Cytotoxicity and radiosensitization of high-grade glioma cells by CI-1033, an irreversible pan-ErbB inhibitor

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ABSTRACT

Background: High-grade gliomas (HGG) are highly infiltrative malignancies, causing considerable mortality in child- and adulthood, necessitating new therapies. Novel therapies directed against multiple epidermal growth factor family (ErbB) members are potentially effective in HGG.

Aim: To assess ErbB family expression in normal brain and pediatric and adult HGG in silico and to determine radiosensitizing property of the pan-ErbB inhibitor CI-1033, in HGG cells in vitro.

Material and methods: In silico mRNA array expression analysis was performed to assess EGFR, ERBB2, ERBB3, ERBB4 gene expression in normal brain, adult and pediatric HGG. ErbB family protein expression was determined in HGG cell lines using Western blot. Sulforhodamine-B assay was used to assess cytotoxicity of CI-1033 and clonogenic assays to determine radiosensitization. The effect on cell cycle distribution and PI3K-Akt/Ras-MAPK signaling of CI-1033 ± radiation was measured using flow cytometry.

Results: EGFR and ERBB2 were significantly overexpressed in datasets of pediatric and adult HGG. Heterogeneous protein expression of EGFR, ErbB2, 3 and 4 was observed in HGG cell lines. CI-1033 IC50 values of 1.0 µM, 2.5µM and 4.3 µM were found in D384MG, U-251 MG and Gli-6 cells, respectively. CI-1033 significantly sensitized Gli-6 and D384MG cells to radiation, with 24 and 48 hrs pre-treatment respectively.

Conclusion: EGFR and ERBB2 are overexpressed in adult and childhood HGG. Irreversible pan-ErbB inhibition by CI-1033 is cytotoxic and radiosensitizes HGG cell lines in vitro, warranting further in vivo studies.
INTRODUCTION

High-grade gliomas (HGG), most commonly glioblastoma multiforme (GBM) and anaplastic astrocytoma (AA), are highly malignant, diffusely growing, infiltrative brain tumors in children and adults. Despite aggressive multimodality treatment strategies, prognosis is still poor in these patients, with 3-year overall survival (OS) rates of 16 (+/- 4)% in adults, and 22 (+/- 5)% in children\(^1,2\). New therapies are therefore warranted. In recent years, monoclonal antibodies and tyrosine kinase inhibitors (TKI), directed against the ErbB growth factor receptor family of tyrosine kinases are increasingly used as novel anti-cancer agents in multiple cancers, including HGG\(^3\). In adult GBM, EGFR is the most commonly amplified gene (in approximately 40% of cases) and EGFR expression has been described as an independent marker for poor prognosis\(^4\). The amplified EGFR gene is frequently rearranged, resulting in a constitutively activated, ligand-independent EGFR receptor, EGFR\(^{vIII}\). In childhood HGG, EGFR expression is observed in up to 58% of cases and recently EGFR\(^{vIII}\) was detected in 4/9 cases of diffuse intrinsic pontine glioma (DIPG)\(^6,7\). EGFR expression and pathway activation has been correlated with radioresistance, via activation of DNA-dependent protein kinase, catalytic subunit (DNA PKcs), either by direct interaction with EGFR or indirect via ErbB family mediated PI3K-Akt signaling. Furthermore, Ras/MAPK pathway deregulation, either ErbB family mediated or in crosstalk with the PI3K/Akt pathway is described to convey radioresistance\(^5,8,9\).

Considering the involvement of EGFR signaling in HGG, these tumors might benefit from enhancement of radiosensitivity by ErbB family interference. CI-1033 (Canertinib) has the distinct advantage of being a highly selective, irreversible pan-ErbB family TKI, with an additional advantage of blocking mutant EGFR variant EGFR\(^{vIII}\), a determinant of radioresistance in GBM cells\(^10\). CI-1033 has already been employed and proven to be well tolerated in multiple phase I and II studies in solid tumors, as monotherapy and in combination with cytotoxic agents\(^11-13\). In vitro and in vivo studies of CI-1033 as single agent or in concert with other chemo- or radiotherapeutic strategies have been performed in other solid tumors\(^14-20\), but only scarcely in HGG. In this study, we therefore study cytotoxic and radiosensitizing properties of CI-1033 on HGG cells in vitro.

MATERIAL AND METHODS

\textit{In silico} analysis

R2, an online microarray analysis and visualization platform, provided by the Department of Oncogenomics of the Academic Medical Centre, Amsterdam, the Netherlands (http://r2.amc.nl), was used to obtain an overview of EGFR, ERBB2, ERBB3 and ERBB4 mRNA expression in adult and pediatric HGG. MAS5.0 normalized datasets of adult HGG (n=153; GSE4290)\(^21\), childhood HGG (n=53; GSE19578)\(^22\) were compared to normal prefrontal cortex (n=44; GSE13564)\(^23\). Datasets of anaplastic astrocytoma (n=19; GSE4290) and glioblastoma multiforme (n=77; GSE4290)\(^21\) were also compared with normal prefrontal cortex.
Cell lines and culture
The established human adult glioma cell line D384MG is a clone derived from anaplastic astrocytoma (AA) cell line G-CCM, whereas U-251 MG and Gli-6 cell lines originate from GBM. All cells were grown in DMEM, supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin (all from Invitrogen NV, Breda, the Netherlands). Cells were transferred to a new 25 cm² flask every 3-4 days and maintained in a humidified incubator at 37°C and 7% CO₂. All cell lines were tested to exclude Mycoplasma infection.

Western Blot analysis
Whole cell lysates were prepared using RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.05% SDS) supplemented with 1 mM pefabloc (Sigma-Aldrich, Zwijndrecht, the Netherlands). Per cell line and condition one lysate was used to detect ErbB family proteins. Protein concentration was measured using the Bio-Rad protein assay kit (Bio-Rad, Veenendaal, the Netherlands). A total of 100 µg protein was loaded per lane, separated by 7.5% SDS-PAGE and transferred to PVDF membrane (Millipore, Amsterdam, the Netherlands). Subsequently the membrane was blocked with TBST (Tris buffered saline with 0.1% Tween 20) + 5% non-fat milk for 1 h at room temperature (RT) and incubated with 1:1000 mouse anti-EGFR (sc-03, Santa Cruz), ErbB2 (sc-284, Santa Cruz), ErbB3 (sc-285, Santa Cruz) or ErbB4 (sc-283, Santa Cruz) and β-actin monoclonal antibody (A5441, Sigma-Aldrich) overnight at 4°C. Protein lysates from cell lines A431, HT-29, Hep-G2 and MCF-7 were used as positive controls for immunoblots of EGFR, ErbB2, ErbB3 and ErbB4 respectively. The blot was washed three times with TBST + 0.5% milk and the membrane was incubated with 1:1000 goat-anti-mouse-HRP antibody (DAKO Cytomation, Glostrup, Denmark) for one hour at RT. Subsequently, the membrane was washed three times with TBST + 0.5% milk and EGFR, ErbB2, ErbB3, or ErbB4 protein and β-actin was visualized on a hyperfilm using an ECL plus system (Amersham Bioscience, England).

CI-1033
The pan-ErbB inhibitor CI-1033 (Pfizer, New York, NY, USA) was dissolved in 100% DMSO (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) and the stock solution of 20 mM was stored at -20 °C. For experiments, CI-1033 was diluted in phosphate-buffered solution (PBS, Invitrogen NV, Breda, the Netherlands).

Proliferation assays
Sulforhodamine B (SRB) cell proliferation assay was used to measure CI-1033 drug sensitivity. U-251 MG, D384MG and Gli-6 cells were plated at a concentration of 2,000 cells/well, 3,000 cells/well and 4,000 cells/well (100 µl), respectively, in 96 wells plates and allowed to attach for 24 hrs. Subsequently, cells were exposed for 24, 48 or 72 hrs to CI-1033 (0 - 9 µM) or vehicle (DMSO 0.045%). Per concentration, six wells were used. Following treatment, cells
were washed, fixed with 30% trichloroacetic acid (TCA), stained with SRB and washed with 1% acetic acid. SRB stain was eluted with unbuffered Tris base (trishydroxymethylaminomethane) (10 mM, pH 10.5) and plates were measured at 540 nm using a spectrophotometer. Triplicate experiments were performed for each cell line.

**Clonogenic assay**

Clonogenic assay was performed as described by Franken et al. In brief, D384MG, U-251 MG and Gli-6 cells were plated in a culture flask and after 24 hrs the sub-confluently growing cells were exposed to different concentrations of CI-1033 or vehicle (0.04% DMSO) for 24 or 48 hrs and irradiated with single doses of gamma-radiation (0-6 Gy) using a 60Co Cobalt source (Gammacell 200, Atomic Energy of Canada Ltd). After treatment cells were plated in 6-wells plates in medium without CI-1033 or vehicle and incubated for 10 to 14 days to form colonies. Colonies were fixed with 10% formaldehyde (Sigma-Aldrich Chemie) and stained with 10% Giemsa solution (Merck, Germany). Clones of more than 50 cells were scored with a microscope. Surviving fractions (SF) were calculated by dividing the plating efficiency (PE) of the CI-1033 treated cells by the PE of the vehicle-treated cells. SF for each radiation dose were corrected for the toxicity of CI-1033 alone.

**Intracellular immunostaining and flow cytometric analysis**

For cell cycle analysis, FACS samples were prepared following 24 or 48 hrs treatment with CI-1033 or vehicle (DMSO). Cells were collected, washed twice in PBS containing 0.1% bovine serum albumin (Invitrogen NV, the Netherlands), centrifuged at 1000 rpm and fixed in 70% ethanol. Cells were washed twice with ice-cold PBS, incubated with 50 µg RNAseA (Sigma-Aldrich, the Netherlands) for 20 min at RT followed by a 30 minute incubation with 50 µg propidium iodide (PI, Sigma-Aldrich, the Netherlands) in the dark on ice.

For the analysis of Akt and ERK phosphorylation after 24 or 48 hrs of treatment with CI-1033 or vehicle (DMSO), cells were fixed with using 4% formaldehyde during 10 min at 37°C and put on ice for one minute. Subsequently, cells were washed twice with PBS and the cell pellet was incubated with 10 µl human IgG for five min at RT, followed by 100 µl 0.1% saponine/PBS for five min at RT for cell permeabilization. Cells were then incubated with primary rabbit anti-human antibodies; 1:100 phospho-Akt (#4058, Cell Signaling Technology, Danvers, MA, USA), 1:50 Akt (#9272, Cell Signaling Technology), 1:50 phospho-ERK (#4377, Cell Signaling Technology) or 1:25 ERK (#9102, Cell Signaling Technology) for two hrs at RT in the dark and washed four times with PBS. Next, cells were incubated at RT for 30 min in 100 µl 0.1% saponine/PBS and 1:200 goat-anti-rabbit-biotine and washed twice with PBS, incubated with 100 µl 0.1% saponine/PBS and 1:20 streptavidin PE for 15 min at RT in the dark and washed four times with PBS. Finally, 200 µl 0.5% paraformaldehyde was added.
Cells were then analyzed by a Becton Dickinson FACS scanner and the data were analyzed by ModFit LT (Verity Software House, USA). Pre-G1 peak analysis of the PI FACS measurements was used to assess apoptosis in the samples.

Statistics
For the in silico analysis of ERBB family members in adult and pediatric HGG, one-sided analysis of variance (ANOVA) was used to compare EGFR, ERBB2, ERBB3 and ERBB4 mRNA expression between the dataset of normal brain tissue and each dataset of adult and pediatric HGG. A p-value < 0.0005 was considered to be statistically significant. Analysis of inhibitory concentrations in the SRB proliferation assay was performed using SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA, USA), in which the IC₅₀s were calculated with a four parameter logistic equation. For clonogenic cell survival, curves were constructed by nonlinear regression according to the linear-quadratic formula using GraphPad Prism software (GraphPad Software Inc., USA). Differences between survival curves were analyzed using Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) statistical software, as previously described. Dose modifying factors at a survival fraction of 0.2 were calculated to quantify radiosensitization, as described by Franken et al.

RESULTS

EGFR and ERBB2 mRNA overexpression in adult and pediatric HGG
ErbB family (EGFR, ERBB2, ERBB3 and ERBB4) mRNA expression was determined in two datasets of HGG (adult and pediatric HGG) and non-malignant brain tissues (Figure 1). EGFR and ERBB2 were significantly overexpressed in both datasets of glioma, as compared to normal brain. ERBB4 was significantly down-regulated in adult and pediatric HGG, as compared to normal cortex. Moreover, additional in silico analysis showed EGFR and ERBB2 to be overexpressed in anaplastic astrocytoma and to higher extent in glioblastoma multiforme (Figure 2).
Figure 1. *In silico* analysis of EGFR, ERBB2, ERBB3 and ERBB4 mRNA expression using R2 analysis software on datasets of non-malignant brain tissues of cerebral cortex (white, n=44) versus datasets of adult (dark grey, n=153) and pediatric HGG (grey, n=53). Y-axis shows expression in arbitrary units. Boxplots represent 25%, 50% (median), and 75% values, with error bars indicating maximum and minimum values for each category. Statistical outliers are indicated by small circles.
High-grade glioma cell lines are differentially sensitive to pan-ErbB inhibition by CI-1033

Western Blot was used to analyze EGFR, ErbB2, ErbB3 and ErbB4 protein expression in U-251 MG, D384MG and Gli-6 HGG cells. In all cell lines moderate/strong EGFR (170 kD) and weak/moderate ErbB4 (185 kD) protein expression was detected. ErbB2 (185 kD) was strongly expressed in U-251 MG and D384MG cells, no ErbB2 was detected in Gli-6 cells. ErbB3 (185 kD) was found only in D384MG cells (Figure 3A).

Figure 2. In silico analysis of EGFR, ERBB2, ERBB3 and ERBB4 mRNA expression using R2 analysis software on datasets of non-malignant brain tissues of cerebral cortex (white, n=44) versus datasets of anaplastic astrocytoma (first grey, n=19) and glioblastoma multiforme (second grey, n=77). Y-axis shows expression in arbitrary units. Boxplots represent 25%, 50% (median), and 75% values, with error bars indicating maximum and minimum values for each category. Statistical outliers are indicated by small circles.
To determine the effect of ErbB family inhibition on cell proliferation, we first exposed the cells to pan-ErbB inhibitor CI-1033 as a single agent. In figure 3B the effect of CI-1033 treatment on glioma cell lines Gli-6, U-251 MG and D384MG is shown. The AA cell line D384MG showed high sensitivity to CI-1033 treatment as compared to the other cell lines, with a 50% inhibitory concentration (IC50) of 1.0 µM. GBM cell lines U-251 MG and Gli-6 were observed to be less sensitive, with IC50 values of 2.5 µM and 4.3 µM, respectively.

Figure 3. (A) Immunoblot of baseline EGFR (170 kD), ErbB2 (185 kD), ErbB3 (185 kD), ErbB4 (185kD) expression in HGG cell lines and control cell lines (A431, HT-29, Hep-G2, MCF-7 cells respectively). Representative beta actin staining from the ErbB3 immunoblot (42 kD) (B) Cell viability assay for HGG cell lines, exposed to the indicated concentrations of CI-1033 (Canertinib). Cell viability was assayed after 72 hrs exposure. Dots indicate mean values of experiments performed in triplicate. Bars indicate standard deviation of the mean (SD). IC50 values are calculated using SigmaPlot and are indicated between brackets.

Differential effects of CI-1033 on cell cycle kinetics in HGG cells.

Cell cycle analysis after 24 and 48 hrs was performed on HGG cell lines Gli-6, D384MG and U-251 MG after treatment with vehicle or 8 µM, 5 µM and 8 µM CI-1033 respectively. With these concentrations, growth was almost completely inhibited (Figure 4A). In U-251 MG and Gli-6 cells an increase of G2/M cell cycle arrest at 24 and 48 hrs CI-1033 treatment was observed, which was partly resolved at 48 hrs in U-251 MG cells (Figure 4B and C). In D384MG cells no apparent effects on cell cycle were detected (figure 3D). However, analysis of sub-G1
populations, showed increased apoptosis in this cell line (up to 35%), contrary to U-251 MG and Gli-6 in which ± 6% apoptotic cells were detected (data not shown).

Pan-ErbB inhibition with CI-1033 differentially sensitizes HGG cells to radiation, dependent on pre-incubation period

To investigate whether irreversible inhibition of ErbB family members enhances radiosensitivity by decreased clonogenic capacity, clonogenic assays were performed in HGG cell lines. Significant enhancement of radiosensitivity upon pretreatment with CI-1033 was observed in Gli-6 and D384MG cells (p < 0.001) (Figure 5). Interestingly, Gli-6 cells showed enhanced radiation sensitivity upon 24 hrs pre-incubation with CI-1033 and not with 48 hrs pre-treatment, whereas in D384MG cells the opposite was observed: only 48 hrs pre-treatment resulted in significant radiosensitization. U-251 MG did not show increased radiosensitivity with CI-1033 treatment in either pre-incubation schedule.
ErbB family members in HGG cells down-regulated after treatment with CI-1033

To investigate the effect of CI-1033 and radiotherapy on ErbB family members protein expression, HGG cell lines U-251 MG, D384MG and Gli-6, were treated with 8 µM, 8 µM and 5 µM CI-1033, respectively and/or radiation. Subsequently, EGFR, ErbB2, ErbB3 and ErbB4 were detected by Western Blot. In all cell lines, CI-1033 treatment resulted in a strong down-regulation of ErbB2, irrespective of radiotherapy (Figure 6). This phenomenon was also observed for EGFR.
albeit to a lesser extent. ErbB4 was strongly reduced by CI-1033 in D384MG cells, and slightly in U-251 MG, where baseline expression was already weak. Upon radiation alone, U-251 MG cells only showed weak up-regulation of ErbB3, which was more pronouncedly observed in D384MG cells, that also showed moderate EGFR up-regulation, whereas CI-1033 efficiently reduced radiation-induced up-regulation.

Akt and ERK pathway inhibition in HGG cells upon treatment with CI-1033
To ascertain downstream effects of irreversible ErbB family tyrosine kinase domain blockage by CI-1033 and radiation in HGG cells, levels of phosphorylated Akt, total Akt, phosphorylated ERK and total ERK were measured by FACS. To this purpose, U-251 MG, D384MG and Gli-6 cells were incubated with either CI-1033 or vehicle for 24 hrs, and irradiated with 4 Gy or not irradiated. Compared to vehicle control, treatment with CI-1033 resulted in a marked reduction of absolute Akt and ERK phosphorylation in both Gli-6 and D384MG cells, which was sustained after radiation (Figure 7). Interestingly, U-251 MG cells, that did not show a radiosensitizing effect with CI-1033 pre-treatment, did not reveal diminished Akt and ERK phosphorylation: even a mild to moderate increase was observed.

Figuur 6. Immunoblot of EGFR, ErbB2, ErbB3 and ErbB4 expression in HGG cells after 24 hrs of treatment with CI-1033 or vehicle, with or without 4 Gy of irradiation. Gli-6 cells were pretreated with 5 µM CI-1033, U-251 MG and D384MG cells with 8 µM CI-1033.
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Figuur 7. Levels of Akt and ERK phosphorylation in HGG cells, compared to baseline (vehicle treated, non-irradiated cells), measured after 24 or 48 hrs pre-treatment CI-1033, with or without 4 Gy of radiation. Bars indicate standard deviation (SD).
DISCUSSION

ErbB family signaling involves highly complex signal transduction cascades between multiple inter-related pathways. Heterogeneity at the cellular and molecular level and redundant or overlapping signaling between these pathways may explain, in part, the therapeutic resistance of HGG to agents directed against single ErbB family members. Indeed, inhibiting a specific ErbB member and pathway may result in activation of compensatory receptors and pathways, allowing glioma cell survival\(^{27}\). Thus, an irreversible, pan-ErbB inhibitor is to be expected to exceed the efficacy of single ErbB family TKI or monoclonal antibodies, overcoming and preventing (mutation-related) resistance to these agents in HGG\(^{28}\).

In our study, we performed in silico analysis of the ErbB family members in mRNA datasets of adult and childhood HGG, which revealed that EGFR and ERBB2 were significantly overexpressed to normal brain in all datasets. ERBB4 however, was markedly down-regulated compared to normal brain tissue. Furthermore, increasing expression of both EGFR and ERBB2 was observed with increasing grade of malignancy in AA and GBM respectively, suggesting a role in malignant transformation (Figure 2). These profiles of overexpression suggest that targeting more than one ErbB family member might be of therapeutic benefit in both AA and GBM. Analogous to the in silico analysis, EGFR and ErbB2 were expressed in all three HGG cell lines tested, but ErbB3 only in AA cell line D384MG. Moderate expression of the ErbB4 receptor was observed in all three cell lines.

Irreversible pan-ErbB family tyrosine kinase inhibition by CI-1033 resulted in differential and potent growth inhibition in the HGG cell lines tested, with IC50 values ranging from 1 – 4.3 µM, which is within the range of in vitro studies in other cancer cell lines\(^{29}\). Interestingly, AA cell line D384MG, harboring all four ErbB family members, showed the highest sensitivity to CI-1033, whereas the higher sensitivity of U-251 MG compared to Gli-6 might be explained by the expression of ErbB2 in the former. The paradigm of ErbB family member expression and sensitivity to ErbB inhibition was previously observed in lung and breast cancer\(^{30,31}\). Cell cycle arrest was observed in U-251 MG and Gli-6, whereas apoptosis contributed to the observed drug sensitivity in D384MG cells. This might be explained by both ErbB family addiction of this cell line, and a strong reduction of Akt pathway signaling upon ErbB family inhibition, abrogating apoptosis inhibition by Akt\(^{32,33}\). Upon CI-1033 treatment, strong ErbB2 down-regulation was observed in all cell lines and EGFR down-regulation in two out of three. This inhibitor-mediated down-regulation is likely to be caused by receptor internalization, ubiquitination, and degradation\(^{34}\).

This study is the first to show both potent cytotoxic effects and radiosensitization in HGG cells by pan-ErbB family inhibitor CI-1033, and its modulation of EGFR downstream signaling components in these cells. Two out of three HGG cell lines could be sensitized to radiation
by CI-1033 pretreatment. These results are in concordance with previously published in vitro studies showing radiosensitization in other tumor types14-16. Interestingly, we found these radiosensitizing effects to be schedule-dependent. No differences in cell cycle distribution were found to account for this observed phenomenon. Baseline ErbB family expression could not predict the differences in radiosensitization observed, although in U-251 MG cells a less profound ErbB2 down-regulation upon CI-1033 treatment might partly explain the lack of radiosensitization.

Our study is the first to show CI-1033 induced alterations of phosphorylation of Akt and ERK in HGG cells. We found reduced levels of pAkt and pERK upon treatment with CI-1033, both as single agent and in concert with radiation in HGG cell lines D384MG and Gli-6. Interestingly, in these cells CI-1033-induced radiosensitization was observed. This suggests that irreversible ErbB family inhibition disrupts PI3K/Akt- and Ras/MAPK pathway signaling, leading to a diminished DNA repair response, hereby enhancing radiosensitivity in these cells. In line with this, U-251 MG cells, in which no radiosensitization was observed, showed strong up-regulation of phosphorylated Akt and to a lesser extent up-regulation of phospho-ERK after CI-1033 treatment. Interestingly, the U-251 MG cell line harbors a homozygous inactivating PTEN mutation (723_724insTT; E242fs*15)35, which might explain this observed Akt activation and lack of radiosensitization, due to increased Akt activity. Activation of both Akt and EGFR as a result of loss of PTEN was described earlier in EGFR inhibitor resistance in GBM and non small-cell lung cancer36,37.

In conclusion, our study is one of the first to show CI-1033 induced cytotoxicity and radiosensitization in glioma cell lines in vitro. This warrants further in vivo orthotopic GBM mice studies, studying timing of the combination of CI-1033 with RT (± temozolomide). More research is needed to define predictors of CI-1033 induced radiosensitization. Ultimately, CI-1033 might be an interesting drug to study in clinical studies in adults and children with HGG, aiming to improve the dismal prognosis of these cancers.
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