Cyclopamíne cooperates with EGFR inhibition to deplete stem-like cancer cells in glioblastoma-derived spheroid cultures

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Putative cancer stem cells have been identified in glioblastoma (GBM), associated with resistance to conventional therapies. Overcoming this resistance is a major challenge to manage this deadly brain tumor. Epidermal growth factor receptor (EGFR) is commonly amplified, over-expressed, and/or mutated in GBM, making it a compelling target for therapy. This study investigates the behavior of 3 primary neurosphere (NS) cell lines and their adherent counterparts originated from human GBM resections, when treated with EGFR–tyrosine kinase inhibitor erlotinib, associated or not with cyclopamine, a hedgehog pathway inhibitor. Adherent cells cultured in the presence of serum expressed the glial fibrillary acidic protein, whereas NS-forming cells cultured in serum-free medium expressed CD133, nestin, and Oct-4, markers of neural stem and progenitor cells. For the 3 adherent cell lines, erlotinib has a moderate effect (50% inhibitory concentration [IC50], >10 μM). Conversely, erlotinib induced a strong cell growth inhibition (IC50, <1 μM) on NS-forming cells, related to the EGFR gene amplification and EGFR protein expression. A short exposure to erlotinib reduced nestin-positive cell proliferation, but NS-initiating activity and self-renewal were not altered. EGFR pathway seems essential for GBM progenitor cell proliferation but dispensable for cancer stem-like cell self-renewal. Inhibition of hedgehog pathway with cyclopamine was evaluated in association with erlotinib on NS growth. Although each drug separately had no effect on sphere initiation, their combination significantly decreased the sphere number (P < .001). Our findings show synergic efficiency for erlotinib-cyclopamine association and provide a suitable in vitro model to explore drug combinations on GBM cells.

Keywords: cancer stem cells, cyclopamine, epidermal growth factor receptor, glioblastoma, tyrosine kinase inhibitor.

Gliomas are the most frequent primary brain tumors. The most malignant form of glioma is glioblastoma (GBM) (WHO grade IV), a very aggressive tumor with <12-month median survival time for patients with GBM.1,2 Despite recent advances in surgery and chemotherapy, patient prognosis has not improved substantially over the past few years. Characteristic features of GBM include diffuse invasion, intense apoptosis resistance, necrosis, angiogenesis, and a multiform histological profile suggesting a tumor cell plasticity.2 Moreover, EGFR gene amplifications are frequently associated with GBM, making this tyrosine kinase receptor a putative target for therapy.3,4 Malignant gliomas are heterogeneous in their cell composition.5 Recently, a range of tumors, including GBM, have been found to contain cancer stem-like cells (CSC).6,7 It has recently been proposed that GBM derive from neural stem or progenitor cells, and CSCs may play a central role in the propagation of several cancer types.7 Thus, signaling pathways playing a key role in primitive neural cells may also be required in tumorigenesis of glial tumors.8–11 CSCs have also been
shown to be responsible for a prevalent radioresistance and chemoresistance.\textsuperscript{12}

The hedgehog pathway is of crucial importance during embryonic development\textsuperscript{13} and is also activated in neural stem cells\textsuperscript{14} and GBM CSC.\textsuperscript{15,16} Its inhibition abolishes GBM cell tumorigenicity in mouse xenograft models\textsuperscript{15,17,18} and enhances their sensitivity to temozolomide therapy,\textsuperscript{19} currently used to treat patients with GBM in association with radiotherapy.

Brain CSC have the aptitude to form NS, undergo self-renewal, and generate brain tumors.\textsuperscript{20} These cells proliferate and differentiate to reproduce the original tumor phenotype.\textsuperscript{21} The study of stem cells in glioma benefits from the competence of these tumors to grow as NS in serum-free media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (FGF), permitting measurement of their clonogenic potential, proliferation, and differentiation capacity and, thus, providing a model to establish therapeutic strategies.\textsuperscript{22}

In this work, we studied the behavior of 3 primary neurosphere (NS) cell lines and their adherent counterparts originated from human GBM resections, when treated with the EGFR-specific tyrosine kinase inhibitor erlotinib associated or not with cyclopamine, a hedgehog pathway inhibitor. Erlotinib alone inhibited glial progenitor proliferation in NS but was unable to inhibit NS generation by CSC or differentiated adherent cell proliferation. Cyclopamine enhanced erlotinib-induced NS generation inhibition.

### Material and Methods

#### Cells

GBM samples were obtained after informed and written consent from patients admitted to the Neurosurgery Department at Rennes University Hospital for surgical resection in accordance with the local ethical committee. All tumors were histologically diagnosed as grade IV astrocytoma according to the WHO criteria.\textsuperscript{2}

For each of the 3 GBM samples used in this study, 2 types of primary cell lines were generated: 1 was maintained in serum-containing medium as adherent cells (rAdh cell lines), and the other was cultured as NSs in an appropriate medium (rNS cell lines). GBM primary cell lines were obtained from GBM samples as described elsewhere.\textsuperscript{23} In brief, after mechanic dissociation of tumor tissues, cells were grown in NS and adherent (Adh) conditions. For NS cultures, cells were grown in Dulbecco’s Modification of Eagle’s Medium/F12 (DMEM; 1/1, Invitrogen) supplemented with B27 and N2 additives (Invitrogen), EGF (20 ng/mL), and basic FGF (20 ng/mL) (Peprotech, Tebu-Bio) at 37°C in a humid atmosphere of 5% CO\textsubscript{2}. For Adh cultures, primary cell lines were obtained as described elsewhere.\textsuperscript{21,24} Cells were grown in DMEM supplemented with 10% fetal calf serum (FCS; Lonza). All GBM NS and Adh cell lines were used between the 10th and 20th passages for the experiments.

Adh and NS cell lines have been characterized elsewhere.\textsuperscript{23} Adherent rAdh85, rAdh125, and rAdh142 cells were 95% positive for GFAP. rNS85, rNS125, and rNS142 expressed CD133,\textsuperscript{24} Nestin, and OCT-4, which are markers of neural stem and progenitor cells. The 6 cell lines expressed the EGFR, and rNS85 also expressed the vIII variant of the EGFR.\textsuperscript{24}

Every 14 days, the NS-forming cell lines were washed and dissociated with diluted trypsin; viable cells were counted and seeded at 5 × 10\textsuperscript{3} cells/mL in 24-well plates. When the effect of drugs on cell proliferation had to be assayed, the rNS cell lines were seeded at the same concentration. When the effect on sphere generation had to be determined, the cells were seeded at 10\textsuperscript{2}/mL to preserve clonal conditions of growth.\textsuperscript{25,26}

For the limiting dilution assay, a small volume of dissociated NS cells was dispersed in 1 mL of NS medium to obtain 8, 6, 4, 2, or 1 cell/100 µL. For each dilution, the wells of a 96-well plate were seeded with 100 µL of the cell suspension. Half the wells (n = 48) were treated with 10 µM erlotinib. After 7 days of culture, the wells without any sphere were scored for each condition. The percentage of empty wells was plotted as a function of seeded cells.

U87-MG– and DBTRG-05MG–adherent cell lines were obtained from the ATCC and originated from patients with GBM. U87-MG cells were cultured in DMEM supplemented with 10% FCS and nonessential amino acids. DBTRG-05MG cells were maintained in Roswell Park Memorial Institute supplemented with 10% FCS. Both cell lines were replated twice a week at 5 × 10\textsuperscript{4} cells per mL.

#### Drugs

Cyclopamine was purchased from Selleck Chemicals. Erlotinib was kindly provided by Roche Genetech.

#### Cytogenetic Analysis

**Array-Comparative Genomic Hybridization (CGH) Analysis.**—Oligonucleotide array-CGH was performed using the Agilent Human Genome CGH microarray 105K (Agilent Technologies). Experiments were performed according to the protocol provided by the manufacturer (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis). Images were analyzed using Agilent Genomic Workbench software. Data graphical overview and analysis were obtained using the Agilent DNA analytics software. As previously described, only imbalances involving ≥3 adjacent probes were retained.\textsuperscript{27} Identification of probes with a significant gain or loss was based on the log\textsubscript{2} ratio plot deviation from 0 with cutoff values of 0.5–1 and −0.5 to −1, respectively.

**Flourescence In Situ Hybridization (FISH) Analysis.**—FISH assays for the locus 7p12 were performed using the Vysis LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen Probe (Abbott Molecular). Probes were hybridized to metaphase or interphase nuclei according to the manufacturer’s instructions. After
hybridization, the slides were counterstained with 4,6-diamino-2-phenyl-indol (DAPI; Qbiogene MP Biomedsicals) and analyzed using an epifluorescence microscope Olympus BX61 equipped with a charge-coupled device camera. Images were captured using Isis software (MetaSystems).

**EGFR Expression**

For NS cell staining, dissociated cells were washed in phosphate-buffered saline 2% FCS and incubated with saturating concentrations of fluorescent-labeled anti-EGFR antibody (Santa Cruz Biotechnology, Tebu-Bio) for 30 min at 4°C. Cells were then washed with phosphate-buffered saline 2% FCS and analyzed using flow cytometry with a FACSCanto II flow cytometer (BD Biosciences). The population of interest was gated on adherent GBM cells,29 and we confirmed here this result which is in agreement with previously described results showing that spheroid cultures maintained better EGFR amplification than did adherent cultures.28 Using flow cytometry, we confirmed that EGFR was expressed at the NS cell line surface after blocking its intercalation by EGF deprivation. Higher expression levels were observed on the rNS85 and rNS142 cell lines corresponding to EGFR amplification (Fig. 1D and E).

**Results**

**EGFR Gene Amplification and Protein Expression in GBM Adh and NS Cells**

Two types of cell lines derived from 3 different GBMs were used in this study. One type was cultured as Adh cells in serum-containing medium, whereas the other type was cultured in serum-free medium supplemented with EGF and basic FGF and generated NSs (NS). For each cell line, genomes of NS and Adh cells were compared using array-CGH analysis. Adh cell genomes were used as references. These assays showed a strong genomic gain of the EGFR gene (7p12) in both rNS85 (Fig. 1A) and rNS142 (Fig. 1B). FISH analysis demonstrated amplification clusters of the EGFR gene in NS85 cells (Fig. 1A), whereas Adh cells exhibited only an EGFR polysomy with 6–8 copies (Fig. 1A). No EGFR amplification was evidenced by array-CGH in the rNS125 cells or rAdh125 cells, and FISH analysis showed an EGFR polysomy with 7–13 copies in both rNS125 (Fig. 1C) and rAdh125 (data not shown) cells. These results were in accordance with previously published results showing that spheroid cultures maintained better EGFR amplification than did adherent cultures.27

**Effect of Erlotinib on GBM Adh and NS Cell Proliferation**

These results led us to test the effect of EGFR inhibition on the proliferation of the different GBM cell lines. Erlotinib has been shown to inhibit the proliferation of adherent cell lines,29 and we confirmed here this result on the 3 adherent cell lines tested (Fig. 2). Erlotinib 50% inhibitory concentration (IC50) was >10 μM, which is in agreement with previously described values,30 and this shows a low efficiency of this inhibitor on adherent GBM cells. In the same conditions of treatment, NS-generating cells were more sensitive to
erlotinib than were their adherent counterparts derived from the same GBM tumors (Fig. 2) showing that the EGFR signaling pathway was necessary for their growth. Of interest, the rNS125 cell line, which did not show EGFR amplification and had a low EGFR expression level, was more resistant (IC₅₀ = 10 μM) than were the rNS85 and rNS142 cells (IC₅₀ < 5 μM). This was confirmed by a lower inhibition by 2 μM erlotinib of EGFR phosphorylation on Tyr1068 residue after EGF ligation in rNS125 than in the other 2 cell lines (Fig. 2D). The EGF-induced phosphorylation of EGFR was however drastically reduced in the 3 cell lines by erlotinib.

**Effect of Erlotinib on Neurosphere Formation in GBM NS Cells**

Owing to the characteristic culture features of these last cells, sphere generation and growth were then separately evaluated (Fig. 3) on the basis of a 14-day culture time. After treatment with erlotinib, a dose-dependent reduction of the NS size was observed (Fig. 3A). Indeed,
although the number of NSs was not modified (Fig. 3B), the total number of living cells was greatly affected with an IC50 < 1 μM for rNS 85 and 142 and 5 μM for rNS 125 (Fig. 3C). This suggests that EGFR inhibition did not alter the NS-generating ability of GBM NS cells but that the EGFR pathway was essential for the proliferation of glial progenitor cells. Here, a good correlation was found between the effect of 10 μM erlotinib and EGF deprivation in the culture medium (Fig. 3E). This experiment confirms that erlotinib had no effect on sphere generation, whereas EGF deprivation only slightly decreased the sphere number (Fig. 3D). In addition, erlotinib is a better cell proliferation inhibitor than EGF deprivation in all the 3 cell lines (P < .05). Moreover, although rNS125 cells (the one without EGFR amplification) were insensitive to EGF deprivation (Fig. 3E), a 90% reduction in proliferation was obtained with 10 μM erlotinib in 14 days (Fig. 3C and E). These last results suggest that, at 10 μM, erlotinib inhibited other essential pathways besides EGFR.

**Effect of Erlotinib on Self-Renewal Capacity of GBM NS Cells**

Because the NS-forming ability has previously been related to neural stem cell self-renewal, the effect of erlotinib on this ability was investigated. After NS formation, the 3 cell lines were treated with 10 μM erlotinib, a high concentration that reduced the cell number by 80% in 3 days (Fig. 4A). Erlotinib induced a significant apoptosis in the 3 cell lines (P < .05, Fig. 4B). When these treated cells were dissociated and replated, the rate of NS generation for 104
viable cells was not decreased, compared with untreated control (Fig. 4C). After the second or third replating this sphere-generation ability was also conserved in erlotinib pretreated cells, showing that the auto-renewal ability of the living stem-like cells was not further altered. However, high seeding concentrations used in these experiments may mask erlotinib toxicity to sphere-generating cells. Indeed, a limiting dilution assay confirmed that erlotinib treatment did not significantly modify the frequency of the untreated control as a function of the erlotinib concentration. The x axis is in a logarithmic scale. Results are expressed as mean ± SD of 3 experiments. (D) rNS-85, -125, or -142 cells were incubated for 3 days without EGF (lanes 1, 4, and 7). Afterward, EGF was added alone (lanes 2, 5, and 8) or in the presence of 2 μM erlotinib (lanes 3, 6, and 9) for 30 min. The samples were submitted to Western blot analysis and probed with an anti-phospho-EGFR (Tyr1068) and an anti-total EGFR antibody. An anti-tubulin antibody was used to assess loading homogeneity.

Effect of Erlotinib on Nestin- and GFAP-Expressing Cell Populations in GBM NS Cells

Neurospheres are heterogeneous in nature, and only a small percentage of cells in each sphere has the ability to form NSs. In our models (Fig. 4D), on the basis of the Poisson distribution, this frequency can be evaluated to 1 of 4 cells. Nestin is expressed in neural stem cells/progenitor cells, and GFAP is progressively expressed during glial differentiation. We investigated by immunofluorescence (Supplementary material, Fig. S1) the distribution of the cells constituting the NSs and their adherent counterparts between nestin/GFAP double negative, nestin positive/GFAP negative, and nestin/GFAP double positive compartments after a 48-h treatment with 10 μM erlotinib (Fig. 5). Experiments were performed in all GBM Adh and NS cell lines. Erlotinib decreased the relative amount of nestin positive/GFAP negative cells in GBM-rNS85, rNS125, and rNS142 cells by 34%, 12%, and 23%, respectively (P < .05), suggesting that progenitor cells were preferentially affected by the EGFR inhibition (Fig. 5A). More differentiated cells (GFAP positive) remained relatively insensitive. In adherent cell lines, which predominantly expressed GFAP, no differential effect of erlotinib was observed in any population (Fig. 5B). These data are in agreement with the results observed in Fig. 2, in which erlotinib was poorly efficient on differentiated adherent GBM cells.

Combined Effects of Erlotinib and Cyclopamine on the Generation of GBM NS Cells

Inhibition of the hedgehog signaling pathway has been previously described to deplete CSCs in GBM. A high dose of cyclopamine (10 μM) significantly inhibited the localization of Gli1 in the nucleus (P = .013), confirming the inhibition of the hedgehog pathways in the 3 rNS-cell lines (Supplementary material, Fig. S2). To enhance the effect of erlotinib on sphere growth, we assessed the cyclopamine in combination with erlotinib on NS-forming cell lines. Both drugs used at 10 μM significantly reduced the cell number in culture by 68 and 16 for erlotinib and cyclopamine, respectively, whereas
Although erlotinib and cyclopamine alone had no significant effect on the mean number of spheres, the combination of both drugs decreased this number by 25 (P = .001) (Fig. 6B). This drug combination effect on the number of NSs was also assessed with increasing concentrations in a constant ratio (5 molecules of cyclopamine for 1 molecule of erlotinib) on the rNS85, rNS125, and rNS142 cell lines using the Chou and Talalay method. The combination index was determined for each cell line with use of the CalcuSyn software (Biosoft) (Fig. 6C). A combination index < 1 is considered as synergistic, combination index of 1 is additive, and combination index > 1 is antagonist. The table on Fig. 6C shows that combination index < 1 was observed at ED75 (P = .03) and ED90 (P = .003), and an additive effect (combination index of 1) was observed at ED50. Sequential addition of the drugs at ED75 concentrations showed that early addition of erlotinib followed by cyclopamine 3 days later was as efficient as both drugs used in association. Conversely, when cyclopamine was added before erlotinib, the effect was greatly reduced (P < .001) (Supplementary material, Fig. S3), suggesting that the EGFR pathway was necessary for the early events of sphere initiation and the hedgehog pathway was not.
Discussion

In this work, we took advantage of NS cultures to investigate the efficiency of erlotinib on EGFR inhibition by affecting the different steps of glial differentiation in glioblastoma cells. Experiments were conducted on NS-initiating cell lines obtained from 3 different patients. The corresponding adherent cell lines, isolated from the same tumors, were also used as a model of more differentiated cells. EGFR pathway activation was found to be necessary for progenitor cell expansion but dispensable for differentiated cell proliferation. In addition, it was also dispensable for cell sphere-initiating activity and CSC auto-renewal. EGFR genomic amplification was associated with EGFR over expression and with a higher sensitivity to EGF deprivation and erlotinib treatment. These in vitro results confirmed that the EGFR expression status must be considered to select a targeted therapy. Some phase I and II clinical studies illustrate these findings. Indeed, EGFR immunoexpression evaluated in children who received a diagnosis of malignant gliomas was correlated with the progression-free survival but not with the overall survival, when treated with erlotinib. Similar to Haas-Hogan et al. have shown that GBM with high EGFR level had a better response to erlotinib. For Mellinghoff et al., the coexpression of

Fig. 4. Effect of erlotinib on NS-initiating cell auto-renewal. After trypsin dissociation, rNS85, rNS125, and rNS142 cells (10^5 cells/well) were treated (Erlo) or not (Ctrl) with 10 μM erlotinib for 3 days. Viable cells were counted (A). The percentage of apoptotic cells was evaluated by flow cytometry on the basis of mitochondrial membrane potential (B). Viable cells (10^5/well) were then plated (P1) for 14 days in the absence of erlotinib, and the number of NSs was evaluated (C). Cells were further serially replated at 10^4 cells/well once (P2, rNS85) or twice (P2, P3, rNS125). Results are expressed as a percentage of the untreated control value for each plating (P1, P2, P3). Results are expressed as mean ± SD of 3 experiments. (D) Control (closed symbols, continuous line) and erlotinib-treated (open symbols, dashed line) cells were plated in serial dilutions in 96-well plates. After 7 days of culture, the wells without sphere were scored for each dilution of each cell line. The percentage of empty wells was plotted as a function of the cell number (mean ± SD of the 3 cell lines).
mutant EGFRvIII and wild-type PTEN was correlated with the response to tyrosine kinase inhibitors. Our results show that EGFR kinase inhibition decreased progenitor cell proliferation on the 3 cell lines, but EGFR inhibitor anti-tumor activity was restricted to a population of proliferating cells during tumor cell differentiation. Erlotinib did not affect the generation of NSs, but a dose-dependent reduction in sphere size was observed. Furthermore, erlotinib effect in each cell line was related to EGF dependence in the culture medium, and erlotinib was more efficient than was EGF deprivation. This last result suggests that erlotinib may inhibit signaling events other than EGFR. Furthermore, erlotinib was toxic for the 3 NS cell lines, inducing a significant apoptosis, but did not alter the self-renewal capacity of sphere-generating cells. Thus, EGFR inhibition is insufficient to abolish the cancer stem-like activity, and we also found that proliferation of differentiated adherent GBM cells was poorly affected by erlotinib. By targeting proliferating progenitor cells and, thus, tumor growth, erlotinib may be of interest if associated with other drugs able to block the tumor-initiating cells.

Sonic hedgehog homolog (SHH) signaling events have been implicated in tumor cell proliferation and survival and participate in the molecular hallmarks of different human tumor entities that include esophageal squamous cell carcinoma, basal cell carcinoma, medulloblastoma, prostate cancer, colon cancer, brain tumors, rhabdomyosarcoma, breast cancer, and pancreatic cancer. Aberrant activation of SHH signaling is thus implicated in many human cancers. Inhibition of SHH signaling has been attempted in multiple human cancers. Multiple lines of evidence support the idea that SHH is a prerequisite to increase the viability of GBM cancer stem cells and that blocking active SHH signaling with a therapeutic inhibitor molecule, such as cyclopamine (targeting SHH receptor smoothened), induced cell death. It has previously been shown that cyclopamine is toxic for GBM cells.
been shown for GBM tumor cells, that hedgehog signal-
ing sustains tumor-initiating cell proliferation\(^\text{18}\) and that its inhibition using high cyclopamine dose depletes CSCs\(^\text{17}\) in GBM. Cyclopamine was also shown to sensitize glioma CD133 stem cells to temozolomide.\(^\text{19}\) Moreover, SHH inhibition has recently been shown to decrease the migration of CD133-positive GBM cells\(^\text{48}\) and, thus, may also restrict their invasive potential.

The attractive option in our NS model, containing GBM cells at different steps of differentiation, was to associate the EGFR inhibitor with cyclopamine. A high concentration of cyclopamine (10 \(\mu M\)) was found to be necessary to significantly inhibit both Gli1 nuclear localization and sphere initiation. Of note, such high doses were described to induce off-target effects independently of the hedgehog pathway.\(^\text{49,50}\) However, it was also found necessary by others\(^\text{17,46,51}\) to inhibit by 50\% the activation of Gli1 in GBM cells. Although each drug separately showed a low activity on NS-initiating cells, their combination abrogated sphere formation and enhanced erlotinib efficiency on progenitor proliferation. Of particular interest, synthetic smoothened antagonist cyclopamine and tyrosine kinase inhibitor erlotinib indicate a synergistic effect, and this combination of the 2 drugs had never been reported to date.

Currently, erlotinib has already been tried to treat GBM and had modest effects, such as all other therapies when they are used in monotherapy. In conclusion, we showed that cyclopamine or analogs could be useful to sensitize GBM progenitor and CSCs to EGFR inhibition. This result might lead to therapeutic implications favoring the use of several drugs, each targeting a different population constitutive of the tumoral tissue but also cooperating with others in a synergistic anti-tumor action.

**Supplementary Material**

Supplementary material is available at *Neuro-Oncology* online (http://neuro-oncology.oxfordjournals.org/).

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**References**


