The Effects of Anti-VEGFR and Anti-EGFR Agents on Glioma Cell Migration Through Implication of Growth Factors with Integrins

KONSTANTINOS DIMITROPOULOS[1], EFSTATHIA GIANNOPOULOU[1], ANDREAS A. ARGYRIOU[1,2], VASSILIKI ZOLOTA[1], THEODORE PETSAS[1], EKATERINI TSIATA[1] and HARALABOS P. KALOFONOS[1]

[1]*Clinical Oncology Laboratory, Division of Oncology, Department of Medicine, University Hospital of Patras, Patras Medical School, Rion, Greece;*
[2]*Department of Neurology, "Saint Andrew's" State General Hospital of Patras, Patras, Greece*

Abstract. *Objective: The aim of this study was to assess the antitumour effect of an anti-VEGFR (sunitinib) and the anti-EGFR multi-targeted agent (lapatinib), applied either alone or in combination on the migration capacity of two glioma cell lines. Furthermore, this study 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. Materials and Methods: U87 and M059K cells were cultured as recommended by the American Type Culture Collection (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 localisation was evaluated using immunofluorescence assay. Results: It was found that both agents, administered either alone or in combination, reduced the ability of U87 and M059K cells to migrate four 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 two h after its application without affecting PDGFR-integrin β_3 complex. The previously described interruption of complexes formation was confirmed with an immunofluorescence assay. Conclusion: The preliminary results of this 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 cell migration, through a mechanism implying interruption of growth factor receptor integrin complexes formation.*

Correspondence to: H.P. Kalofonos, MD, Ph.D., Division of Oncology, Department of Medicine, University Hospital of Patras, Rion, 26504, Greece. Tel: +30 2610999535, Fax: +30 2610994645, e-mail: kalofon@med.upatras.gr

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Malignant gliomas (MG) are the most common and aggressive primary brain tumours. Despite advances in treatment using modern molecularly-targeted treatment options, the outcome of patients with MG, particularly with glioblastomas, remains poor (1). To date, research has been focused on investigating whether targeting multiple signalling pathways by multi-targeted kinase inhibitors or combinations of single-targeted kinase inhibitors increases treatment efficacy (2).

Sunitinib, recently approved for the treatment of advanced renal carcinoma and refractory gastrointestinal stromal tumours, is an orally administered, 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 (3). However, its efficacy in patients with glioblastoma remains to be clarified in both the preclinical and clinical setting (4).

Epidermal growth factor receptor (EGFR) is amplified in around half of patients with glioblastoma, thus significantly contributing to signal transduction, metabolism and overall oncogenic activity of these brain tumours (5). 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 (6).

It has been previously demonstrated that sunitinib and lapatinib have an inhibitory effect on U87 and M059K glioma cell lines (7). The current study investigated 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 (8), this study focused on the complex formation between integrin subunit β_1 with EGFR and integrin subunit β_3 with VEGFR or PDGFR.

It was hypothesized that the tested agents interrupt these complexes and inhibit cell migration. To test this hypothesis, the effect of lapatinib in the formation of EGFR-integrin β1 complex, as well as the effect of sunitinib in the VEGFR-integrin β_3 and PDGFR-integrin β_3 complexes formation were evaluated. 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 β_1 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% foetal bovine serum, 100 U/ml penicillin-streptomycin and 50 μg/ml gentamycin at conditions of 37˚C, 5% CO_2 and 100% humidity. The tested agents were applied in cells at the dose of 1 μM as previously described (7).

Immunoprecipitation. U87 and M059K cell lines were plated at 1×10^6 cells per flask in 75 cm^2 flasks 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, 2 mM Na-orthovanadate and 10mM leupeptin). The total amount of protein was determined by Bradford assay and 1mg of total protein was immunoprecipitated with a mouse monoclonal anti-EGFR antibody (Millipore, Upstate, Temecula, CA, USA), 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, Uppsala, Sweden) were added and samples were incubated for four hours, 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 analysed with Western blotting (9).

Western blot analysis. Immunoprecipitates were loaded in 8% SDS-PAGE gels, analysed and transferred to nitrocellulose membrane (Schleicher and Schuell Bioscience, GmbH, Germany). For the detection of integrins, subunits β_1 and β_3 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 one hour at room temperature and under continuous agitation. The membranes were then incubated with a mouse monoclonal anti-β_1 (1:1000, SantaCruz, USA), a mouse monoclonal anti-β_3 (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 two hours, 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, USA) 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 WestPico (Pierce, Rockford, USA), according to the manufacturer's instructions.

Immunofluorescence assay. Both glioblastoma cell lines were treated with sunitinib or lapatinib as previously described (7). At the indicated time points, the medium was removed and cells were washed twice with PBS. Cells were fixed with a 4% paraformaldehyde in PBS buffered solution for ten minutes at room temperature and then they were rinsed 3×5 min with PBS. An incubation of one hour 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 five minutes 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-β_3 (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 visualised using a Leica microscope (LEICA, Germany) (10).

Results

The interaction of lapatinib with the β_1 integrin subunit - EGFR complex. U87 and M059K cells were treated with lapatinib 1 μM and cells were collected at the indicated time points. The applied dose of lapatinib as well as sunitinib was chosen according to previously published data (7). Immunoprecipitation and Western blot analysis in U87 cells revealed that lapatinib interrupts the formation of β_1 subunit –EGFR up to 30 min after the treatment of cells (Figure 1). In M059K cells, lapatinib exerted a similar effect at 30 min (Figure 2). The disruption of the complex was reversed at later time points for both cell lines.

The interaction of sunitinib with the β_3 integrin subunit - VEGFR complex. As previously, U87 and M059K cells were treated with sunitinib 1 μM 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 β_3 subunit –VEGFR 60 min after treatment of cells (Figure 3). The results were confirmed using an immunofluorescence assay (Figure 4). Double staining of β_3 integrin subunit and VEGFR revealed a translocation of β_3 subunit from the cell membrane to the nucleus. The same effect was observed in M059K cells. The inhibition of integrin β_3 subunit with VEGFR was reversed at later time points for both cell lines (Figure 5).

The interaction of sunitinib with the β_3 integrin subunit - PDGFR complex. It was found that sunitinib did not affect the integrin β_3 subunit –PDGFR complex in M059K at any

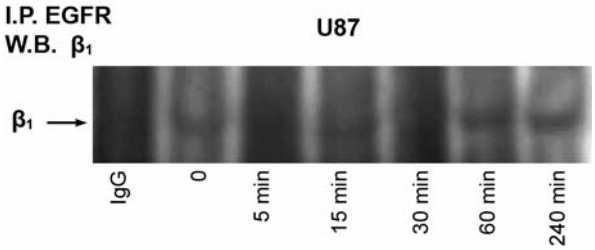

Figure 1. *Lapatinib intercepted the formation of the integrin β_1-EGFR complex in U87 cell line up to 30 min after the agent application to cells. An IgG antibody was used as a negative control. The figure represents three independent experiments.*

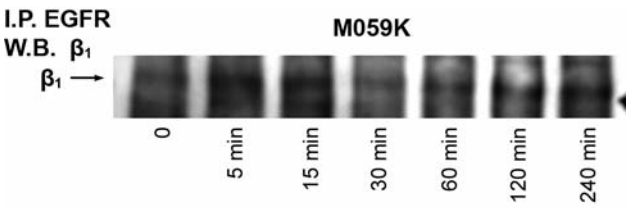

Figure 2. *Lapatinib intercepted the formation of the integrin β_1-EGFR complex in M059K cell line 30 min after the agent application to cells. The figure represents three independent experiments.*

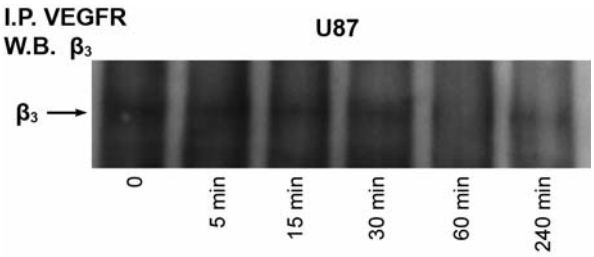

Figure 3. *Sunitinib intercepted the formation of integrin β_3-VEGFR complex in U87 cell line 60 min after the agent application to cells. The figure represents three independent experiments.*

of the time points tested. Double staining of β_3 integrin subunit and PDGFR did not show any change in location of the two receptors up to two h after the treatment of cells with sunitinib (Figure 6).

The effect of lapatinib in p-FAK levels. To clarify whether FAK acts as an intermediate molecule after disruption of β_1 subunit –EGFR complex, U87 cells were treated with lapatinib at the indicated time points. It was found that lapatinib induced a decrease in phosphorylated levels of FAK and this effect occurred five min after treatment of cells (Figure 7).

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 (11). Particularly for glioblastomas, integrins participate in 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 up-regulated in a variety of tumour types, including glioblastomas (12). Therefore, the intracellular inhibition of integrin function and signalling might represent an alternative option for the therapeutic inhibition of glioblastoma cell migration (13).

The results of the current study are in line with the aforementioned published data, as the main finding was that both agents administered either alone or in combination, inhibited the ability of glioma cells to migrate, through the interruption of complex formation between integrins and growth factor receptors.

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 in both cell lines. Previous data in A431 cells have shown that EGFR is co-precipitated with β_1 integrin subunit and this co-localisation is located at the cell-cell contact sites (14). In the same study, it was described that EGFR, which is co-localised 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 not yet understood, 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. The current study demonstrated that it interfered with the complex formation of VEGFR-integrin β_3, but not with PDGFR-integrin β_3 complex. In addition, there was not any change in the complex between PDGFR and integrin β_3. The interruption of sunitinib with the complex VEGFR-integrin β_3 was observed within two h after its application. Previous data show that in endothelial cells there is an interaction between integrin β_3 and VEGFR (15). In this study it was found that the interaction of integrin with the VEGFR lead to an activation of VEGFR in the absence of VEGF. Considering that sunitinib may cause the accumulation of VEGFR in endosomes (16), the translocation of VEGFR from membrane to the cytosol might suggest that the receptor was degraded in lysosomes.

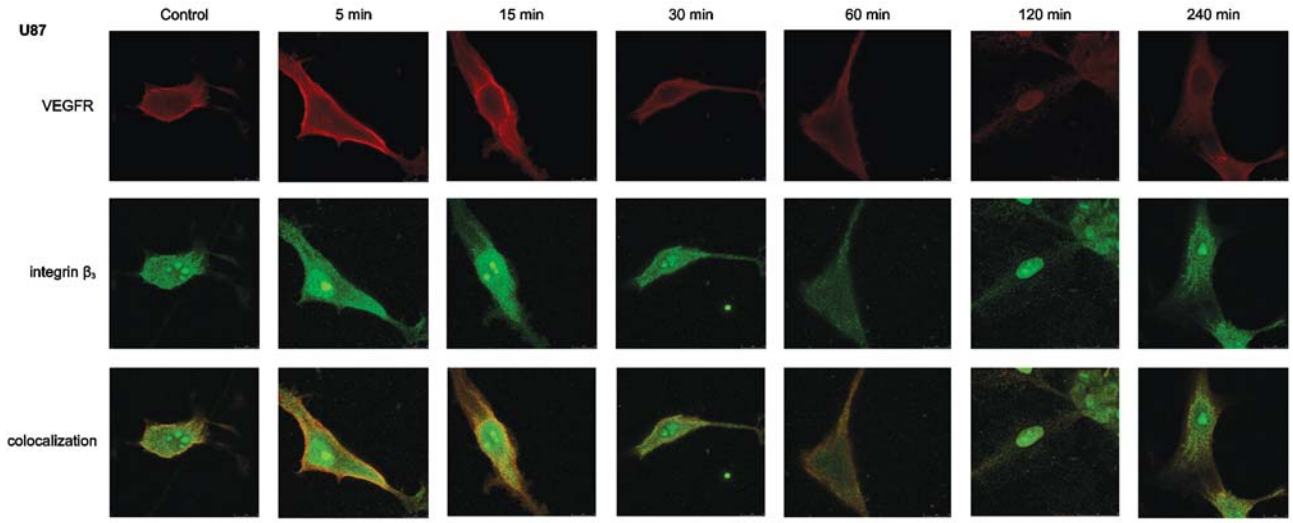

Figure 4. *Sunitinib induced the movement of VEGFR from cell membrane to the cytoplasm in U87 cells up to 60 min after the agent application. The figure represents three independent experiments (magnification ×3.5).*

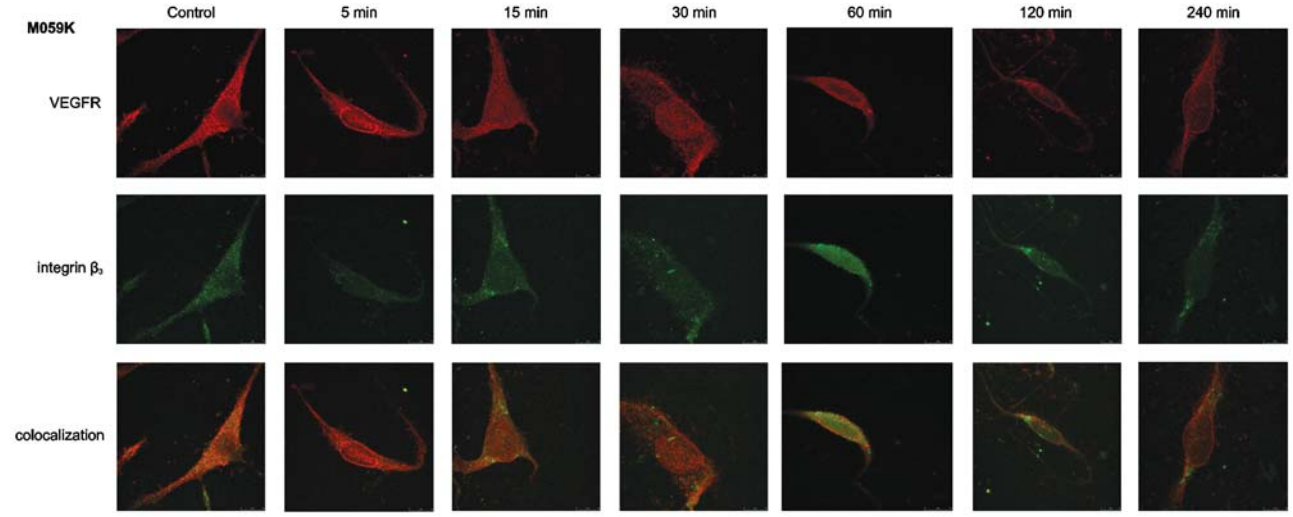

Figure 5. *Sunitinib induced the movement of VEGFR from cell membrane to the cytoplasm in M059K cells up to 30 min after the agent application. The figure represents three independent experiments (magnification ×3.5).*

It might be hypothesized that the interaction of integrins with growth factors receptors may promote cell migration without the presence of any ligand being necessary. The results from the current study are consistent with this hypothesis and support previously published data supporting that anti-β_1, anti-$\alpha_v\beta_3$ and anti-β_3 antibodies have induced potent inhibition of glioma cell migration through various ECM substrates (17).

Concerning the translocation of integrin β_1 subunit in nucleus without observing the same effect in β_3 subunit, it is known that integrin-linked kinase (ILK) is able to bind to the cytoplasmic tail of integrin β_1 subunit and may also translocate to the nucleus, thus affecting the nuclear integrity and function (18, 19). As a result it seems that the translocation of integrin β_1 subunit to the nucleus might be mediated by ILK. However, further studies are needed to support this hypothesis.

FAK, a non-receptor cytoplasmic-tyrosine kinase, is activated by several different cell surface receptors that are shown to be up-regulated 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 increased phosphorylated levels, may contribute to an increased ERK activity and cell proliferation of these brain tumours (20). In the current study, lapatinib decreased the phosphorylated levels of

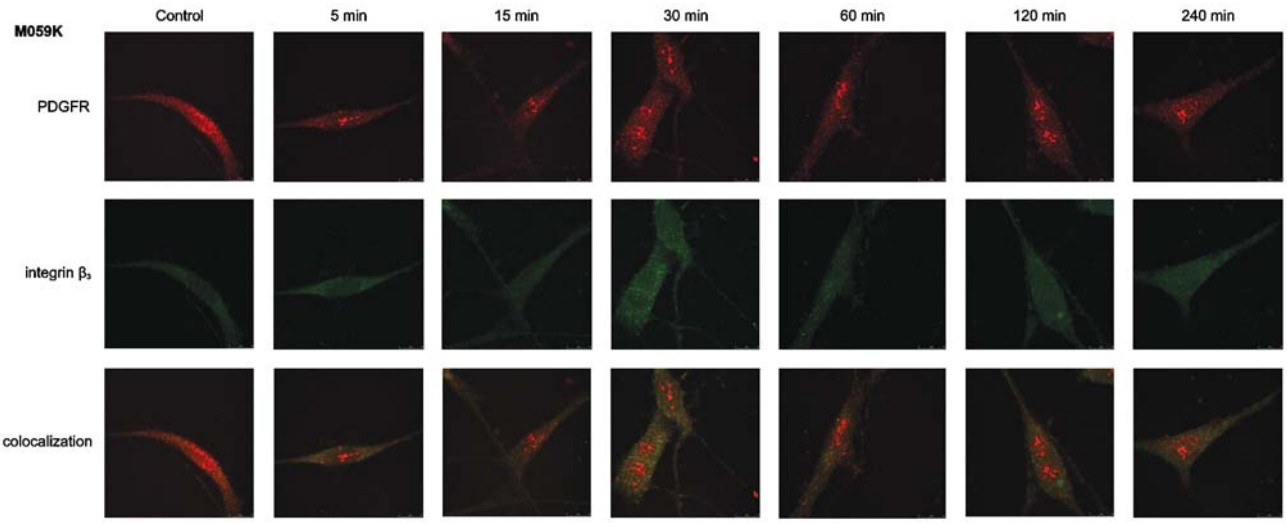

Figure 6. *Sunitinib did not affect the location of PDGFR and integrin subunit β3 in M059K cells at any time point tested after the agent application. The figure represents three independent experiments (magnification ×3.5).*

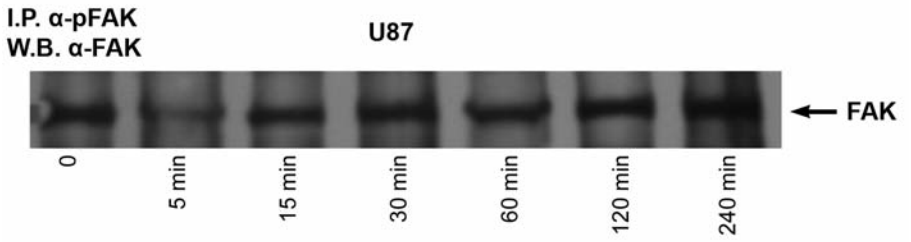

Figure 7. *Lapatinib inhibited the FAK phosphorylation five min after its application in U87 cells. The figure represents three independent experiments.*

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 signalling and affect cell migration through a different pathway, possibly as a downstream target of growth factor signalling (21).

It has been described that integrins may activate the non-receptor tyrosine kinase SRC, which leads to the activation of a FAK independent pathway (22). Alternatively, the rapid FAK dephosphorylation might be the result of EGFR inhibition by lapatinib since inhibition of the receptor causes inactivation of Src, which in turn reduces FAK phosphorylation (23). Furthermore, at later time points, the re-phosphorylation of FAK might indicate an inactivation of protein tyrosine phosphatises, as there is evidence that PTP-1B is up-regulated in HER-2 transformed cell lines (24).

In agreement with the results of the current study 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 (25, 26). 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 (27).

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 (28, 29). 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 (30).

In conclusion, the results of this 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 these results, it is proposed 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 these *in vitro* results are valid for glioblastoma cell migration *in vivo*.

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