The effects of anti-VEGFR and anti-EGFR agents on glioma cell migration through implication of growth factor with integrins

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Objective: The aim of this study was to assess the antitumor effect of an anti-VEGFR (sunitinib) and the anti-EGFR multitargeted agent (lapatinib), applied either alone or in combination on the migration capacity of two glioma cell lines. Furthermore, we sought to evaluate the effect of lapatinib in the formation of EGFR-integrin β_{1} complex, as well as the effect of sunitinib in the VEGFR-integrin β_{3} and PDGFR-integrin β_{3} complexes formation.

Material and methods: U87 and M059K cells were cultured as recommended by ATCC. Migration assays were performed in boyden chambers, using uncoated polycarbonate membranes. Immunoprecipitation and western blot analysis were used for studying the complex formation of EGFR, PDGFR and VEGFR with integrins. The protein localization was evaluated using immunofluorescence assay.

Results: We found that both agents administered either alone or in combination, reduced the ability of U87 and M059K cells to migrate 4 h after their application. The time course study of the effect of lapatinib on EGFR-integrin β_{1} complex revealed an inhibition in complex formation up to 30 min after the application of the agent. Likewise, sunitinib inhibited complex formation of VEGFR-integrin β_{3} complex within 2h after its application without affecting PDGFR-integrin β_{3} complex. The previously-described interruption of complexes formation was confirmed with an immunofluorescence assay.

Conclusions: The preliminary results of our study are the first to support the implication of a dual anti-EGFR/HER-2 agent, lapatinib and a multi-targeted agent, sunitinib in glioma cells migration, through a mechanism implying interruption of growth factor receptor - integrin complexes formation.
Key words: Lapatinib, sunitinib, malignant glioma, migration, MMPs, growth factors, integrins
**Introduction**

Malignant gliomas (MG) are the most common and aggressive primary brain tumors. Despite treatment advances with the use of modern molecularly-targeted treatment options, the outcome of patients with MG, particularly with glioblastomas, remains dismal (Argyriou et al, 2009a). At present, research has been focused on elucidating the hypothesis concerning whether targeting multiple signalling pathways by multitargeted kinase inhibitors or combinations of single-targeted kinase inhibitors may increase treatment efficacy (Argyriou and Kalofonos, 2009).

Sunitinib, recently approved for the treatment of advanced renal carcinoma and refractory gastrointestinal stromal tumours, is an oral, small-molecule, multi-targeting receptor tyrosine kinase inhibitor (TKI), including platelet-derived growth factor receptors (PDGFR) and vascular endothelial growth factor receptors (VEGFR). It also inhibits other important growth factor receptors, such as cKIT, FLT3 and RET (Gan et al, 2009). However, its efficacy in patients with glioblastoma remains to be clarified in both the preclinical and clinical setting (Argyriou et al, 2009b).

Epidermal growth factor receptor (EGFR) is amplified in about half of patients with glioblastoma, thus significantly contributing to signal transduction, metabolism and overall oncogenic activity of these brain tumours (Nakamura, 2007). Lapatinib is an ATP-competitive dual TKI for epidermal growth factor receptor (EGFR) and HER2/neu (ErbB-2), with some evidence of inhibitory effect in certain cell lines, including glioblastomas (Guo et al, 2009).

Our group has previously demonstrated that sunitinib and lapatinib have an inhibitory effect on U87 and M059K glioma cell lines (Giannopoulou et al, 2010). In the current setting, we studied the effect of each agent in cell migration of these two glioma cell lines. Considering that cell migration is promoted by the cooperation of
integrins with growth factor receptors (Hynes, 2002), we focused on the complex formation between integrin subunit β₁ with EGFR and integrin subunit β₃ with VEGFR or PDGFR.

We have hypothesized that the tested agents interrupt these complexes and inhibit cell migration. To test our assumption, we evaluated the effect of lapatinib in the formation of EGFR-integrin β₁ complex, as also the effect of sunitinib in the VEGFR-integrin β₃ and PDGFR-integrin β₃ complexes formation. A mediator that might also participate in this pathway is Focal Adhesion Kinase (FAK) and therefore its role as an intermediate molecule after disruption of β₁ subunit –EGFR complex was also assessed.

Materials and Methods

Cell culture and reagents

The U87 and M059K glioblastoma cell lines were cultured in DMEM with 2 mM L-glutamine and supplemented with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin and 50 μg/ml gentamycin at conditions of 37 °C, 5% CO₂ and 100% humidity. The tested agents were applied in cells at the dose of 1 μM as previously described (Giannopoulou et al, 2010).

Immunoprecipitation

U87 and M059K cell lines were plated at 1x10^6 cells per flask in 75 cm² flask in culture media at 37 °C. Tested agents were added as described above and incubation of cells was terminated at several time points (5, 15, 30, 60, 120 and 240 min) later by adding lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 10% glycerol, 1mM phenylmethyl-sulphonyl-fluoride, 2mM Na-
orthovanadate and 10 mM leupeptin). Total amount of protein was determined by
Bradford assay and 1 mg of total protein was immunoprecipitated with a mouse
monoclonal anti-EGFR antibody (Millipore, Upstate, Temecula, CA), a rabbit
polyclonal anti-VEGFR2 (Flk-1) antibody (SantaCruz, USA) and a rabbit polyclonal
anti-pFAK (R&D, Germany) overnight at 4°C, under continuous agitation. In each
sample, 50 μl of protein-A sepharose beads (Sigma, Amersham biosciences) were
added and samples were incubated for 4 h, at 4°C, under continuous agitation.
Precipitates were washed twice with ice-cold lysis buffer and sepharose beads were
re-suspended in 50 μl 2X sample buffer (0.5 M Tris-HCl pH 6.8, 20% glycerol, 2%
SDS and 2% bromophenol blue, 10% β-mercaptoethanol). Samples were heated for 5
min at 95°C and analyzed with Western blotting (Giannopoulou et al, 2009).

Western blot analysis

Immunoprecipitates were loaded in 8% SDS-PAGE gels, analyzed and transferred to
nitrocellulose membrane (Schleicher and Schuell Biosciene, GmbH, Germany). For
the detection of integrins, subunits β₁ and β₃ and FAK proteins blocking was
performed by incubation of the membranes in 5% (w/v) non-fat dry milk in Tris-
buffered saline pH 7.4 containing 0.05% Tween 20 (TBS-T), for 1 h at room
temperature and under continuous agitation. The membranes were then incubated
with a mouse monoclonal anti-β₁ (1:1000, SantaCruz, USA), a mouse monoclonal
anti-β₃ (1:500, SantaCruz, USA) and a sheep polyclonal anti-pFAK (1:1000, R&D,
Germany) in 3% (w/v) non-fat dry milk in TBS-T, for 2 h, at room temperature, under
continuous agitation. After three washes in TBS-T, membranes were further incubated
with horseradish peroxidase conjugated goat anti-mouse IgG (Millipore, Upstate,
Temecula, CA) or donkey anti-sheep IgG (R&D, Germany), in 3% (w/v) non-fat dry
milk in TBS-T, for 1.5 h, at room temperature, under continuous agitation. Detection
of the immunoreactive proteins was performed by chemiluminescence horseradish peroxidase substrate SuperSignal (Pierce), according to the manufacturer’s instructions.

**Immunofluorescence assay**

Both glioblastoma cell lines were treated with sunitinib or lapatinib as previously described (Giannopoulou et al., 2010). At the indicated time points, medium was removed and cells were washed twice with PBS. Cells were fixed with a 4% paraformaldehyde in PBS buffered solution for 10 min at room temperature and then they were rinsed 3X5 min with PBS. An incubation of 1 h was followed by a 3% BSA solution supplemented with 10% FBS at 37 °C. After the incubation with blocking solution, cells were rinsed once with PBS for 5 min and they were treated overnight at 4 °C with a rabbit polyclonal anti-VEGFR2 (1:250, SantaCruz, USA), a rabbit polyclonal anti-PDGFR (1:100, Upstate, Millipore, Temecula, CA), and a mouse monoclonal anti-β3 (1:50, SantaCruz, USA) diluted in blocking solution. Cells were rinsed 3X5 min with PBS and then a donkey anti-rabbit antibody Alexa Fluor 594 or chicken anti-mouse Alexa Fluor 488 (Invitrogen, Molecular probe) diluted in blocking solution was added for 30 min at 37 °C. Cells were rinsed 3X5 min with PBS and mounted on glass sides. Fluorescence was visualized using a Leica microscope (Koutras et al., 2009).

**Statistical analysis**

Differences between groups and controls were tested using the one way analysis of variance (ANOVA). Each experiment included at least triplicate measurements for each condition tested. All results are expressed as mean ± SEM from at least three independent experiments.
Results

The interaction of lapatinib with the $\beta_1$ integrin subunit -EGFR complex

U87 and M059K cells were treated with lapatinib 1 $\mu$M and cells were collected at the indicated time points. The applied dose of lapatinib as well as sunitinib has been chosen according to our previous published data (Giannopoulou et al, In press). Immunoprecipitation and western blot analysis in U87 cells revealed that lapatinib interrupts the formation of $\beta_1$ subunit –EGFR up to 30 min after the treatment of cells (fig1). In M059K cells, lapatinib exerted a similar effect at 30 min (fig2). The disruption of the complex was reversed at later time points for both cell lines.

The interaction of sunitinib with the $\beta_3$ integrin subunit -VEGFR complex

As previously, U87 and M059K cells were treated with sunitinib 1 $\mu$M at the indicated time points and cell pellets were collected. Western blot analysis of the U87 immunoprecipitates revealed that sunitinib inhibited the complex formation of integrin $\beta_3$ subunit –VEGFR 60 min after treatment of cells (fig3). The results were confirmed using an immunofluorescence assay (fig4). Double staining of $\beta_3$ integrin subunit and VEGFR revealed a translocation of $\beta_3$ subunit from the cell membrane to the nucleus. The same effect was observed in M059K cells. The inhibition of integrin $\beta_3$ subunit with VEGFR was reversed at later time points for both cell lines (fig5).

The interaction of sunitinib with the $\beta_3$ integrin subunit -PDGFR complex

It was found that sunitinib did not affect the integrin $\beta_3$ subunit –PDGFR complex in M059K at any time points tested. Double staining of $\beta_3$ integrin subunit and PDGFR did not show any change in location of two receptors up to 2h after the
treatment of cells with sunitinib (fig6). The experiments with U87 cells are still ongoing.

**The effect of lapatinib in p-FAK levels**

To clarify whether FAK acts as an intermediate molecule after disruption of β1 subunit –EGFR complex, U87 cells were treated with lapatinib at the indicated time points. We found that lapatinib induced a decrease in phosphorylated levels of FAK and this effect occurred 5 min after treatment of cells (fig7).

**Discussion**

Integrins are cell surface migration-promoting receptor glycoproteins that mediate various intracellular signals through interaction with the extracellular matrix (ECM). Integrins also play a significant role in the attachment of cells to ECM, through the formation of cell adhesion complexes, consisting of integrins and many cytoplasmic proteins (Stupack, 2007). Particularly for glioblastomas, integrins participate into the regulation of complex processes, such as angiogenesis, tumour growth and metastasis.

Current knowledge shows that the turnover of adhesions is critical for effective cancer cell migration, which is considered to typically be regulated by integrins, matrix-degrading enzymes and cell to cell adhesion molecules. Several cytokines and growth factors have been shown to stimulate migration and be upregulated in a variety of tumour types, including glioblastomas (Hood and Cheresh, 2002). Therefore, the intracellular inhibition of integrin function and signalling might represent an alternative option for the therapeutic inhibition of glioblastoma cells migration (Rüegg and Alghisi, 2010).
The results of the current experimental study advocate in favor of the latter view as our main finding to emerge was that both agents administered either alone or in combination, inhibited the ability of glioma cells to migrate, through the interruption of complexes formation between integrins with growth factor receptors.

The effect of lapatinib on EGFR-integrin β1 complex revealed an inhibition in complex formation up to 30 min after the application of the agent in both cell lines. Previous data in A431 cells have shown that EGFR is coprecipitated with β1 integrin subunit and this colocalisation is located at the cell-cell contact sites (Yu et al, 2000). In the same study, it has been described that EGFR, which is colocalized with integrin is phosphorylated without the presence of any ligand. The phosphorylation of EGFR is induced by its association with integrins. Although, the role of EGFR in cell-cell contact sites is so far unknown, it might be implicated in cell migration after the formation of the complex with integrins.

Sunitinib was able to exert pharmacological inhibition of vascular integrins. Our experiments demonstrated that it interfered with the complex formation of VEGFR-integrin β3 but not with PDGFR-integrin β3 complex. In addition, we did not find any change in the complex between PDGFR and integrin β3. The interruption of sunitinib with the complex VEGFR-integrin β3 was observed within 2h after its application. Previous data show that in endothelial cells there is an interaction between integrin β3 and VEGFR (Mahabeleshwar et al, 2009). In this study the authors support that the interaction of integrin with the VEGFR leads to an activation of VEGFR in the absence of VEGF.

We might imply that the interaction of integrins with growth factors receptors may promote cell migration without being necessary the presence of any ligand. Our results are consistent and support previously published data supporting that anti-β1,
anti-αβ3 and anti-β3 antibodies have induced potent inhibition of glioma cell migration through various ECM substrates (Friedlander et al, 1996).

FAK, a non-receptor cytoplasmic-tyrosine kinase, is activated by several different cell surface receptors shown to be upregulated on glioblastoma cells. Phosphorylated FAK can signal through several different signalling pathways in glioblastomas, thereby stimulating glioma cell proliferation and invasion on various ECM substrates. In addition, increased levels of FAK protein, together with its overactivity, may contribute to an increased ERK activity and cell proliferation of these brain tumors (Natarajan et al, 2003). In the current setting, lapatinib decreased the phosphorylated levels of FAK. However, this occurred at an earlier time point compared to the interruption of the complexes integrins-growth factor receptors, thereby indicating that FAK pathway acts independently of integrin-growth factor receptor signaling and affect cell migration through a different pathway, possibly as a downstream target of growth factor signalling (Riemenschneider et al, 2005). It has been described that integrins may activate the non-receptor tyrosine kinase SRC, which leads to the activation of a FAK independent pathway (Desgrosseliers and Cheresh, 2010).

In line with our results are the results of previous experimental studies that showed that inhibition of FAK phosphorylation by cerivastatin or geldanamycin decreases migration of several glioma cell lines (Obara et al, 2002; Zagzag et al, 2003). Moreover, there is evidence to indicate that the complex formation of PI3K and FAK in glioblastoma cells correlates with the ability of PI3K inhibitors to block cell migration (Ling et al, 1999).

In the clinical setting, multiple-targeting treatment approaches combining both drugs might be more effective than the application of each agent alone, as in recently
published small-sized phase I/II trials, lapatinib and sunitinib administered alone did not show significant activity in recurrent glioblastoma patients (Scott et al, 2010; Thiessen et al, In press). Other preliminary clinical data on the efficacy of these agents in terms of less CNS progression in patients with renal and breast cancer are more promising (Geyer et al, 2006).

Summarizing, the results of our study are the first to support the implication of a dual anti-EGFR/HER-2 agent (lapatinib) and a multi-targeted agent (sunitinib) in the migration of glioma cells, through a mechanism implying interruption of growth factor-integrin complexes formation. Considering that the malignant phenotype of glioblastomas are not dependent on a single pathway and in view of our results we could propose that the multiple-targeting treatment approaches might be more effective than the application of each agent alone. In any case, further studies should be performed to clarify whether our results in vitro on the efficacy of the sunitinib and lapatinib are valid for glioblastoma cell migration in vivo.
References


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Figure 1: Lapatinib intercepted the formation of the integrin β₁ – EGFR complex in U87 cell line up to 30 minutes after the agent application to cells. An IgG antibody was used as a negative control. The picture is a representative of three independent experiments.
Figure 2: Lapatinib intercepted the formation of the integrin β₁ – EGFR complex in M059K cell line 30 minutes after the agent application to cells. The picture is representative of three independent experiments.
**Figure 3:** Sunitinib intercepted the formation of integrin $\beta_3$-VEGFR complex in U87 cell line 60 minutes after the agent application to cells. The picture is representative of three independent experiments.
Figure 4: Sunitinib induced the movement of VEGFR from cell membrane to the cytoplasm in U87 cells up to 60 minutes after the agent application. The picture is representative of three independent experiments (magnification X3.5).
Figure 5: Sunitinib induced the movement of VEGFR from cell membrane to the cytoplasm in M059K cells up to 30 minutes after the agent application. The picture is representative of three independent experiments (magnification X3.5).
Figure 6: Sunitinib did not affect the location of PDGFR and integrin subunit β3 in M059K cells at any time point tested after the agent application. The picture is representative of three independent experiments (magnification X3.5).
**Figure 7:** Lapatinib inhibited the FAK phosphorylation 5 minutes after its application in U87 cells. The picture is representative of three independent experiments.