Combined Inhibition of HER1/EGFR and RAC1 Results in a Synergistic Antiproliferative Effect on Established and Primary Cultured Human Glioblastoma Cells

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Abstract

Glioblastoma is the most frequent brain tumor of glial origin in adults. With the best available standard-of-care, patients with this disease have a life expectancy of only approximately 15 months after diagnosis. Because the EGF receptor (HER1/EGFR) is one of the most commonly dysregulated oncogenes in glioblastoma, HER1/EGFR–targeted agents, such as erlotinib, were expected to provide a therapeutic benefit. However, their application in the clinical setting failed. Seeking an explanation for this finding, we previously identified several candidate genes for resistance of human glioblastoma cell lines toward erlotinib. On the basis of this panel of genes, we aimed at identifying drugs that synergistically enhance the antiproliferative effect of erlotinib on established and primary glioblastoma cell lines. We found that NSC23766, an inhibitor of RAC1, enhanced the antineoplastic effects of erlotinib in U87MG, T98MG, and A172MG glioblastoma cell lines for the most part in a synergistic or at least in an additive manner. In addition, the synergistic antiproliferative effect of erlotinib and NSC23766 was confirmed in primary cultured cells, indicating a common underlying cellular and molecular mechanism in glioblastoma. Therefore, agents that suppress RAC1 activation may be useful therapeutic partners for erlotinib in a combined targeted treatment of glioblastoma.

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Introduction

Glioblastoma is the most common primary brain tumor in adults. It is a highly proliferating neoplasia characterized by intense neoangiogenesis as well as adjacent and distant infiltration of the brain parenchyma (1, 2). After surgical resection, these tumors almost always recur and develop resistance toward established adjuvant therapeutic measures. The biologic features of glioblastoma translate into a poor prognosis, which is reflected by a median overall survival of only approximately 15 months after diagnosis (3). Despite great efforts aiming at the refinement of standard therapies, only minor improvements were achieved. Therefore, novel therapeutic strategies were developed and evaluated, among them EGF receptor (HER1/EGFR)–targeted therapies.

The HER1/EGFR is overexpressed in more than 50% of glioblastoma, which was shown to be associated with enhanced tumorigenicity and tumor growth in preclinical models of this disease (4–7). For that reason, HER1/EGFR was considered a promising molecular target for glioblastoma therapy, and a variety of HER1/EGFR–targeted agents were developed, including small-molecule tyrosine kinase inhibitors such as erlotinib (Tarceva; Genentech Inc.; reviewed in ref. 4). Erlotinib competitively inhibits the binding of ATP to the intracellular catalytic tyrosine kinase domain of the receptor and suppresses subsequent autophosphorylation and downward signaling via phosphoinositide 3-kinase/murine thymoma viral oncogene homolog (PI3K/AKT) and mitogen-activated protein kinase (MAPK) pathways (8, 9). Despite encouraging results derived from preclinical studies with erlotinib, translation into the clinical setting has failed so far (10–12).

To partially reconcile the paradox that inhibition of HER1/EGFR, a proto-oncogene dysregulated so frequently in glioblastoma, has failed in the clinical setting, we previously identified a panel of candidate genes for resistance toward erlotinib (13). These genes were shown to share the common features of increased expression in erlotinib-resistant glioblastoma cell lines and decreased expression in erlotinib-sensitive glioblastoma cell lines. Among these candidate resistance genes, RAC1 was identified.
RAC1 is a member of the rho family of GTPases and serves as a molecular switch to regulate diverse cellular functions including migration, survival, and malignant transformation (14). Ligand binding to tyrosine kinase or G-protein–coupled receptors induces the activation of RAC1, which occurs in terms of a cycling process mediated by guanine nucleotide exchange factors (GEF), generating the active GTP-bound form of RAC1 by catalytically exchanging GDP for GTP (15). Subsequently, RAC1 effectors are activated that regulate downstream signaling via c-Jun-NH2-kinase (JNK) or MAPK cascades. NSC23766 represents a small-molecule inhibitor that by specific binding to RAC1 interferes with the interaction of RAC-specific GEFS such as Tiam1 or Trio and RAC1, thereby preventing RAC1 activation (16). It has been shown that NSC23766 specifically inhibits RAC1 activation while not affecting the activity of other closely related Rho GTPases such as RhoA or Cdc42 (16). To date, the use of NSC23766 is restricted to preclinical applications only. The presence of aberrant RAC1 activity has been shown in various malignant diseases, including cancers of the breast, lung, and colon (17–19). Moreover, activation of RAC1 was shown to promote malignant transformation, tumor progression, and increased invasiveness of tumor cells (20–24).

In glioblastoma, inhibition of RAC1 activity was shown to induce apoptosis and to inhibit survival of a variety of established and primary cultured cell lines, suggesting that RAC1-dependent signal transduction represents an important survival pathway in this disease (25). In addition, downregulation of RAC1 expression was reported to cause impaired maintenance of glioma stem–like cell characteristics such as anchorage-independent growth, the expression of self-renewal–related proteins, and neurosphere formation, indicating that RAC1 might be essential for the preservation of the phenotype of glioma stem–like cells (26). Recently, in a transgenic glioma model in zebrafish, progression of gliomas induced by AKT1 overexpression was shown to be greatly accelerated by coexpression of RAC1 (27). Moreover, RAC1 expression was implicated in the invasive behavior of glioma cells (28). In conclusion, the fact that dysregulated RAC1 was shown to be associated with various proneoplastic biologic effects, highlights its promising role as a molecular target in cancer therapy including glioblastoma.

In this work, we experimentally confirmed RAC1 to represent a highly effective target for a rationally designed combination therapy, inhibition of which enhances the antiproliferative effect of erlotinib on different established and primary glioblastoma cell lines in a synergistic manner. Moreover, we investigated the effects of the combined treatment with erlotinib and NSC23766 on cellular features such as migration, locomotion, tumorigenicity, and three-dimensional tumor growth. Our data conclusively show that treatment with both compounds results in antineoplastic effects that are superior to the treatment with either agent alone.

Materials and Methods

Reagents
Erlotinib was kindly provided by OSI Pharmaceuticals, Inc. NSC23766 was purchased from Calbiochem (EMD Chemicals, Inc.). Of note, 50 mmol/L working solutions were prepared for erlotinib with dimethyl sulfoxide (DMSO) and for NSC23766 with sterile water. Both working solutions were stored at −20°C. For all experiments, final concentrations of DMSO were below 0.1% (v/v).

Cell cultures and growth conditions
U87MG, A172MG, and T98MG human glioblastoma cell lines were obtained from the American Type Culture Collection. The initial stocks were expanded, frozen, and stored in liquid nitrogen. Fresh aliquots were thawed every 6 weeks. PC38 and PC40 are primary cultured human glioblastoma cells derived from tumor resections carried out at our institution and were generated as previously described (29). Patient’s or next of kin’s consent was obtained, and procedures were carried out in accordance with the local ethics committee. Unless indicated otherwise, all cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco, Invitrogen) containing 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 4 mmol/L glutamine, 1 mmol/L sodium pyruvate (Gibco, Invitrogen) and were incubated at 37°C in a water-saturated atmosphere containing 5% CO2.

Cell viability assays
To examine cellular proliferation, MTT assays and cell counts were conducted. For MTT assays, 1.5 × 10⁴ cells per well were seeded in 96-well flat-bottomed plates and allowed to attach overnight at 37°C before changing the medium to DMEM supplemented with 1.5% FBS and treatment with compounds or the corresponding vehicle. At defined time points, the medium was aspirated, and 100 μL of MTT solution was added to the wells before incubation for 3 hours at 37°C. The reaction was stopped by adding 100 μL of 100% isopropanol (Sigma-Aldrich), and optical densities (OD) were measured at 550 nm using an automated microplate reader (ELx800; BioTek). Percentage viability was expressed as (ODcompound − ODblank)/(ODvehicle − ODblank). For cell count determinations, 1 × 10⁶ cells per well were seeded in 12-well plates. Similar to the MTT assay, cells were allowed to attach, and medium was changed before adding the different compounds. At distinct time points, the medium was aspirated, the cells were enzymatically detached (Trypsin/EDTA; Biochrom AG), and the cell numbers were determined with a CASY1 DT cell counter (Schärfe System). Microphotographs were taken using a BIORÉVO BZ 9000 microscope (Keyence).

Cell migration/motility assays
Cell migration was examined by a transmembrane migration (Transwell) assay as well as by in vitro scratch assays. Cell motility was evaluated by time-lapse live cell microscopy imaging.
For the Transwell assay, 3 × 10^4 cells were seeded in DMEM containing 10% FBS onto Transwell membranes (Corning Incorporated) with a pore size of 8 µm, and intrinsically migrated toward medium containing 20% FBS. Experiments were carried out according to the manufacturer’s recommendations. After 24 hours, the upper side of the membrane was wiped and washed with PBS for three times. The cells on the bottom side of the membrane were then fixed with methanol and stained with 4',6-diamidino-2-phenylindole (DAPI) before mounting. The number of migrated cells was determined by counting one high-power field (hpf) at ×10 magnification in triplicate for each treatment condition.

Scratch assays were conducted as previously described (30). In brief, subconfluent cell layers in 12-well plates sustained a scratch across the well carried out with a 200 µL pipet tip. Sequential microscopic images were taken at defined time points at ×4 magnification, and the area of the scratch was further analyzed with the BZ-II Analyzer software (Keyence). For time-lapse live cell microscopy imaging, 4 × 10^4 cells per well were seeded onto 12-well plates, and microscopic images at ×10 magnification were taken with a live-imaging inverted video microscope (IX81; Olympus) every 30 minutes for a total observation time of 24 hours. During this period, cells were kept at standard culture conditions (37°C, 5% CO₂, water-saturated atmosphere). Single-cell tracking was carried out with the MtrackJ plugin (ref. 31; www.imagescience.org/meijering/software/mtrackj/) for the NIH ImageJ software (Bethesda, Maryland; http://imagej.nih.gov/ij/). Normalized “wind-rose” plots were generated with the chemotaxis and migration tool from Integrated BioDiagnostics (Ibidi; www.ibidi.com).

Cell adhesion assay

The 96-well plates were precoated overnight with collagen and washed three times with PBS. Then, 3 × 10^4 cells per well were seeded and treated as described for the Transwell assay. After 24 hours, wells were rinsed three times with DMEM, representative microscopic images were taken, and MTT assays were conducted.

Soft agar assay

Anchorage-independent growth was assessed as previously described (32). Briefly, 6-well plates were coated with a base layer of 0.9% low-melting agarose (Biozym Scientific GmbH) containing 10% FBS, antibiotics, and the respective reagents or corresponding solvents. A layer of 0.35% agarose containing the same supplements as the base layer plus 6 × 10^5 cells per well was placed on top of the base layer before incubation for 21 days at standard culture conditions. Microscopic images were taken at ×4 magnification with a CK40 inverted microscope and a CC-12 imaging system (Olympus). The largest diameter of the colonies was measured, and colonies with a diameter exceeding 50 µm were counted.

Chorioallantoic membrane assay

To assess potential effects of the respective compounds and their combination on tumor cell invasion into a biologic membrane and on tumor formation in a three-dimensional environment, we conducted chorioallantoic membrane (CAM) assays as previously described (33, 34). A 1:1 mixture of serum-free medium and Matrigel (BD Biosciences) containing 1 × 10^6 cells was seeded onto the CAM of 1-week old fertilized chicken eggs. The experimental treatment was started after 24 hours and consisted of the local application of the respective agents twice a day. The tumor and its adjacent CAM were harvested 4 days after seeding and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and analyzed microscopically (AX70 “Provis,” Olympus). The tumor area was quantified with the BZ-II Analyzer software (Keyence). For semiquantitative analysis of invasion, a scoring system was defined as follows: (i) tumor laying on the CAM; (ii), less than 50% of the tumor engulfed by the CAM; (iii), more than 50% of the tumor engulfed by the CAM; and (iv), tumor completely engulfed by the CAM. Analysis was conducted by two independent and blinded examiners.

Human orthotopic glioblastoma xenograft model

Human xenografts were stereotactically implanted into 6- to 8-week-old male mice (NOD.Cg-Prkdcscid Il2rgtm1WjI/SzJ) weighing 22 to 30 g. All tumors used to establish xenografts were histologically diagnosed as glioblastoma. Patients’ consent was obtained and the study was approved by the local Animal Care and Use Committee (Regierungspräsidium Tübingen, Germany). To prepare the xenografts, patient-derived tumor material was minced and taken up in ice-cold PBS before centrifugation at 13,000 rpm for 5 minutes at room temperature. Then, the supernatant was discharged and the pellet was incubated for 5 minutes with TrypLE Express (Gibco, Life Technologies). Subsequently, the tumor specimen was passed through a cytosieve (pore size, 70 µm) and resuspended in DMEM/F12 (HAM) medium (Gibco, Life Technologies) supplemented with 20 ng/mL EGF (Biomol GmbH), 20 ng/mL basic fibroblast growth factor (bFGF; Miltenyi Biotec GmbH) and B27 (Gibco, Life Technologies), 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL amphotericin B (Gibco, Life Technologies) and allowed to grow in spheres. For the generation of intracranial tumors, 1 × 10^5 cells, in 5 µL DMEM/F12 (HAM) medium (Gibco, Life Technologies) without supplements, were implanted into the right basal ganglia of anesthetized mice using a small animal stereotactic frame (Stoelting). Motorized injections were performed at a rate of 2.5 µL/min. Treatment was started intraperitoneally 5 days after tumor implantation and carried out daily for 9 days. Survival was assessed by calculating Kaplan–Meier curves.

Western blot analysis

Specific protein expression in cell lines was determined by Western blot analysis using the following primary
antibodies: rabbit polyclonal anti-phosphorylated (p) EGFR Tyr1068, rabbit polyclonal anti-EGFR, rabbit polyclonal anti-pAKT Ser273, rabbit polyclonal anti-pAKT Thr308, mouse monoclonal anti-AKT (clone 55/PKBα/Akt), mouse monoclonal anti-pERK1/2 Thr202/Tyr204 (clone E10), rabbit polyclonal anti-pS6, mouse monoclonal anti-S6 (clone 54D2; Cell Signaling Technology), mouse monoclonal anti-β-actin (clone AC-15), rabbit polyclonal anti-ERK (both from Sigma-Aldrich), mouse monoclonal anti-pan-pTyr (clones PY20 and PY99; Santa Cruz Biotechnology), and mouse monoclonal anti-RAC1 (Pierce Biotechnology). Secondary horseradish peroxidase (HRP)-linked antibodies were purchased from Santa Cruz Biotechnology. Briefly, cells were washed twice with ice-cold PBS and frozen at −80°C for at least 24 hours before lysing the cells using a lysis buffer containing 30 mmol/L Tris–HCl, 150 mmol/L NaCl, 1% Triton X-100, and 10% glycerol at pH 7.4 supplemented with the Complete Protease Inhibitor Cocktail (Roche Diagnostics GmbH), 50 mmol/L NaF, 1 mmol/L Na3VO4, 2 mmol/L dithiothreitol, 200 μmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L 3-glycerophosphat for 10 minutes at 4°C. After centrifugation for 15 minutes at 4°C and 16,000 × g (Eppendorf 5417R, Eppendorf AG), supernatants were transferred and protein concentrations were assessed using the BCA Protein Assay reagent (Pierce Biotechnology). Fifty micrograms of each protein sample were resolved on SDS-PAGE (10% for EGFR and pEGFR or 15% for the other proteins), transferred to a nitrocellulose membrane (Amersham) and incubated with the respective antibodies. Detection was carried out by using the Pierce enhanced chemiluminescence (ECL) Western blotting substrate.

Affinity precipitation of GTP-RAC1

The presence of the GTP-bound, active form of RAC1 was determined by using a commercially available pull-down assay (active RAC1 pull-down and detection kit; Pierce Biotechnology) according to the manufacturer’s instructions with some modifications with respect to the cell lysis. Briefly, cells were incubated for 1 hour at −80°C before lysis on ice in 750 μL lysis/binding/washing buffer [25 mmol/L Tris–HCl (pH 7.2), 150 mmol/L NaCl, 5 mmol/L MgCl2, 1% NP-40, and 5% glycerol] supplemented with Halt Protease Inhibitor Single-Use Cocktail (Pierce Biotechnology) and centrifuged at 16,000 × g for 15 minutes. Protein concentrations in the supernatants were determined and adjusted to 500 μg of total protein. Subsequently, the samples were incubated with 20 μg of glutathione S-transferase fused to the p21-binding domain of human p21-activated kinase 1 (GST-human Pak1-PBD) for 1 hour at 4°C in tubes coated with glutathione agarose beads. The precipitated complexes were washed three times with lysis/binding/washing buffer before resuspension of the complexes in 50 μL of 2 × reducing sample buffer [125 mmol/L Tris–HCl (pH 6.8), 2% glycerol, 4% SDS, 0.05% bromophenol, 4.8% β-mercaptoethanol]. Western blotting as described previously was used to analyze total RAC1 and GTP-RAC1 expression.

Statistical analysis

Statistical significance was assessed by Mann–Whitney U test using Prism version 5.04 (GraphPad). A P ≤ 0.05 was considered statistically significant.

Bliss analysis was conducted to detect synergistic, additive, or antagonistic effects. For two agents, the expected total response to the combination treatment was calculated as fractional response to drug A (F_a) + fractional response to drug B (F_b) − F_a × F_b. If the ratio of the actual total response and the expected total response equaled 0.9 to 1.1, additivity was assumed. If this quotient was less than 0.9, the effect was described as antagonistic. Synergism was stated if the quotient was greater than 1.1. For three substances, the expected total response was calculated as F_a + F_b + F_c − F_a × F_b − F_a × F_c − F_b × F_c. Further analysis was conducted as described previously for two agents.

Results

RAC1 inhibition effectively enhances the antiproliferative effect of erlotinib

We previously identified several genes that were overexpressed in erlotinib-resistant glioblastoma cell lines, whereas a decreased expression of these genes was shown in erlotinib-sensitive glioblastoma cells (13). To determine whether inhibition of the products of these candidate resistance genes enhances the antiproliferative effect of erlotinib on established glioblastoma cell lines, we conducted MTT assays for screening purposes. Therefore, inhibitory concentrations yielding 75% (IC50) or 50% (IC25) of remaining viable cells were measured for U87MG, A172MG, and T98MG glioblastoma cell lines and each agent used (Supplementary Fig. S1). Erlotinib was combined with up to two other inhibitors at IC25 and IC50 to identify the most promising combination of compounds. In this approach, combined treatment with erlotinib and NSC23766, an inhibitor of RAC1, proved to be most efficient (Figs. 1 and 2A). Statistical analysis using the Bliss equation revealed that dual therapy with these agents resulted in a synergistic antiproliferative effect on all three established glioblastoma cell lines under investigation (Fig. 2A and B), an effect confirmed by methodologically independent cell count analysis (Fig. 2C). Furthermore, in the primary glioblastoma cell cultures PC38 and PC40, combined treatment with erlotinib and NSC23766 also exhibited synergistic antiproliferative effects (Fig. 2B and C). Similar results were obtained when combining NSC23766 with lapatinib (Tykerb; GlaxoSmithKline), a dual inhibitor of HER1/EGFR and HER2 directed against HER1/EGFR’s inactive conformation of the catalytic tyrosine kinase domain (Supplementary Fig. S2).

NSC23766 enhances the antimigratory effect of erlotinib on established glioblastoma cell lines

The antiproliferative effect of the combined treatment with erlotinib and NSC23766 on glioblastoma cell lines
and primary cultured glioblastoma cells was accompanied by another prominent finding that was revealed by microscopic imaging.

A marked change in cell morphology was observed in cell lines treated with both agents, sharing as the most pronounced common feature a reduction of cytoplasmic process formation (Fig. 2D, black arrowheads). The morphologic effect exerted by each agent was apparently enhanced by the combination treatment and, although difficult to quantify, led to the hypothesis of an increased antimigratory effect. To further address this issue, a scratch-induced migration assay was conducted by monitoring the width of a freshly inflicted linear defect within the cellular monolayer after certain periods of time. As shown in Fig. 3A and B, U87MG cells subjected to treatment with both agents showed significantly reduced migration into the scratch after 18 hours when compared with control cells and cells treated with either agent alone. In T98MG cells, this effect was less apparent. However, T98MG cells treated with both agents showed less cytoplasmic process formation and cellular elongation directed toward the scratch (white arrowheads in Supplementary Fig. S3A), an observation entirely absent in A172MG cells. For further verification of this finding, we examined the capacity of the cells to traverse a collagen-coated porous membrane along a chemoattractant gradient formed by DMEM containing an increasing concentration of FBS. As anticipated, treatment with both agents resulted in a significantly reduced transmigration of U87MG cells in comparison with control cells and cells treated with either agent alone. In T98MG cells, this effect was less apparent. However, T98MG cells treated with both agents showed less cytoplasmic process formation and cellular elongation directed toward the scratch (white arrowheads in Supplementary Fig. S3A), an observation entirely absent in A172MG cells. For further verification of this finding, we examined the capacity of the cells to traverse a collagen-coated porous membrane along a chemoattractant gradient formed by DMEM containing an increasing concentration of FBS. As anticipated, treatment with both agents resulted in a significantly reduced transmigration of U87MG cells in comparison with control cells and cells treated with either agent alone (Fig. 3C and D). In A172MG, a less pronounced but still significant reduction of the migratory capacity was noted (Fig. 3C and D), an effect which was absent in T98MG (Supplementary Fig. S3C and S3D). Differences in cellular adhesion or proliferation were excluded as the reason for the varying transmigration of the three cell lines subjected to the combination treatment in that MTT assays and microscopic imaging of cells seeded in collagen-coated 96-well plates showed similar results after 24 hours of treatment across the different treatment groups after multiple washes (Supplementary Fig. S4A and S4B). We also examined the effects of the combined treatment with erlotinib and NSC23766 on random cellular movements (i.e., locomotion) using time-lapse live cell microscopy imaging. Single-cell tracking revealed that all three established glioblastoma cell lines we examined were significantly inhibited in locomotion, that is, randomly moved over a significantly shorter overall distance when treated with both erlotinib and NSC23766 compared with cells treated with either agent alone or vehicles (Fig. 4A). Moreover, conversion of these data into wind-rose diagrams, which depict the paths taken by the single cells starting from the same zeroed position, showed that the distance of movement was markedly reduced by the combination therapy (Fig. 4B). Taken together, these results indicate that NSC23766 clearly enhances the inhibitory effect of erlotinib on the migratory phenotype of glioblastoma cell lines.

NSC23766 enhances the antitumorigenic effect of erlotinib on established glioblastoma cell lines

Next, we sought to investigate whether the combined treatment with erlotinib and NSC23766 is superior to the treatment with either agent alone in terms of inhibition of the tumorigenicity of glioblastoma cells. For this purpose, a soft agar assay was used in which U87MG cells were treated with 3 μmol/L erlotinib, 1.5 μmol/L NSC23766, both agents, or only with the respective solvents. After 21 days of incubation under continuous drug exposure, representative microscopic images were taken and subjected to further analysis. Only colonies larger than 50 μm in diameter were counted. Typically, U87MG cells formed a relatively high number of large colonies in soft agar under control conditions (Fig. 5A). Although treatment with either erlotinib or NSC23766 alone resulted in significant reductions of colony numbers, it was notable that cells treated with NSC23766 formed markedly smaller colonies. Moreover, concomitant treatment with both compounds almost completely suppressed anchorage-independent growth of U87MG cells (Fig. 5B). This observation was reproducible in a similar manner using the glioblastoma cell line T98MG (Fig. 5A and B).

NSC23766 increases the inhibitory effect of erlotinib on tumor growth in the CAM environment

We used the CAM assay to further assess whether the combination treatment with erlotinib and NSC23766 also yields superior inhibitory effects on tumor formation,
**Figure 2.** Diagrams showing that combined treatment with erlotinib and NSC23766 results in synergistic antiproliferative effects across the complete set of established and primary glioblastoma cell lines under investigation. A, consolidated representation of qualitative combined antiproliferative effects of erlotinib and one or two of five inhibitors of candidate erlotinib resistance proteins on three established glioblastoma cell lines. U87MG, A172MG, and T98MG glioblastoma cells were treated for 144 hours with erlotinib in combination with inhibitors of nuclear factor of activated T cells, cytoplasmic 1 (cyclosporine A), HSP70 (pifithrin), FGF receptor 1 (PD173074), smoothened (HhAntag), RAC1 (NSC23766), or the neurotrophic tyrosine kinase receptor type I (K252a) at IC50 and IC25 (refer to Supplementary Fig. S1 for the respective concentrations used). (Continued on the following page.)
tumor growth, and the invasive phenotype of U87MG cells in a three-dimensional "in vivo-near" setup compared with the effects of either agent alone. Therefore, U87MG cells were seeded onto the CAM of fertilized chicken eggs and allowed to spread for 24 hours before treatment twice daily with erlotinib, NSC23766, both drugs, or solvents for the following 3 days. As shown in Fig. 6A, the most striking phenotypic difference among the treatment groups was an enhanced reduction in size of those tumors that were treated by the combinatorial regimen, which is

Figure 3. Combined treatment with erlotinib (E) and NSC23766 (N) enhances the inhibition of the migration of U87MG and A172MG cells. A, monolayers of subconfluent U87MG cells were scratched before treatment with either erlotinib or NSC23766 or both. Microscopic images were taken at time zero and 18 hours after infliction of the scratch (magnification, ×4; scale bar, 500 μm). B, graph showing the quantitative assessment of the scratch closure after 18 hours compared with time 0. Columns, mean of three independent biologic experiments each carried out in technical triplicates; bars, SEM. C, 3 × 10⁴ cells were seeded onto a Transwell membrane and treated with erlotinib, NSC23766, or both agents. Medium containing 20% FBS served as a chemoattractant. After 24 hours, cells on the upper side of the membrane were wiped off, and the cells on the lower side of the membrane were stained with DAPI before mounting. Representative microscopic images are shown for three separate experiments each carried out in triplicate (magnification, ×10; scale bar, 200 μm). D, diagrams showing mean numbers of cells per field migrated to the lower side of the membrane expressed as percentage of control; bars, SD. Experiments were carried out in technical and biologic triplicates. One representative hpf per Transwell membrane was counted (in total 9 hpf per treatment condition).

(Continued.) The antiproliferative effect of the different drug combinations was assessed by an MTT assay before conducting Bliss analysis to determine whether the combined treatments yielded synergistic, additive, or antagonistic effects as described in Materials and Methods. The effects of the respective combination therapy on either one of the three cell lines are displayed in the field at the intersection of the vertically and horizontally depicted agents. B, U87MG, A172MG, T98MG and two primary glioblastoma cell lines (PC38 and PC40) were treated with erlotinib or NSC23766 or both agents under serum starvation (1.5% FBS). After 144 hours, a MTT assay was conducted. Data are presented as mean of three independent experiments and SEM. C, growth profiles for U87MG, A172MG, T98MG, PC38, and PC40 cells subjected to treatment with either erlotinib (green) or NSC23766 (blue) or both agents (red) or solvents (black). Cells were enzymatically detached and counted at the indicated time points. Error bars show mean values ± SEM. Means were derived from three replicates (n = 3). D, representative microscopic images of U87MG and PC40 cells at ×10 and ×20 magnification plus ×1.5 digital zoom after 144 hours of treatment with erlotinib (E), NSC23766 (N) or both. Morphologic changes such as decreased lengths and numbers of cell processes are seen in those cells treated with both agents. Characteristic examples are highlighted by black arrowheads. ×10 Magnification; scale bar, 300 μm; ×20 magnification plus ×1.5 digital zoom; scale bar, 100 μm.
reflected by a significant decrease of the tumor area when compared with treatment with each agent alone (Fig. 6B). However, semiquantitative analysis of histologic slides revealed that the invasive capacity of U87MG cells treated with both substances was not impaired to a higher extent in comparison with single-agent treatment (Fig. 6C).

Combined treatment with erlotinib and NSC23766 extends survival in vivo

Finally, we examined the effects of the combination therapy with erlotinib and NSC23766 in a murine orthotopic glioblastoma xenograft model. Human xenografts derived from glioblastoma tumor specimens were implanted into the cerebrum of mice 5 days before intraperitoneal
treatment with erlotinib (100 mg/kg), NSC23766 (3 mg/kg), or both. Controls were treated with the respective vehicles. As shown in Fig. 6D, combined treatment with erlotinib and NSC23766 resulted in a marked prolongation of survival when compared with single-agent treatment or control. These data also imply that the antitumorigenic effect of the combination treatment outweighs potentially toxic side effects.

**Combined treatment with erlotinib and NSC23766 decreases EGFR-mediated signaling and suppresses activation of RAC1**

We have so far presented experimental results that show the supportive influence of NSC23766 on erlotinib’s inhibitory effect on important biologic features of glioblastoma cell lines, such as proliferation, migration, and tumorigenicity. In an attempt to elucidate the molecular mechanism underlying this observation, Western blot analyses were conducted to determine whether EGFR expression, its activation, and/or further downstream signaling are altered by the combined treatment with erlotinib and NSC23766. As shown in Fig. 7A, protein expression of EGFR was decreased after 72 hours and markedly reduced after 144 hours of continuous exposure to the combination regimen in all three established glioblastoma cell lines as compared with single-agent treatments. In addition, pEGFR levels were reduced in a similar manner. We also examined the levels of pAKT, pERK, and pS6 as important effectors of the PI3K/AKT and MAPK pathways (Fig. 7B). Erlotinib and NSC23766 act synergistically to downregulate pAKT, pERK, and pS6. In addition, the total levels of AKT and S6 were also highly reduced by the combination treatment after 72 and 144 hours. However, the expression of ERK was affected to a much lower extent, especially in A172MG and T98MG.

Next, we analyzed the effects of erlotinib and NSC23766 on RAC1 activation to determine whether the biologic effects exerted by the combination treatment may be attributable to alterations of RAC1 activation in addition to interference with EGFR. For this purpose, we used a RAC1-GTP pull-down assay in U87MG, T98MG, and A172MG glioblastoma cell lines. Our data clearly show that treatment with both substances combined suppresses RAC1-GTP protein expression in all three cell lines (Fig. 7C); total RAC1 expression was not affected by any treatment modality when compared with control. Although single treatment with NSC23766 did not result in a decrease of RAC1-GTP, our experiments show that erlotinib and NSC23766 cooperate to inhibit RAC1 activation.

Taken together, our data suggest that the synergistic or under some experimental conditions, additive antineoplastic effects of the combined treatment with erlotinib and NSC23766 on glioblastoma cell lines are due to enhanced inhibition of EGFR activation, subsequent downregulation of the PI3K/AKT and MAPK pathways as well as suppression of RAC1 activation.
Discussion

The gene encoding HER1/EGFR has been reported as one of the most frequently amplified genes in glioblastoma, and a large body of evidence suggests that this alteration is associated with malignant progression and glioblastoma growth (1). As a consequence, high hopes accompanied the development of HER1/EGFR–targeted agents such as erlotinib for the treatment of this disease. However, despite a solid theoretical background, translation of HER1/EGFR inhibition into clinical practice failed altogether in the setting of glioblastoma (11, 12). Because the responsiveness of glioblastoma cells toward erlotinib shows a high variability (13, 35), we previously hypothesized that gene expression analysis of glioblastoma cells with an erlotinib-responsive or -resistant phenotype might unveil potential molecular targets allowing the design of a multitargeted approach to suffocate escape mechanisms or cross-talk between oncogenic pathways.

Figure 6. Combined treatment with erlotinib (E) and NSC23766 (N) inhibits tumor formation on the CAM and prolongs survival in an orthotopic murine xenograft model. A, U87MG cells were seeded on the CAM of fertilized chicken eggs. After 24 hours, the cells were treated twice daily with erlotinib, NSC23766, or both agents. On day 5, the tumors were harvested, and photographs were taken before fixation in 10% formalin and histologic processing. Left, representative photographs of tumors located on the CAM. The circle has a diameter of 5 mm. Middle, four technical replicates are shown for each treatment group, which are representative for two biologically independent experiments. Dashed circles, tumor circumferences. Right, representative histologic images stained with H&E are shown (magnification, ×2; scale bar, 1,000 μm). Dashed lines, tumor–CAM interfaces. B, quantitative analysis of the tumor area. Data are presented as mean of at least 9 tumors per treatment group and SD. C, histologic slides of at least 9 tumors per treatment group were graded for invasion. Data are presented as box plots showing median, minimal, and maximal scores. D, as detailed in Materials and Methods, cells from human glioblastoma xenografts were stereotactically injected into the right basal ganglia of mice before randomization into the different treatment groups. Intraperitoneal administration of 100 mg/kg erlotinib or 3 mg/kg NSC23766 or both was started 5 days after the tumor implantation and was continued for 9 consecutive days. Concomitant treatment with erlotinib and NSC23766 resulted in a survival benefit when compared with control or single-agent treatment as depicted by Kaplan–Meier survival analysis.
ultimately conveying a therapeutic benefit to patients with glioblastoma (13). In this study, combined treatment with erlotinib and inhibitors of those previously identified candidate genes for resistance toward erlotinib revealed that NSC23766, an inhibitor of RAC1, represents the most promising partner for enhancing the antineoplastic effects exerted by erlotinib. Our data show that combined inhibition of HER1/EGFR and RAC1 results in a synergistic antiproliferative effect on both established and primary glioblastoma cell lines. This finding was not only observed in standard monolayer cell culture experiments, but also in a three-dimensional tumorigenicity assay in soft agar, glioblastoma cell lines. A, U87MG, A172MG, and T98MG cells were treated for 144 hours with erlotinib, NSC23766, both agents, or solvents under serum starvation. Whole-cell extracts were examined by Western blot analysis for pEGFR, EGFR, and pan-P-Tyr protein levels. Actin Western blot analysis was conducted to confirm equal protein loading. B, protein levels for pAKT ser473, pAKT thr308, AKT, pERK1/2, ERK1/2, pS6, S6, and actin were determined by Western blot analysis as described in Materials and Methods. Total RAC1 and actin protein expression was determined using a pull-down assay and subsequent Western blot analysis as described in Supplementary Fig. S5).

Our data also show that NSC23766 significantly enhances the inhibitory effect of erlotinib on random movement (motility) of all three established glioblastoma cell lines examined. Enhancement of antimigratory effects exerted by erlotinib due to combined treatment with NSC23766 was only observed in U87MG cells and, to a lower extent, in A172MG cells. However, in T98MG cells, the antimigratory effect of erlotinib was not significantly enhanced by the additional treatment with NSC23766, which contradicts the assumption made by Ramis and colleagues that erlotinib-induced alterations of the migratory capacity of these cell lines is mediated through RAC1 activation (36). Moreover, we show that the combined treatment of U87MG cells seeded on the CAM with erlotinib and NSC23766 markedly impaired tumor formation when compared with single-agent treatments. In this model, the invasiveness of U87MG cells was similar among single-agent and combined treatment groups. Although previous findings indicate that erlotinib inhibits
the invasiveness of glioblastoma cell lines in a Matrigel-coated Transwell assay (36), additional inhibition of RAC1 did not yield further suppression of the invasive phenotype of U87MG cells, suggesting that RAC1 does not contribute to this characteristic biologic feature of glioblastoma cells.

In this study, we showed that not only erlotinib but also lapatinib, a dual HER1/EGFR and HER2 inhibitor, exerted a synergistic antiproliferative effect on different glioblastoma cell lines. This finding is of particular interest because recently, two studies addressed the question why non–small cell lung cancer (NSCLC) and glioblastoma respond differently toward HER1/EGFR–targeted agents (38, 39). Vivanco and colleagues showed that glioblastoma cell lines carrying glioma-specific HER1/EGFR mutations were responsive toward an HER1/EGFR inhibitor directed against the inactive conformation of the catalytic tyrosine kinase domain, that is, lapatinib, an observation that contrasts with the findings in NSCLC cell lines (38). The authors concluded that the clinical failure of first-generation HER1/EGFR inhibitors in glioblastoma may be due to differences among NSCLC and glioblastoma in the conformational state of the receptor that is required for efficient inhibition. In concordance with these findings, Barkovich and colleagues showed that treatment of glioblastoma cells bearing a common glioma-derived HER1/EGFR mutation (EGFRVIII) with erlotinib resulted in a lower occupancy of the kinase–active site by erlotinib when compared with NSCLC bearing NSCLC–typical HER1/EGFR mutations (39). However, the use of HER1/EGFR inhibitors targeting the inactive conformation of the receptor should be regarded with caution. So far, two clinical studies have failed to show a clinical benefit of lapatinib in the setting of recurrent glioblastoma (38, 40). These results may be partly explained by the fact that mean tumor tissue concentrations of lapatinib were more than 10 times lower than those reported for erlotinib, which has a molecular size that is approximately half of that of lapatinib (38, 41). Whether the combined treatment with lapatinib and NSC23766 exceeds the antineoplastic effects of erlotinib plus NSC23766 on glioblastoma cell lines harboring EGFRVIII is the subject of current investigations in our laboratory.

As far as potential plausible mechanisms underlying the synergistic effects of HER1/EGFR and RAC1 inhibition, further exploration of the known roles of RAC1 in tumor biology points to the inhibition of survivin that was shown to be related to increased apoptosis in breast cancer cells treated with NSC23766 (42). Moreover, sensitivity of glioma cells toward YM-155, an inhibitor of survivin, was shown to inversely correlate with the expression of pEGFR (43).

Overall, this study offers important insights into the cellular and molecular effects of a novel therapeutic strategy involving combined HER1/EGFR and RAC1 inhibition. One impediment to making predictions about any clinical success of this combined targeting in glioblastoma is the fact that the in vivo model that we used does not accurately reflect the human blood–brain and blood–tumor barriers. Therefore, clinical validation of this regimen is warranted. This may include evaluating whether drugs that are already approved for nononcologic indications, for example, statins, can be safely and effectively repurposed as RAC1 inhibitors in conjunction with erlotinib.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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