Small Molecule Therapeutics

NT113, a Pan-ERBB Inhibitor with High Brain Penetration, Inhibits the Growth of Glioblastoma Xenografts with EGFR Amplification

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Abstract

This report describes results from our analysis of the activity and biodistribution of a novel pan-ERBB inhibitor, NT113, when used in treating mice with intracranial glioblastoma (GBM) xenografts. Approaches used in this investigation include: bioluminescence imaging (BLI) for monitoring intracranial tumor growth and response to therapy; determination of survival benefit from treatment; analysis of tumor IHC reactivity for indication of treatment effect on proliferation and apoptotic response; Western blot analysis for determination of effects of treatment on ERBB and ERBB signaling mediator activation; and high-performance liquid chromatography for determination of NT113 concentration in tissue extracts from animals receiving oral administration of inhibitor. Our results show that NT113 is active against GBM xenografts in which wild-type EGFR or EGFRvIII is highly expressed. In experiments including lapatinib and/or erlotinib, NT113 treatment was associated with the most substantial improvement in survival, as well as the most substantial tumor growth inhibition, as indicated by BLI and IHC results. Western blot analysis results indicated that NT113 has inhibitory activity, both in vivo and in vitro, on ERBB family member phosphorylation, as well as on the phosphorylation of downstream signaling mediator Akt. Results from the analysis of animal tissues revealed significantly higher NT113 normal brain-to-plasma and intracranial tumor-to-plasma ratios for NT113, relative to erlotinib, indicating superior NT113 partitioning to intracranial tissue compartments. These data provide a strong rationale for the clinical investigation of NT113, a novel ERBB inhibitor, in treating patients with GBM.

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Introduction

ERBB family tyrosine kinases, especially EGFR, continue to attract substantial attention as therapeutic targets for treating various forms of cancer, including glioblastoma (GBM), the most common and malignant form of primary brain tumor in adults (1). EGFR is amplified and/or rearranged in up to 40% of GBM (2–4), and as a result, it is widely considered a key oncogenic driver of the aggressive biologic behavior of a sizeable subgroup of GBM.

First-generation EGFR inhibitors erlotinib and gefitinib, as well as dual EGFR + ERBB2/HER2 inhibitor lapatinib, have been used, and continue to be used, in clinical neuro-oncology, despite widespread appreciation of their shortcomings, which include limited central nervous system penetration (5–7), conformational limiting effects of GBM-specific mutant EGFR on inhibitor activity (8), and GBM adaptation to EGFR inhibition through activation of alternative receptor tyrosine kinases (9, 10). Contemporary clinical trials for treating patients with GBM with EGFR-directed therapeutics are seeking to enrich for responders by using patient tumor EGFR status as a clinical trial.
inclusion/exclusion criterion (http://clinicaltrials.gov/show/NCT01475006). Despite the use of responder enrichment strategies, there is yet to be clear indication of consistent and/or substantial benefit from the use of EGFR-targeted therapies, as single agents, in treating patients with GBM (11, 12). More recently, preclinical and clinical investigations of ERBB inhibitors for treating GBM have shifted focus to combination therapy approaches that are intended to address resistance mechanisms to EGFR-directed monotherapy (13, 14).

An additional concept that is currently being investigated for exploiting EGFR as a therapeutic target in cancer, including GBM, involves the use of second-generation irreversible ERBB and EGFR inhibitors (15). A concern for the use of such inhibitors, especially the pan inhibitors that act against multiple ERBB family members, is achieving a therapeutic window necessary for maximizing antitumor activity, while minimizing adverse events. A trial of afatinib in recurrent GBM may have been negative for these reasons (16).

In the current study, we have conducted preclinical analysis of a novel irreversible pan-ERBB inhibitor, NT113, for activity against orthotopic GBM xenografts. Our interest in testing this therapeutic stems from its preferential partitioning in brain, combined with favorable in vivo stability (17). Our results indicate high-level expression of wild-type or vIII mutant EGFR as identifying NT113-responsive GBM, using an NT113 administration regimen that is well tolerated and without indication of adverse events in animal subjects. In aggregate, our results support NT113 clinical investigation in patients with GBM whose tumors express high levels of EGFR, a molecular characteristic that is invariably associated with corresponding gene amplification (2–4).

Materials and Methods

Investigational agent

NT113 is a quinazolinyl acrylamide-based pan-ERBB irreversible inhibitor, and was provided by NewGen Therapeutics. For oral administration to animal subjects, NT113, as well as erlotinib and lapatinib (LC Laboratories), were dissolved in 2% N,N-Dimethylacetamide and 40% 2-Hydroxypropyl-beta-cyclodextrin at concentrations of 10, 100, and 150 mg/mL, respectively. For addition to cell cultures, stock solutions of NT113 and erlotinib were prepared by dissolution in DMSO at 10 mmol/L.

GBM cell sources

The U87 cell line was obtained from ATCC (catalogue identifier HTB14). U87 modification by retroviral introduction of EGFRvIII, in developing the derivative cell line U87vIII, has been described (18). Human GBM tissues, GBM6, GBM12, and GBM39, are maintained as serially passaged subcutaneous xenografts in athymic mice (19). Information about the EGFR status of these xenografts, and available clinical characteristics of the patients from which they were derived, have been previously described (20). Each of these, as well as the U87 and U87vIII cell lines, has been modified by lentiviral infection for stable expression of firefly luciferase to enable in vivo bioluminescence imaging (BLI; ref. 21). The procedure for the preparation of tumor cells from subcutaneous xenografts for transfer to the intracranial compartment, has been previously described (22, 23).

Cell viability assay

Cells were seeded in 96-well plates: 2,000 cells/well in 200 μL, in hexuplicate. One day after seeding, 1 μL of DMSO, with or without NT113, was added to cells to achieve NT113 concentrations between 0.01 and 20.0 μmol/L. Seventy-two hours later, WST-1 reagent (Roche) was added, and sample 450 nm absorbance determined using a microplate reader (Gen5, BioTek), with background reading at 800 nm subtracted.

Western blot analysis

Cells were serum starved overnight before being treated with 1 μmol/L EGFR inhibitor for 2 hours followed by 5 nmol/L EGF stimulation for 10 minutes. Cells and tissues were lysed in buffer (Cell Signaling Technology) supplemented with protease (Roche) and phosphatase (Sigma) inhibitor cocktails. Proteins in lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After probing with primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody, and visualized by ECL (Pierce). Antibodies specific for total and phospho EGFR, ERBB2, ERBB4, Akt, ERK, and β-actin were obtained from Cell Signaling Technology.

Intracranial tumor establishment in athymic mice

Five-week-old female athymic mice (nu/nu, homozygous; Simonsen Laboratories), housed under aseptic conditions, received intracranial tumor cell injection, as approved by the University of California San Francisco Institutional Animal Care and Use Committee (San Francisco, CA). In brief, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and then were injected with 3 μL of tumor cell suspension (300,000 cells total) into the right caudate putamen (22, 23).

Bioluminescence monitoring of intracranial tumor growth

In preparation for BLI, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), then administered 150 mg/kg of luciferin (D-luciferin potassium salt; Gold Biotechnology) via intraperitoneal injection. Ten minutes after luciferin injection, mice were examined for tumor bioluminescence using an IVIS Luminata imaging station (Caliper Life Sciences). Regions of interest, defined using Living Image software (Caliper), were recorded as photons per second per steradian per
square centimeter. Beginning at 1 week after intracranial tumor cell injection, mice were imaged once or twice weekly.

**IHC**

Resected mouse brains were fixed in 10% buffered formalin, then paraffin embedded and sectioned for hematoxylin and eosin (H&E) staining and IHC analysis. To determine cleaved caspase-3 reactivity, unstained sections were processed with a Ventana BenchMark XT automated system and a protocol consisting of pretreatment with 3% ethanolic hydrogen peroxide for 32 minutes at room temperature, epitope retrieval in Tris buffer (pH 8) for 8 minutes at 90°C, and incubation with primary antibody to cleaved caspase-3 (Cell Signaling Technology) at 0.2 mg/mL for 1 hour at 37°C. Total and activated caspase-3–positive cells were counted in 10 high-powered fields within the tumor, with percent positive cells averaged for all fields and subjected to statistical analysis as described below.

**Biodistribution studies**

Mice with intracranial GBM12 tumors were administered NT113, at 10 mg/kg/d for 3 days, with blood and intracranial tissue samples obtained 2 hours following the third administration. Plasma was separated from whole blood, and frozen at −80°C. After brain resection, tumor tissue was immediately dissected from tumor-bearing hemisphere, then snap frozen and stored at −80°C, as was contralateral hemisphere without tumor. NT113 was extracted from homogenized tissues using a Bullet Blender (Next Advance, Inc.). Homogenates were extracted with organic solvent and further processed before transfer to an autosampler for high-performance liquid chromatography (HPLC) analysis (Shimadzu VP Series 10 System), and determination of NT113 content (Integrated Analytical Systems).

**Statistical analysis**

PRISM 5, version 5.03 (GraphPad Software), was used to conduct all statistical analyses. For survival analysis, significance was determined by the log-rank (Mantel–Cox) test. Animals that died during anesthesia or as a result of oral gavages were excluded from survival analyses. For all other statistical analyses, a two-tailed unpaired t test was applied.

**Results**

**NT113 in vivo antitumor activity**

Our initial experiment with NT113 (Supplementary Fig. S1), which shows specificity for inhibiting EGFR, including EGFR kinase domain mutants that are common in lung cancer (24), ERBB2, and ERBB4 (Supplementary Table S1), utilized an EGFRvIII amplified cell source, GBM39, which we have previously shown to be highly responsive to erlotinib treatment (25). Results from BLI and survival analysis revealed an initial decrease in weight associated with NT113 administration (Fig. 1D), which stabilized at day 4 of treatment, and remained stable until treatment was completed, at which time NT113-treated animals showed rapid weight gain. Mild skin rash was also observed in some NT113-treated animals while on therapy, but resolved quickly upon therapy completion (data not shown).

Because of the observation of skin rash combined with body weight decrease of animal subjects, we investigated lower daily dose administrations of NT113, so that in subsequent experiments, animal subjects could receive continuous daily administration of NT113, without periodic interruption, and without indication of adverse effects. This analysis revealed that a 10 mg/kg daily administration was well tolerated by athymic mice for up to 28 consecutive days. Similar analyses were conducted for erlotinib and the dual ERBB inhibitor lapatinib, which revealed that 100 mg/kg/d and 150 mg/kg b.i.d., respectively, were well tolerated by animal subjects, over extended periods of time.

**Effect of EGFRvIII expression on NT113 activity**

Amplification and overexpression of EGFRvIII has previously been associated with GBM response to EGFR inhibition (25, 26). To address the importance of EGFRvIII expression for intracranial GBM xenograft response to NT113 treatment, we used the isogenic cell pair U87-U87vIII, the latter of which represents a derivative of the parental line that was developed by EGFRvIII retroviral modification (18), and that expresses EGFRvIII at a high level (Supplementary Fig. S2A), comparable with that seen in GBM with endogenous EGFRvIII gene amplification. For parental U87, 50% growth inhibition of cell cultures was observed at an NT113 concentration of 8.67 μmol/L (Supplementary Fig. S3A), and unmodified U87 revealed no response to NT113 (10 mg/kg/d) as intracranial xenografts, as indicated by BLI and survival analysis results (Figs. 2A and B). In contrast, U87vIII cells were 50% growth inhibited at an NT113 concentration 0.19 μmol/L (Supplementary Fig. S3B and S3C), and U87vIII intracranial xenografts experienced growth delay with NT113 treatment (10 mg/kg/d; Fig. 2C) that significantly extended animal subject survival (P < 0.001; Fig. 2D).

Comparison of NT113 effects on ERBB family member and downstream signaling mediator activation also revealed distinct responses between U87 and U87vIII cells. In parental U87 cells, endogenous EGFR, as well as ERBB2 and ERBB4, showed no phosphorylation following EGF treatment (Fig. 3), most likely as a consequence of an insufficient level of Erf receptor expression for activating ligand to promote dimer formation and receptor transphosphorylation. In contrast, U87vIII cells, with high-level expression of constitutively active and virally...
transduced EGFRvIII (Supplementary Fig. S2A), showed detectable p-EGFRvIII, p-ERBB2, p-ERBB4, and p-Akt, the levels for all of which were reduced as a result of NT113 treatment (Fig. 3). Erlotinib treatment of U87vIII cells also inhibited EGFRvIII, ERBB2, and Akt phosphorylation, though to a lesser extent than observed with NT113. Moreover, and in contrast with NT113, erlotinib did not inhibit ERBB4 phosphorylation.

**NT113, erlotinib, and lapatinib in vivo comparison**

GBM12 intracranial tumors, which have amplified wild-type EGFR (19), and that we previously found as being responsive to erlotinib treatment (25), though less than GBM39, were next used for evaluating NT113 anti-tumor activity, and for comparing NT113 efficacy with that of erlotinib, as well as the EGFR-ERBB2 inhibitor lapatinib. For the comparison with lapatinib, mice with intracranial GBM12 began receiving daily administrations on day 6 after tumor cell implantation, with resultant BLI growth curves indicating delayed tumor growth from treatment with either inhibitor, albeit much more substantially delayed from NT113 (Fig. 4A). Consistent with the BLI results, mice receiving NT113 treatment survived significantly longer than mice receiving lapatinib (Fig. 4B, \( P < 0.001 \)). IHC analysis of tumors in mice that were euthanized following one week of treatment showed significant antiproliferative (Fig. 4C) as well as proapoptotic (Fig. 4D) response to each inhibitor, with responses to NT113 significantly greater than those observed from lapatinib.

Mice receiving intracranial injection with GBM12 were also used to compare the efficacy of NT113 versus that of erlotinib, but with treatments initiated on day 23 after tumor cell implantation. As for the previous comparison, BLI results revealed that each inhibitor delayed tumor growth, to a significant extent, with the growth delay from NT113 significantly more than from erlotinib (Fig. 5A). Survival results were again consistent with BLI in showing significant survival benefit from treatment with either inhibitor, and that survival benefit from NT113 was significantly greater than that from erlotinib (\( P = 0.047 \), Fig. 5B).
Analysis of NT113 antitumor activities and biodistribution

Mice with intracranial GBM12 were additionally used for analysis of inhibitor signaling mediator effects, in vivo, and for analysis of inhibitor biodistribution. With respect to the former, one mouse from the NT113 and erlotinib treatment groups were euthanized following one week of therapy, with fresh tumor tissue dissected from resected brain, and dissected tumor used for obtaining protein extracts for Western blot analysis. Included in this analysis was a protein extract from intracranial GBM12 obtained from a mouse following one week of lapatinib treatment. Results for EGFR phosphorylation indicated NT113 as having the most substantial inhibitory effect, whereas NT113 and erlotinib had similar inhibitory effect on Akt phosphorylation (Fig. 5C). Little phosphorylation effect was evident from lapatinib treatment, despite indication of lapatinib antiproliferative effect against intracranial GBM12, as well as survival benefit for mice receiving lapatinib treatment (Fig. 4).

To assess brain and tumor biodistribution, 4 mice each from the NT113 and erlotinib treatment groups were euthanized at 2 hours following their third treatment with inhibitor. Blood was drawn from each mouse immediately before euthanasia, and following euthanasia, brains were immediately resected, with tumor tissue dissected, and contralateral brain, without tumor, separated for obtaining tissue extracts subject to HPLC analysis for inhibitor content. Results from the HPLC analysis (Supplementary Table S2) showed significantly greater tumor-to-plasma and normal brain-to-plasma content for NT113, thereby indicating superior partitioning of NT113 to normal brain as well as to intracranial tumor (Figs. 5D and E). Moreover, in 4 of 4 mice, intracranial tumor NT113 concentration was greater than that for corresponding contralateral brain (mean tumor-to-normal brain = 10.5; Fig. 5F), indicating preferential NT113 sequestration in tumor.

With erlotinib doses 10 times greater than that for NT113 (100 mg/kg/d vs. 10 mg/kg/d), it was not necessarily unexpected that the average amount of erlotinib in intracranial tumor tissue was greater than the average amount for NT113 (330 vs. 157 mg/kg; Supplementary Table S2). Consequently, the heightened inhibitory effect of NT113 on EGFR phosphorylation (Fig. 5C) is presumably not attributable to a reduced amount of erlotinib, relative to NT113, reaching intracranial tumor.

Generalization of NT113 activity to EGFR-amplified GBM

In our previous analysis of GBM xenografts for response to erlotinib, we concluded that tumor $EGFR$ amplification and maintenance of wild-type PTEN expression were necessary but not sufficient for identifying GBM that should be erlotinib sensitive (25). An example of a GBM xenograft with appropriate molecular characteristics for anticipating sensitivity, but that proved to be nonresponsive to erlotinib, is GBM6, which has amplified $EGFRvIII$ and expresses wild-type PTEN. To address the possibility of NT113 having broader spectrum activity against GBM than erlotinib, we conducted an additional therapy-response experiment, using mice that had received intracranial injection with GBM6, and then treated with either NT113 or erlotinib. BLI results obtained on day 16 after tumor cell injection, and following 5 days of
 treatment, showed no significant antitumor effect from erlotinib, whereas 5 days of treatment with NT113 had significantly slowed tumor growth, both in relation to control mice, as well as with respect to mice receiving erlotinib treatment (Fig. 6A). Imaging results at day 20 after tumor cell implantation and following day 9 of treatment confirmed a significant difference in bioluminescence for NT113 and erlotinib treatment groups (Fig. 6B). Consistent with our previous results for erlotinib treatment of mice with intracranial GBM6, there was no indication of survival benefit from administration of this EGFR inhibitor, whereas mice receiving NT113 survived significantly longer than mice receiving no treatment, or mice receiving treatment with erlotinib (P < 0.001 for NT113 vs. erlotinib comparison; Fig. 6C). Importantly, immunoblot analysis of GBM6 total protein, as well as total protein for GBM39 and GBM12, revealed readily detectable ERBB2 in GBM6 only (Supplementary Fig. S2B).

Discussion

The results presented highlight several points of interest about NT113. First, they indicate a broader spectrum of GBM as being responsive to NT113 than erlotinib, which has seen extensive, and predominantly negative clinical trial evaluation for improving the outcomes of patients with GBM. Examples in support of NT113 being active against a larger fraction of GBM include the results from the GBM6 and U87vIII intracranial xenograft models. GBM6, though of appropriate genotype for anticipating sensitivity to EGFR small-molecule inhibition, is not responsive to erlotinib, but shows distinct growth suppression from NT113 treatment, and mice with intracranial GBM6 experience significant survival benefit from treatment with NT113 (Fig. 6). U87 and its EGFRvIII derivative are PTEN deficient, and intracranial U87vIII shows suppressed growth from treatment with NT113 (Fig. 2C). In our previous study testing GBM intracranial xenograft response to erlotinib (25), we concluded that tumor maintenance of wild-type PTEN expression is a GBM molecular characteristic that may be required for tumor response to this inhibitor, which is an interpretation that is consistent with clinical trial results (26).

Three of the five xenograft models used in our study involve cell sources that highly express EGFRvIII, and their collective results strongly implicate EGFRvIII in and of itself, as a biomarker predictive of GBM response to NT113 treatment. This relationship is perhaps most strongly supported by the comparison of parental U87 versus derivative U87vIII xenograft response to NT113 treatment (Fig. 2). An alternative approach, involving the use of isogenic cell pairs for testing the importance of EGFRvIII to NT113 response, would be to develop derivatives of GBM6 or GBM39 cells, in which shRNA expression was used to suppress endogenous EGFRvIII expression, and determine whether NT113 responsiveness was diminished in association with suppression of EGFRvIII expression.

In addition to the EGFRvIII-NT113 response relationship, results from our use of the GBM12 model, with amplified wild-type EGFR and positive for expression of wild-type PTEN (19, 25), showed this tumor as being responsive to NT113. The unmodified parental U87 model, with low-level endogenous EGFR expression (Supplementary Fig. S2A), was the only type of GBM xenograft tested that failed to show response to NT113 treatment. Collectively, our results indicate that patients whose tumors have amplified wild-type EGFR and/or amplified EGFRvIII, which invariably result in elevated expression of encoded protein (2–4, 27), are candidates for benefiting from NT113 treatment.

As well as being an irreversible ERBB inhibitor, as based on chemical structure homology comparisons, the pan-ERBB inhibitory activity of NT113 (EGFR, ERBB2, and ERBB4 shown here; Fig. 3) provides a mechanistic rationale for its heightened antitumor activity, in comparison with erlotinib, with NT113 interference of downstream Akt activation (Fig. 3) potentially being of importance to the antitumor effects of this inhibitor. The determination of elevated ERBB2 expression in GBM6,
relative to other tumor cell sources used in this study (Supplementary Fig. S2B), combined with the distinct response of intracranial GBM6 to NT113 versus erlotinib, suggests that the pan-selectivity of NT113 (see U87vIII results in Fig. 3) is a key contributor to its efficacy against tumors that express multiple ERBB family members. In addition to the impact of ERBB family members, other than EGFR, the activities of non-ERBB receptor tyrosine kinases, as determinants of GBM response and resistance to EGFR therapeutic targeting, are thought to be of importance (9, 10).

Excellent intracranial biodistribution of NT113, in relation to erlotinib (Fig. 5D and E), is also likely to contribute to the anti-GBM xenograft activity of NT113. It bears mentioning that our analysis of mouse brain for NT113 and erlotinib content is based on a single time point measurement, and therefore does not allow for evaluation of temporal changes in tumor exposure to either drug. Nonetheless, at 2 hours subsequent to inhibitor administration, the mean concentration of erlotinib in GBM12 intracranial tumor was approximately 2-fold greater than that of NT113 (Supplementary Table S2),

Figure 4. Comparing the in vivo efficacy of NT113 versus lapatinib. Mice were intracranially injected with GBM12 cells that were obtained from disaggregated subcutaneous tumor, with NT113 and lapatinib treatments beginning on day 6 after tumor cell injection, and mice receiving continuous daily administration of inhibitor until required euthanasia due to increasing tumor burden. Both lapatinib (150 mg/kg b.i.d.; see ref. 31) and NT113 (10 mg/kg/d) treatments slowed intracranial tumor growth rate (A, no significant difference was determined for control vs. lapatinib bioluminescence values at days 10, 13, 16, 20, and 23, whereas significant differences of $P < 0.037$ were determined in comparing NT113 vs. control, and $P < 0.003$ in comparing NT113 vs. lapatinib, for these same days) and significantly extended animal subject survival (B, $n = 10$ for control and NT113 treatment groups; $n = 8$ for lapatinib treatment group), though the antigrowth effect of NT113 was significantly more pronounced than that of lapatinib. Median group survivals: control $= 29.5$ days, lapatinib $= 34.5$ days, NT113 $= 63$ days. The significance of each two-group survival comparison is shown in the graph in B: C, control; L, lapatinib; and N, NT113. IHC analysis of tumors from mice that were euthanized following one week of treatment with each inhibitor showed significant reductions in Ki-67 positivity, relative to tumor from an untreated control mouse (C), and additionally showed Ki-67 labeling of NT113-treated tumor as being significantly less than that of tumor from the lapatinib treatment group mouse. Representative images of control (left), lapatinib (center), and NT113 (right) Ki-67–stained tumors are shown, and quantitative analysis of tumor staining results are shown to the right (mean values from positive cells in 10 high-powered fields: *, comparisons with Student $t$ test values of $< 0.05$). IHC analysis of tumors for apoptotic response (D, cleaved/activated caspase-3 staining), with the same sample sequence as indicated for Ki-67 results, showed each inhibitor as promoting increased apoptosis, relative to untreated tumor, and also revealed apoptotic response in NT113-treated tumor as being significantly greater than that of lapatinib-treated tumor.
Figure 5. Comparison of NT113 versus erlotinib efficacy. The same cohort of mice injected with GBM12 were used to compare antitumor effects from NT113 versus erlotinib treatment, but waiting until day 23 after tumor cell injection to initiate inhibitor administration. Results from BLI of luciferase-modified tumor cells, and associated tumor growth curves (A), revealed delayed tumor growth resulting from erlotinib as well as NT113 treatment, though growth delay from NT113 was substantially longer than that resulting from erlotinib (significant differences in group bioluminescence values of $P < 0.01$ existed between control vs. erlotinib at day 34 and between control and NT113 at days 27 and 34). NT113 versus erlotinib BLI comparisons revealed significant differences ($P < 0.042$) at days 27, 34, 37, 41, and 44. Consistent with the BLI results, survival analysis (B) showed significant extension of survival from NT113 treatment, in comparison with untreated control mice ($P < 0.001$), as well as with respect to erlotinib-treated mice ($P = 0.047$, $n = 8$, all groups). Group median survivals: control = 34 days, NT113 = 62.5 days, erlotinib = 48 days. The significance of each two-group survival comparison is shown in the graph in B: C, control; E, erlotinib; and N, NT113. One mouse each from the erlotinib and NT113 treatment groups was euthanized following one week of treatment, with brains resected and tumors dissected immediately following euthanasia. A tumor from a lapatinib-treated mouse was acquired to include for Western blot analysis of extracted proteins for effect of treatment on signaling mediator phosphorylation. The results show NT113 as having the most substantial inhibitory effect on EGFR phosphorylation, with NT113 and erlotinib having similar inhibitory effect on Akt phosphorylation (C). Four additional mice receiving erlotinib treatment, and 4 receiving NT113 treatment, were euthanized 2 hours following their third administration of inhibitor, with plasma, dissected intracranial tumor, and normal brain examined for inhibitor content. Lapatinib was excluded from the brain and plasma analysis because of its relatively low antitumor activity against GBM12 intracranial xenografts (Fig. 4). The results of this analysis showed significantly higher tumor:plasma and normal brain:plasma ratios for mice receiving NT113 (D and E), indicating superior partitioning of NT113 to intracranial tumor and to normal brain. In addition, NT113 concentration was higher in tumor than in paired contralateral brain for all four mice receiving NT113 (F), indicating preferential sequestration of NT113 in tumor.
suggesting that, at this time, GBM12 tumor is experiencing a higher concentration of erlotinib than NT113, though responding less to the erlotinib regimen than to the NT113 regimen. Additional NT113 pharmacokinetic results, from a multi-timepoint analysis of NT113 concentration in plasma, from rats receiving oral administration of NT113 at 5 mg/kg, indicate that maximal NT113 plasma concentration is achieved by 4 hours after oral administration, and that at least 77% of this maximal level is maintained between hours 1 and 8, p.o. (Supplementary Fig. S4). This, in combination with the IC50 and tumor-to-plasma partitioning results (Supplementary Fig. S3 and S5D, respectively), support that an antiproliferative concentration of NT113 is reached and sustained in intracranial tumor for several hours following oral administration of 10 mg/kg NT113. Although not tested, we expect that U87vIII intracranial xenograft tumors would have even higher concentration of NT113 than GBM12 tumors because of the circumscribed nature of U87vIII intracranial xenografts, the growth of which is more disruptive of the blood–brain barrier than GBM12 intracranial tumors, which grow in an infiltrative manner, as is also the case for GBM6 intracranial xenografts (22).

Proapoptotic activity of pan-ERBB inhibitors, in preclinical studies, has been previously noted (28, 29), and was evident for NT113-intracranial GBM xenografts, as indicated by increased activated caspase-3 staining (Fig. 3D). Therefore, NT113 acts to slow tumor growth through combined antiproliferative and proapoptotic effects.

Despite the favorable results, supporting a possible clinical trial evaluation of NT113, it is clear that a more detailed analysis of NT113 and other in-class inhibitors, such as dacomitinib, which was recently shown to have activity against subcutaneous GBM xenografts with EGFR alterations (30), is needed to facilitate straightforward comparison of in-class inhibitor pharmacokinetics, biodistribution, tumor exposure, toxicology, and molecular as well as biologic indicators of antitumor activities.

As noted, a major concern for ERBB inhibitors as monotherapies for treating cancer, including GBM, is tumor adaptation through various mechanisms, including activation of compensating receptor tyrosine kinases (9, 10). Indeed, comparison of bioluminescence growth curves for two of the tumor models used here indicate complete stasis for GBM39 (Fig. 1A), during the 2-week course of treatment, whereas GBM12 intracranial tumors, subjected to continuous daily administration of NT113, seem to begin growing after 2 to 3 weeks of treatment (Figs. 4A and 5A). Although GBM adaptation to any monotherapy is to be expected, the variability and duration of tumor responsiveness to NT113 are yet to be determined, as are GBM adaptive mechanisms to sustained NT113 treatment. Further investigation will resolve these important issues, as will studies aimed at identifying optimal therapeutic partners for combination treatments with NT113.
Disclosure of Potential Conflicts of Interest

W. Shen has ownership interest in NewGen Therapeutics. D. Brown has ownership interest (including patents) in NewGen. J.J. Raizer received commercial research grant from Novartis and Celldex, has received speakers’ bureau honoraria from Genentech, and is a consultant/advisory board member for Genentech, Stemline, Agensus, Novartis, and Novocure. J.N. Sarkaria received commercial research supports from Basilea, Genentech, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

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