cells in 2 µl PBS) into the left forebrain (2 mm lateral, 1 mm anterior to bregma, at 3 mm depth from skull surface). Animals were randomized into four treatment groups (control, LY2109761, radiation, radiation plus LY2109761, n=13 each, with four mice scheduled for histology). LY2109761 treatment (50 mg/kg twice daily) started on day 1 after tumor inoculation and was administered 5 days weekly until the end of observation. Radiation was delivered on day 4 to the entire head of anesthetized mice (7Gy single dose) using the 6 MV LINAC. On day 15 animals were examined by magnetic resonance imaging (MRI) at 1.5-T (Siemens Magnetom Vision, Erlangen, Germany) using a custom-made small animal solenoid Tx/Rx radiofrequency coil. Tumor volumes were estimated using Gadolinium enhanced T1 weighted spin-echo images. A three-dimensional reconstruction of the tumor surface and calculation of the tumor volume were performed with the QuickVol software as previously described (22). For survival studies, moribund mice or mice with severe neurologic symptoms were euthanized.

**Tumor histology and immunohistochemistry**

Four animals per group were sacrificed on day 16 and exsanguinated by transcardial perfusion first with ice cold PBS. Brains were dissected, partially cryofixated or embedded in paraffin
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

After fixation in 4% paraformaldehyde for 24 h. Sections of paraffin-embedded blocks were stained with hematoxylin-eosin. Immunohistochemistry was carried out with frozen sections as previously described (23). Primary antibodies included: anti-Vimentin (rabbit polyclonal, Abcam, 1:200), anti-CD31 (rabbit polyclonal, BD Biosciences PharMingen, 1:100), anti-Fibronectin (rabbit polyclonal, Abcam, 1:400), anti-COL5A1 (rabbit polyclonal, Santa Cruz, 1:100) and anti-YKL-40 (rabbit polyclonal, Quidel, 1:200). After incubation with primary antibodies, the appropriate fluorescence-labeled secondary antibodies were applied. Images were captured using a Nikon Eclipse E600 microscope equipped with a Nikon digital sight DS-U1 camera and subsequently analyzed using ImageJ software (NIH, Bethesda, Maryland). For each treatment condition, the analysis was performed in at least 5 randomly chosen fields from 3 to 5 sections.

**Statistical Analysis**

The unpaired two-tailed t test was used for the comparison of parameters between groups. The Kaplan–Meier method was used to determine the median survival time and a log-rank test was used to compare the differences between survival curves. A value of *P* < 0.05 was considered significant. The statistical analysis was performed using the software package Statistika 6.0 (Statsoft).

**RESULTS**

**LY2109761 enhanced radiosensitivity of glioblastoma cells**

To determine the effects of LY2109761 on GBM tumor cell radiosensitivity, clonogenic survival analysis was performed. LY2109761 pretreatment reduced clonogenic survival in cell cultures of U87MG (Fig. 1A) and T98 (Fig. 1B) following radiation, resulting in an increase in the radiosensitivity with a DEF0.1 of 1.30 and 1.37, respectively. Likewise, TGBFR1 siRNA reduced TGBFR1 mRNA expression and inhibited SMAD2 phosphorylation (Supplementary Fig.1), associated with an increase in the radiosensitivity with a DEF0.1 of 1.34 in U87MG cells (Fig. 1C), supporting the notion that LY2109761 exhibits its radiosensitizing effect via TGF-β signaling blockage.
LY2109761 reduced GBM derived CSLCs self-renewal and proliferation and sensitizes them to radiation

Considering that glioblastoma stem cells have been linked to radioresistance, we explored whether LY2109761 would inhibit the self-renewal of GBM CSLCs and increase the sensitivity of these tumor-initiating cells towards radiation. We employed neurosphere formation as a surrogate marker of the clonogenic survival of glioblastoma CSLC given they grow in suspension (24). We found that LY2109761 (10 µmol/L) or radiation (4 Gy) alone reduced neurosphere forming efficiency in NMA-23 cells. The combination of LY2109761 plus radiation had supra-additive effects in neurosphere formation and limiting dilution assays (Fig. 2A, B and D). Likewise, blockage of TGF-β signaling via TGBFR1 siRNA alone or combined with radiation also reduced clonogenicity of NMA-23 cells (Fig. 2C). Furthermore, a proliferation assay revealed a reduction of NMA-23 cells proliferation/viability after LY2109761 or radiation treatment alone, and their combination resulted in a further reduction of the cell count (Fig. 2E).

LY2109761 enhanced radiation-induced DNA damage and apoptosis rates in glioblastoma cells and GBM derived CSLCs

DNA damage and repair are important components of radiation-induced cytotoxicity. As a marker of recognized DNA double-strand breaks (DSB), we evaluated the induction of nuclear foci of phosphorylated histone H2AX (γH2AX). U87MG and NMA-23 cells were pretreated with LY2109761 (10 µmol/L) for 2 hours and irradiated (2 Gy). In both cells, LY2109761 significantly increased the number of radiation-induced γH2AX foci at 30 min and 24h post-irradiation (Fig. 3A, B and C) while LY2109761 monotherapy did not significantly alter (p>0.5) the number of γH2AX foci. These data suggested that LY2109761 increased radiation-induced DSBs and inhibited DNA damage repair.

Next, we analyzed whether LY2109761 also affected apoptosis. We found in both U87MG cells and NMA-23 cells that 2 h pretreatment with LY2109761 before irradiation increased apoptosis rates in a supra-additive manner in both sub-G1 and caspase-3 activity assays at 24
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

h after radiation (Fig 3D, E).

**LY2109761 suppressed SMAD2 phosphorylation in both glioblastoma cells and GBM derived CSLCs**

Because SMAD proteins are central mediators of signals from TGF-β receptors, we evaluated the effect of LY2109761 on the phosphorylation of SMAD2 (p-SMAD2), one of their immediate downstream targets. As expected, LY2109761 (10 µmol/L for U87MG and NMA-23; 5 µmol/L for T98) alone or combination with radiation (4 Gy) effectively suppressed SMAD2 phosphorylation (Fig.4).

**LY2109761 altered gene expression and pathway analysis in glioblastoma cells**

To further investigate the potential molecular basis of the interaction of LY2109761 and radiation, gene expression analysis was performed on U87MG cells using microarrays. A heatmap was generated (Fig 5A) representing 988 transcripts comparing significantly differentially regulated genes for each treatment condition (LY, RT, LY+RT) versus untreated controls. Selected genes were found similarly regulated in NMA-23 cells as in U87MG cells, which was confirmed using qRT-PCR (Fig. 5B and Supplementary Fig. 2), including ID1, HEY1, ANGPT2, BIRC2 (down-regulated by LY2109761 treatment) and RHOB, LRIG1, ACTG2 (up-regulated by LY2109761 treatment) as well as AKT2, ATF5, LMO2 (up-regulated by radiation and reversed by LY2109761 pretreatment).

Further analysis using Ingenuity Pathway Analysis (IPA) of differentially regulated genes with at least 2-fold change (LY: 111 genes; RT: 36 genes; LY+RT: 157 genes) revealed eight functional categories with highly affected and enriched transcripts (Fig. 5C). The associated molecular and cellular functions are related to cellular movement, cellular growth and proliferation, gene expression, cell death, cell cycle, cell-to-cell signaling and interaction, cell signaling, and molecular transport. For example, the top function for LY2109761 and radiation alone or their combination was associated with cellular movement, cell death and cellular growth and proliferation.

Venn diagrams revealed the treatment specific up-regulated and down-regulated genes
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

(Fig.5D) and also the commonly affected genes (Fig 5D). A significant number of genes were commonly affected under the treatment of LY2109761 and radiation plus LY2109761, whereas considerably fewer genes were commonly affected by radiation and radiation plus LY2109761, suggesting a comprehensive impact of LY2109761 on radiation response at the gene expression level when combined with radiotherapy.

**LY2109761 enhanced radiation-induced tumor growth delay in a U87MG subcutaneous xenograft tumor model in Balb c nude mice**

A tumor growth delay experiment was performed using U87MG tumors growing s.c. in the hind leg of nude mice. As shown in Fig. 6A, the time for 5-fold increase in tumor size (from 150 mm$^3$ to 750 mm$^3$) was calculated using the tumor volumes from the individual mice in each group. The mean time for 5-fold increase in tumor size increased from 11.4 days for sham-treated mice to 17.2 days for LY2109761 treated mice and 23.9 days for irradiated mice. Radiation plus LY2109761 increased the time to 36.4 days. The tumor growth delay versus controls was 5.8 d for LY2109761 alone, 12.5 d for radiation alone, and 25.0 days for the combination, indicating a supra-additive effect between LY2109761 and radiation on growth delay of U87MG xenografts.

**LY2109761 increased survival in an orthotopical CSLCs glioblastoma model and enhanced antitumor activity of radiation**

The effect of LY2109761+/-radiation on intracranial CSLCs (NMA-23) tumor growth was monitored by noninvasive magnetic resonance imaging (MRI). By day 15, both LY2109761 and radiation monotherapy markedly inhibited tumor growth by 43.4% and 54.3% vs. controls (Fig.6B and C), which was further reduced by the combination to 76.3%. The Kaplan–Meier survival curves showed that, LY2109761 alone increased modestly but statistically significantly animal survival compared to controls, with a median survival time (MST) of 20 days vs. 18 days (p<0.05, logrank; Fig. 6D); radiotherapy alone prolonged the MST to 22 days. Importantly, the combination treatment with LY2109761 plus radiotherapy further increased the MST to 28 days (p<0.05 vs. respective monotherapies) indicating a supra-additive effect on tumor growth and animal survival between LY2109761 and
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

Radiotherapy in the orthotopic glioblastoma model.

**LY2109761 inhibited tumor invasion and reduced tumor microvessel density**

Previous studies have shown that glioblastoma cells can express the intermediate neurofilament vimentin, including those tumor cells which infiltrate into normal parenchyma. To evaluate tumor invasiveness in intracranial tumor sections hematoxylin-eosin staining and immunohistochemical staining for vimentin was done. As shown in Fig. 7A and B, sham-treated intracranial xenografts displayed peripheral invasion of the surrounding brain as single cells and cell clusters, frequently surrounded by numerous small-satellite tumors. Interestingly, a more invasive growth pattern was observed after radiotherapy alone compared to controls. In contrast, tumors from mice receiving LY2109761 monotherapy or LY2109761 plus radiotherapy displayed a significantly reduced invasion growth pattern compared with the control and radiotherapy group.

Next we investigated tumor angiogenesis as another important process of tumor growth particularly pertinent in glioblastoma using CD31 endothelial staining and microvessel density (MVD) analysis. We found that LY2109761 alone (MVD: 39.2±8.4) and radiotherapy alone (47.7±10.5) reduced MVD vs. controls (62.2±10.55, p<0.05, Fig 7B) More importantly, LY2109761 plus radiation resulted in a further reduction in MVD (23.4±5.9) versus monotherapies (p<0.05), suggesting a beneficial combination effect on reducing tumor angiogenesis in the orthotopic CSLC model (Fig.7B and C).

**LY2109761 inhibited mesenchymal change in the CSLC orthotopic glioblastoma model**

A mesenchymal character has been associated with increased malignancy, treatment resistance and poor prognosis in glioblastoma. We found here that LY2109761 inhibited the mesenchymal character in our orthotopic CSLCs model. Immunohistochemical staining showed that LY2109761 alone and in combination with radiotherapy reduced the expression of established mesenchymal marker proteins including fibronectin, COL5A1 and YKL-40 (23, 25) (Fig. 7B).
DISCUSSION

The pivotal role of TGF-β in promoting cellular processes that are important for glioblastoma progression suggests that this pathway may be a promising target for therapy. In the present study, we provide evidence that TGF-β signaling blockade by the small-molecule TβRI kinase inhibitor, LY2109761, is an effective treatment approach and can moreover augment radiation response in human glioblastoma. This conclusion is supported by several pieces of evidence. First, LY2109761 significantly enhanced radiation–induced cytotoxicity and cooperated with radiation to suppress clonogenic survival of established glioblastoma cell lines. Mechanistic studies revealed that LY2109761 may act in concert with radiation to enhance radiation-induced DNA damage and apoptosis. Second, LY2109761 also increased the sensitivity of primary GBM CSLCs derived from surgical specimens towards radiation. Third, LY2109761 enhanced radiation-induced tumor growth delay in two independent models: in U87MG tumors growing subcutaneously in balb c nu/nu mice and in orthotopic CSLC tumors growing in brains of SCID mice. In this orthotopic brain tumor model LY2109761 increased animal survival alone and increased the radiation-induced prolongation of the animal life span.

The interesting finding that TGF-β inhibition impedes the cellular DNA damage stress response and results in increased radiosensitivity had been shown in mammary epithelial cells (26). While these authors reported a reduced γH2AX foci number following TGF-β inhibition, we found here an increase of radiation-induced γH2AX foci number which may be due to the difference in cell types or TGF-β inhibitors used.

Targeting cancer stem-like cells in human tumors including glioblastoma is an evolving concept for developing new treatment options in cancer. Stem cells have been reported to be responsible for the initiation, propagation, recurrence, and radioresistance of gliomas (6). Notably, the most effective of targeted therapies may have activity against cancer stem-like
cells. Encouragingly, we found that LY2109761 reduced the self-renewal and proliferation capability of GBM CSLCs and also enhanced the radiosensitivity of GBM CSLCs. More importantly, LY2109761 further increased the anti-tumor effects of radiation and increased animal survival when GBM CSLCs were injected orthotopically into mice brains.

Invasion is another important feature of human glioblastoma responsible for their dismal outcomes. Although ionizing radiation is the mainstay of nonsurgical treatment in GBM, radiation may also promote migration and invasiveness of glioma cells (27) which has been attributed in part to increased levels of TGF-β (28). Accordingly, our results indicated that radiation may promote glioblastoma cell invasion while the blockade of TGF-β signaling using LY2109761 inhibited both constitutive and radiation-provoked tumor cell invasion in the orthotopic model.

Activated tumor angiogenesis is another characteristic feature of GBM, contributing to tumor invasiveness and radioresistance. The antiangiogenic effects of targeting TGF-β (29, 30) could be corroborated in our study, because LY2109761 alone and in combination with radiation reduced tumor microvessel density, indicating that LY2109761 has direct anti-migratory and anti-angiogenic properties, which can be beneficial in the context of an attenuation of unwanted side effects of radiotherapy such as certain pro-migratory and pro-angiogenic effects.

The fundamental role for mesenchymal change in promoting invasion, treatment response and even cancer stem cell function in human carcinoma and GBM invasion is increasingly recognized (6, 31). Recent studies have established that TGF-β is a master regulator of epithelial to mesenchymal transition (EMT) in carcinoma (32). To our best knowledge, its relevance to mesenchymal change in GBM models has not been reported. Here we found that the blockade of TGF-β signaling using LY2109761 markedly reduced the expression of mesenchymal markers in the orthotopic glioblastoma model. It is conceivable that this inhibitory effect on mesenchymal change of LY2109761 at least partly contributes to its anti-migratory capacity.
Considering that TGF-β is involved in multiple signaling pathways genome wide microarray analysis was performed to investigate potential mechanisms of LY2109761, TGF-β signaling, and radiation interactions. The regulated genes represent functional classes involved in diverse biological processes including cellular movement, cellular growth and proliferation, cell death, cell cycle, cell-to-cell signaling and interaction and cell signaling. ID1 (along with other ID family members ID2, ID3 and ID4, data not shown), HEY1, ANGPT2, BIRC2 (cIAP1, Inhibitor of apoptosis protein 1) were among the genes down-regulated by LY2109761. ID1 has been reported to promote invasion, to mediate tumor angiogenesis by production of vascular endothelial growth factor, to be involved in the resistance of cancer cells against cytotoxic drugs (33), and has also been implicated in cancer stem cell function (34) as well as EMT process (35). Our data are here in line with a very recent report focusing on the effects of LY2109761 on ID1 (36). Angiopoietins (ANGPTs) are ligands of the endothelial cell receptor TIE2 and have crucial roles in the tumor angiogenic switch, inflammation, metastasis and lymphangiogenesis. Increased expression of ANGPT2 and higher ANGPT2/ANGPT1 ratios in tumors correlate with poor prognosis in many cancers. Agents specifically targeting ANGPT1 and ANGPT2 are currently in Phase II clinical trials (37). High expression levels of IAPs which inhibit apoptosis have been associated with poor treatment response and prognosis (38). In addition, we also indentified and confirmed several genes up-regulated by LY2109761 treatment, including RHOB, LRIG1 and ACTG2, which act as tumor suppressors and have putative roles in tumor therapy resistance and DNA damage response (39-41).

Radiotherapy is an effective treatment modality for glioblastoma. To evade radiation toxicity, certain pathways might be activated which contribute to tumor invasiveness, angiogenesis and radioresistance (42, 43). The set of such genes which were upregulated by radiation and reversed by LY2109761 further included: AKT, ATF5, and LMO2. AKT is a major downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway, a potent prosurvival pathway, associated with radioresistance of cancer cells with AKT inhibition
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

having been shown to increase radiation effects on tumors (44). Activating transcription factor 5 (ATF5) is highly expressed in malignant glioma and plays an important role in promoting cell survival by stimulating transcription of MCL1, an anti-apoptotic BCL2 family member (45). ATF5 has been shown to be a potent repressor of p53, and elevated expression of ATF5 in tumor is related to radioresistance and greater cell motility (46). The LIM-domain protein LMO2 is a specific regulator of tumor angiogenesis, which has been suggested an attractive drug target in cancer (47). Together these array data may provide additional molecular signaling hints for the beneficial effects observed after combined TGF-β signaling inhibition with LY2109761 and radiation in the treatment of glioblastoma.

In summary, we have provided evidence that LY2109761, a specific TβRI kinase inhibitor, is active against glioblastoma alone and enhances the antitumor efficacy of radiation both in vitro and in vivo, in particular in GBM CSLCs. Our findings rationalize further translational studies of LY2109761 or agents with similar properties alone and in combination with radiotherapy in the treatment of glioblastoma.
References

LY2109761 and radiation in an orthotopic glioblastoma model Zhang et al.

LY2109761 and radiation in an orthotopic glioblastoma model   Zhang et al.


Figure legends

**Fig. 1.** The effects of LY2109761 or TGFBR1 siRNA on radiosensitivity of glioblastoma cell lines measured by clonogenic survival assay. U87MG and T98 cells were pretreated with 10 µmol/L and 5 µmol/L of LY2109761, respectively (which resulted in a surviving fraction of about 80%, data not shown), 2 h before irradiation or transfected with siRNA targeting TGBFR1 or negative nonsense siRNA for 48 h before radiation. Colony-forming efficiency was determined 10 to 14 d later and survival curves were generated after correction for the cytotoxicity induced by LY2109761 alone and linear-quadratic (LQ) equation was fitted to data sets. A, U87MG treated with/without LY2109761. B, T98 treated with/without LY2109761. C, U87MG treated with siRNA or negative control (DEF0.1=1.34 when siRNA vs. negative control). Points, mean; bars, SD; DEF, dose enhancement factor; LY, LY2109761.

**Fig. 2.** LY2109761 or TGFBR1 siRNA reduces NMA-23 self-renewal and proliferation and sensitizes them to radiation. A, representative images (40 x) of neurospheres formed by NMA-23 cells treated with LY2109761 (LY, 10 µmol/L) or radiation (RT, 4 Gy) or their combination. B, the number of neurospheres per flask, compared between the control group and cells treated as indicated. C, the number of neurospheres per flask, compared between the untreated group and cells treated with negative control, siRNA, RT, or siRNA+RT. D, NMA-23 cells self-renewal capacity was evaluated in a limiting dilution assay under each treatment condition. The data are presented as the percentage of wells not containing neurospheres for each cell-plating density. E, NMA-23 cells proliferation was determined by cell count after 72 h exposure to each treatment. Relative numbers of cells are shown as histogram. Columns, mean; bars, SD; *, P < 0.05 vs. control (or negative control); **, P < 0.05 vs. control (or negative control) and each monotherapy; #, P > 0.05 vs. untreated.

**Fig. 3.** LY2109761 increases radiation-induced γH2AX foci and increases radiation-induced apoptosis in U87MG and NMA-23 cells. A, representative images of γH2AX foci immunofluorescent staining obtained from control and treated cells at 24 h after radiation in U87MG. B, representative results of the foci number distribution using an automated image-analysis system for treated and control cells at 24 h after radiation in U87MG. The histogram depicts the frequency of the cells with a specific number of foci and the line-scatter plot shows the accumulated percentage of cell frequency. C, the median number of foci compared between controls and treated groups in U87MG (left) and NMA-23 (right). Columns, mean; bars, SD; *, P < 0.05. D, combined treatment with LY2109761 and irradiation substantially enhanced apoptosis rates in a supra-additive manner. The percentage of apoptotic cells was determined by flow cytometric analysis of cells with sub-G1 DNA content and active caspase-3 at 24 hours after treatments in U87MG (left) and NMA-23 (right). U87MG cells and NMA-23 cells were pretreated with LY2109761 (10 µmol/L) for 2 hours and irradiated (8 Gy). Columns, mean; bars, SD; *, P < 0.05 vs. control, **, P < 0.05 vs. control and respective single treatment. E, representative results of flow cytometric analysis for active caspase-3 using Flowjo software at 24 hours after treatments.
LY2109761 and radiation in an orthotopic glioblastoma model  Zhang et al.

Fig. 4. The effects of LY2109761 on the activation of SMAD2-dependent downstream pathway of TGF-β in U87MG, T98 and NMA-23 cells. Cells were treated with LY2109761 (10 μmol/L for U87MG and NMA-23; 5 μmol/L for T98), or radiation (4 Gy), or their combination, 6 h later cells were lysed and whole cell lysate were subjected to western blot with anti-phospho-SMAD2 and anti-total-SMAD2 antibodies. β-actin monoclonal antibody was used as an internal loading control.

Fig. 5. The effects of each treatment on gene expression profiles generated from U87MG cells. A, A heatmap representing 988 transcripts was generated using 4-class t test (P=1.0E-004). Sets of differentially regulated genes from 3 biological replicates were indentified for each treatment condition (LY : P =1.0E-004; RT: P =2.0E-003; LY+RT: P =1.0E-003) compared with the gene expression profile from untreated control using 2-class t test. Color intensity is assigned to ratios of gene expression; shades of red, genes that are up-regulated; shades of green, genes that are down-regulated; black, genes that are unchanged. B, Fold-changes in expression levels of genes regulated by LY2109761 (upper) and those up-regulated by radiation and reversed by radiation plus LY2109761 (lower) as determined by microarray and qRT-PCR methods. C, Bar graph representing functions significantly different between treatments from Ingenuity Pathway Analysis derived from genes with >2 fold change. X-axis indicated the significant score in each functional category. Given the degree of enrich in the functions, which is reflected by the significance score (negative logarithm of the P value), the results indicate that the genes affected by treatments do not simply comprise a random list and may therefore be of potential functional relevance. D, Venn diagrams depicting the number of genes that were commonly up-regulated or down-regulated by different treatments.

Fig. 6. In vivo anti-tumor activity of LY2109761 ± radiation in a subcutaneous model and in an independent orthotopic glioblastoma model. A, BALB/c nu/nu mice with U87MG human glioblastoma growing s.c. were treated as described in Materials and Methods. Tumor volumes were calculated using the formula v=length x width x width x 0.5. Data, means +/-SE; n=10 animals in each group. Statistical significance is designated as: *, P < 0.05 vs. control; **, P < 0.05 vs. control and each monotherapy. B, Representative GD-enhanced T1-weighed images of orthotopic xenograft from sham-treated control animals and animals that had received LY2109761 (LY) or radiation (RT) or their combination examined by MRI 15 days post tumor injection. C, Tumor volumes estimated from MRI. @, P < 0.05 vs. control; @@, P < 0.05 vs. control and each monotherapy. D, Kaplan-Meier survival analysis of SCID Beige mice transplanted intracranially with NMA-23 cells treated with LY2109761 (LY) or
LY2109761 and radiation in an orthotopic glioblastoma model

Zhang et al.

radiation (RT) or their combination. Differences between survival curves were compared using a log-rank test. *, P < 0.05 for median survival time (MST).

**Fig. 7.** Histological studies of glioblastoma orthotopic xenografts from mice treated with LY2109761 (LY), or radiation (RT), or both. Original magnification 200x. **A**, Representative examples for the hematoxylin-eosin staining. **B**, Representative examples for the immunofluorescence staining of human vimentin, CD31, fibronectin, COL5A1 and YKL-40. T, tumor; B, normal brain. **C**, Quantitative analysis of CD31 staining in tumors given as microvessel density (MVD). For quantification of MVD, vessels on each section were counted in five high power fields (magnification 200 x). Columns, mean; bars, SD. *, P < 0.05 vs. control; **, P < 0.05 vs. control and respective single treatment.

**Supplementary Fig.1.** TGBFR1 mRNA and p-SMAD2 protein level of U87MG cells and NMA-23 cells after siRNA transfection. **A** and **B**, TGBFR1 mRNA levels of untreated cells and cells treated with siRNA targeting TGBFR1 or negative nonsense siRNA were measured by quantitative real-time PCR 48 h after transfection. TGBFR1 mRNA values were normalized to those of untreated. **C**, p-SMAD2 protein levels of untreated cells and cells treated with siRNA targeting TGBFR1 or negative nonsense siRNA were examined by Western blot with a SMAD2 phosphorylation specific antibody 48 h after transfection. β-actin served as an internal loading control.

**Supplementary Fig.2.** Fold-changes in mRNA expression levels of genes in NMA-23 cells regulated by LY2109761 (A) and those up-regulated by radiation and reversed by radiation plus LY2109761 (B) as determined by quantitative real-time PCR methods.
Fig. 1

A

U87MG

Surviving fraction

0 2 4 6 8 10 Radiation dose (Gy)

Control
LY 10 umol/L
DEF0.1=1.30

B

T98

Surviving fraction

0 2 4 6 8 10 Radiation dose (Gy)

Control
LY 5 umol/L
DEF0.1=1.37

C

U87MG

Surviving fraction

0 2 4 6 8 10 Radiation dose (Gy)

Untreated control
Negative control
TGFB1 siRNA
DEF0.1=1.34
Fig. 2

Zhang et al

A

control

LY

RT

LY+RT

B

Number of spheres

Control LY RT LY+RT

C

Number of spheres

untreated negative control siRNA RT siRNA+RT

D

% wells without neurospheres

Number of cells per well

Control LY RT LY+RT

E

Relative proliferation

Control LY RT LY+RT

Downloaded from cancerres.aacrjournals.org on April 19, 2017. © 2011 American Association for Cancer Research.
Fig. 3

A

B

H2AX foci number

Frequency of cells

Accumulated frequency of cells (%)
Fig. 3

C

U87MG

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LY</th>
<th>RT</th>
<th>LY+RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Median number of foci

NMA-23

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LY</th>
<th>RT</th>
<th>LY+RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Median number of foci

D

Active caspase-3

U87MG

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LY</th>
<th>RT</th>
<th>LY+RT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Apoptotic cells (%)

NMA-23

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>LY</th>
<th>RT</th>
<th>LY+RT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Apoptotic fraction (%)

E

Sample Name

- Ly+radiation-1.010
- radiation-1.007
- Ly-1.004
- ctrl-1.001

Downloaded from cancerres.aacrjournals.org on April 19, 2017. © 2011 American Association for Cancer Research.
### Fig. 4

<table>
<thead>
<tr>
<th></th>
<th>U87MG</th>
<th>T98</th>
<th>NMA-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY2109761</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Radiation</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>p-SMAD2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total-SMAD2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Image of Western Blots](image-url)
Fig. 5
Fig. 5

C

© 2000-2010 Ingenuity Systems, Inc. All rights reserved.
Fig. 5

D

Up-regulated

Down-regulated
Fig. 6

A

![Graph showing tumor volume over time with different treatments.](image1)

B

![Images of tumors with control and treatment groups.](image2)

C

![Box plots of tumor volume with different treatments.](image3)

D

![Survival curve with different treatment groups.](image4)
Fig. 7

A

control

LY

RT

LY + RT
Fig. 7B

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>LY</th>
<th>RT</th>
<th>LY+RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL5A1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YKL-40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7C

![Graph showing MVD (Microvessel Density) for CD31+ per field](image)

- **Control**: 60 ± 5
- **LY**: 45 ± 5
- **RT**: 50 ± 5
- **LY+RT**: 30 ± 5

* p < 0.05
** p < 0.01
Blockade of TGF-beta signaling by the TGFβR-I kinase Inhibitor LY2109761 enhances radiation response and prolongs survival in glioblastoma

Mengxian Zhang, Susanne Kleber, Manuel Roehrich, et al.

Cancer Res Published OnlineFirst October 17, 2011.