An in vitro study, evaluating the effect of sunitinib and/or lapatinib on two glioma cell lines.

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Running title: effect of sunitinib and/or lapatinib on glioma cells.

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Aim: We sought to assess the effect of sunitinib and lapatinib applied either alone or in combination, on U87 and M059K glioma cells.

Methods: Both cell lines were cultured as recommended by the manufacturer. Sunitinib and lapatinib were applied, either separately or in combination, in the cultured cells after cell attachment at doses of 10nM, 100nM, 1 μ M and 10 μ M. To determine whether the agents affect the proliferation of glioma cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5 dimethyltetrazolium bromide assay was used. Apoptosis was detected using annexin V/propidium iodide detection assay, migration assay was performed in 24-well microchemotaxis chambers. The release of MMPs into the culture medium of U87 and M059K cells was measured by zymography.

Results: Both agents, administered either alone or in combination, decreased cell proliferation in a dose-dependent manner 48 h after their application in both cell lines. The inhibition of their combination was statistically different than the inhibition of each agent alone. Apoptosis was increased and migration of U87 and M059K cells was inhibited either by each agent alone or their combination. MMPs levels remained unaffected by the application of both agents in U87 cells. However, MMP-9 and MMP-2 levels were decreased 48h after treatment of M059K cells with sunitinib either alone or in combination with lapatinib.

Conclusion: Sunitinib and/or lapatinib appear to exhibit significant effects on proliferation, apoptosis and migration of glioma cells. When applied alone, sunitinib appears to be a more potent inhibitor than lapatinib.

Keywords

Lapatinib, sunitinib, malignant glioma, proliferation, apoptosis, migration, MMPs.

Introduction

Malignant gliomas (MGs), including glioblastomas (GBMs) and anaplastic astrocytomas (AAs) are the most common and aggressive primary brain tumors. Despite treatment advances, the outcome of patients with MG remains dismal [1]. Recent advances, mostly in the thorough understanding of the complex molecular pathogenesis of MGs, have led to the arousal of rational development of molecularly targeted treatment options [1,2]. Currently, monoclonal antibodies (MAbs) targeting angiogenesis, several multitargeted kinase inhibitors and combination of single-targeted kinase inhibitors of ErbB family receptors have been tested in experimental models and clinical trials for their ability to establish future therapeutic directions in MGs [2].

Sunitinib (Sutent) is an oral, small-molecule, multi-targeted receptor TKI, including platelet-derived growth factor receptors (PDGFR) and vascular endothelial growth factor receptors [3]. Sunitinib exhibits potent antiangiogenic and antitumor activity, particularly evident in advanced renal cell carcinoma (RCC). The inhibitory effect of sunitinib has previously been shown in several RCC and gastrointestinal stromal malignant cell lines [4], as well as in breast [5] and neuroendocrine cancer cell lines [6]. To our knowledge, only one study has examined the inhibitory effect of sunitinib in glioma cell lines [7]. Additionally, sunitinib has also shown survival benefits against a subcutaneous glioma xenograft [8].

Lapatinib (Tykerb) is an ATP-competitive epidermal growth factor receptor (EGFR) and HER2/neu (ErbB-2) dual TKI. An important inhibitory effect of lapatinib has been demonstrated in certain cell lines, including human breast and prostate cancer [9,10].

To our knowledge, there is a lack of experimental studies in the literature on the inhibitory effect of lapatinib in MGs. However, with a great deal of evidence-based empirical expectations of benefits, an in vitro study of lapatinib in glioma cell lines appears to be justified.

Although the efficacy of each one of the TKIs combined with other conventional cytotoxic agents, such as sunitinib and lenalidomide plus low-dose metronomic cyclophosphamide [11] or lapatinib and tamoxifen [9] has been studied, little is currently known about the efficacy of the combination of these two TKIs. Additionally, considering the lack of studies directly comparing the effect of sunitinib versus lapatinib on human cancer cell lines, another issue that remains to be clarified is which of these agents is a more potent inhibitor when applied alone.

The aim of the current in vitro study was to assess the effect of sunitinib and lapatinib applied either alone or in combination on proliferation, apoptosis, migration and release of MMP-2 into the culture medium of U87 human glioblastoma cells.

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 37 °C, 5% CO₂ and 100% humidity. The tested agents were applied in cells at doses of 0; 0.01; 0.1; 1 and 10 µM. Both agents were diluted

in phosphate buffered saline pH 7.6 (PBS) with dimethyl sulfoxide (DMSO). The final concentration of in the culture medium was 0.5%.

Cell proliferation assay

To determine whether the aforementioned agents affect the proliferation of U87 and M059K cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay was used, as previously described [12]. Proliferation assay was performed in serum free medium 48 h after the addition of the tested agents.

Apoptosis assay

Apoptosis was detected using annexin V/propidium iodide detection assay as previously described [12,13]. Tested agents were added in U87 and M059K cells as described above, 24 and 48 h later cells were analyzed by flow cytometry (EPICS-XL of Coulter) according to the manufacturer's instructions (rh Annexin V/FITC kit, Bender MedSystems). The experiments were performed in serum free medium.

Migration assay

Migration assays were performed as previously described [14], in 24-well microchemotaxis chambers (Costar, Avon, France), using uncoated polycarbonate membranes with 8 μ m pores. Briefly, U87 and M059K cells were harvested and resuspended at 3×10^4 cells/0.1ml in serum free medium containing 0.25% bovine serum albumin. The bottom chamber was filled with 0.6 ml of serum free medium with the tested agents. The upper chamber was loaded with 3×10^4 cells and incubated for 4 h at 37 °C. After completion of the incubation, the filters were fixed with saline-buffered formalin and

stained with 0.33% toluidine blue solution. The cells that migrated through the filter were quantified by counting the entire area of each filter, using a grid and a microscope at a 20x magnification.

Zymography

The release of MMP-9 and MMP-2 into the culture medium of U87 and M059K was measured by zymography as previously described [14]. In brief, U87 cells were plated in 24-well plates at a density of 3×10^4 cells per well in serum free medium. The cells were treated with the tested agents, as described above for the proliferation studies. At 24 and 48 h later, 50 μ l of the medium from each group were diluted with 2x Laemmli sample buffer without β -mercaptoethanol. Alternatively, samples of M059K cells were concentrated at 12 fold prior the addition of Laemmli buffer. Protein concentration was detected using Bradford assay and equal amounts of total proteins were analysed. Samples from both cell lines were analyzed in 10 % SDS-PAGE gels containing 0.1 % gelatin. Following electrophoresis, gels were washed four times in 2.5 % Triton-X 100 for 15 min at room temperature and incubated for 24 h at 37 °C in developing buffer (50 mM Tris-HCl pH 7.6, 200 mM NaCl and 10 mM CaCl_2). The gels were stained with Coomassie Brilliant Blue R for 4 h at room temperature, de-stained in methanol acetic acid-water (4.5:1:4.5 v/v) for 2 h and photographed using a digital camera.

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 $\pm$ SEM from at least three independent experiments.

Results

Effects on cell proliferation and apoptosis

Initially, we studied the effect of sunitinib and lapatinib in U87 and M059K cell proliferation. We found that both agents decreased cell proliferation in a dose dependent manner 48 h after their application in cells (Fig. 1A and B). At 1 μ M, sunitinib as well as lapatinib inhibited cell proliferation, **without being toxic for cells**. In U87 cells, the inhibitory effect was not statistically significant between agents (sunitinib vs lapatinib) and was reached at 15% $\pm$ 2 and 24% $\pm$ 3, respectively. However, in M059K cells, lapatinib was more effective than sunitinib. The inhibitory effect for sunitinib and lapatinib was 26% $\pm$ 3 and 42% $\pm$ 5, respectively. The difference was statistically significant and p value was <0.05 . In contrast, at 10 μ M both agents **were toxic for both cell lines** (Fig. 1A and B).

The inhibitory effect of the combination of sunitinib and lapatinib was also studied. We found that at 1 μ M, the inhibition was reached at 47% $\pm$ 7 and 87% $\pm$ 5 in U87 and M059K cells, respectively (Fig.1C and D). The inhibition of agent's combination was statistically different than the inhibition of each agent alone. In U87 cells, the p values was <0.001 and <0.01 for sunitinib and lapatinib, respectively, compared to their combination. Moreover, in M059K cells, the p values were <0.001 and <0.001 for sunitinib and lapatinib, respectively. According to the formula of Fischel et al [15], the effect of agent's

combination is synergistic. The combination of sunitinib and lapatinib at 10 μ M was toxic for cells since the number of cells was lower than cells were used for the initiation of proliferation assay (Fig. 1C and D).

Furthermore, we studied the effect of sunitinib and lapatinib in cell apoptosis. We found that 24 and 48 h after agent's application, apoptosis was increased either by each agent alone or their combination in both cell lines (Fig. 2). The percentage of apoptotic cells in untreated U87 cells was $12\% \pm 2$ and $7\% \pm 2$ at 24 and 48h respectively, after the addition of sunitinib 1 μ M the percentages were elevated to $87\% \pm 2$ and $65\% \pm 11$, respectively, while lapatinib 1 μ M alone increased the apoptotic cells to $32\% \pm 1$ and $20\% \pm 0.5$, respectively. The combination of sunitinib and lapatinib at 1 μ M increased the percentage of apoptotic cells up to $99\% \pm 0.1$ and $92\% \pm 2$ at 24 and 48 h after the addition of agents (Fig. 2A). In M059K cells, the percentages of apoptotic cells were $21\% \pm 7$ and $19\% \pm 2$ at 24 and 48 h, respectively. Sunitinib increased the percentages up to $98\% \pm 0.5$ and $80\% \pm 10$ at the same time points. Lapatinib, did not affect the percentage of apoptotic cells at 24 h ($26\% \pm 0.6$), however the percentage of apoptotic cells was increased up to $36\% \pm 4$ at 48 h. The combination of agents increased the percentage of apoptotic cells up to $83\% \pm 10$ and $79\% \pm 6$, at 24 and 48 h, respectively (Fig. 2B).

Effects on U87 migration capacity

We found that each agent alone as also their combination inhibited the migration of U87 (Fig. 3A) and M059K (Fig. 3B) cells. We found that sunitinib and lapatinib at 1 μ M reduced migration of U87 cells at $45\% \pm 11$ and $21\% \pm 13$, respectively, while their combination caused a reduction in migration up to $42\% \pm 3$. In M059K cells, both 1 μ M

sunitinib and 1 μ M lapatinib reduced cell migration at $51\% \pm 15$ and $28\% \pm 9$, respectively. Their combination reduced cell migration at $62\% \pm 5$.

Effects on secretion of MMPs

U87 and M059K cells were treated with sunitinib 1 μ M or lapatinib 1 μ M or their combination. Twenty four and 48 h later we studied the secretion of MMPs in culture medium. We found that neither each agent alone nor their combination alter MMP-9 and MMP-2 levels in U87 as shown in figure 4 compared to untreated cells. However, sunitinib decreased MMP-9 and MMP-2 levels, 48 h after its application to M059K cells alone or in combination with lapatinib.

Discussion

Multiple or compensatory pathways are involved in the molecular pathogenesis of MGs, whereas multiple receptor tyrosine kinases (RTKs) are coactivated in MGs [16]. MGs are highly dependent on angiogenesis for growth, and therefore, the application of anti-angiogenic molecularly targeted treatment strategies with MAb or TKIs appears to have a solid rationale [17].

To date, a limited number of publications have supported the hypothesis that small TKIs may cross the blood-brain barrier [1]. In any case, the antitumor and antiangiogenic potential of these agents is not widely tested either in experimental or in clinical models of MGs. We therefore tested the antitumor effect of two multi-targeted receptor TKIs, applied

alone or in combination on U87 and M059K cell lines, by evaluating the changes in cell proliferation, apoptosis, migration and MMPs secretion.

Our main finding to emerge was that both sunitinib and lapatinib, when applied alone, decreased cell proliferation in a dose-dependent manner, increased apoptosis and inhibited the migration of both cell lines. This effect was even stronger when the combination of these two drugs was applied. However, when we applied sunitinib alone, we found that it exhibits a stronger antitumor effect compared to lapatinib in the culture medium of U87 and M059K.

Interestingly, no difference in MMPs secretion was detected by zymography analysis of U87 cells conditioned medium following either the application of sunitinib and lapatinib alone or their combination. Therefore, one could assume that the changes in glioma cell invasion may occur without being associated with the alterations in MMPs levels and this event could be potentially attributed to heparanase elevation [18].

Alternative, sunitinib and lapatinib [19] may decrease cells migration through decrease of phosphorylated FAK [7] without necessarily affecting MMPs secretion from U87 cells.

This may imply that MMPs is an essential but not a sufficient factor contributing to migration ability of cells [20]. Downregulation of the status of cell surface $\alpha_5\beta_3$ integrin after drug application may also be implicated and due to that tumor cell surface recruited much less MMPs from surroundings. In contrast, both MMP-9 and MMP-2 levels were decreased after treatment of M059K cells with sunitinib either alone or in combination with lapatinib.

In this study, sunitinib exhibited in a dose-dependent manner a potent antitumor activity and this is primarily attributed to its ability to inhibit angiogenesis, targeting both

VEGFRs and PDGFRs. Additionally, sunitinib may also be able to exert a direct antiproliferative effect on PDGFR-dependent glioma cells [21]. As mentioned earlier, another study previously investigated the inhibitory effect of sunitinib in human glioblastoma cell lines, and in agreement with our results, reached to the conclusion that sunitinib exhibited a potent antiangiogenic activity on U87MG cells. However, this effect was evident and limited to the application of high-dose (10 μ M) sunitinib, whereas this high concentration was non-toxic for cells [7]. In our study, the antitumor effect of both sunitinib and lapatinib was evident at 1 μ M, without **being toxic for cells, while in contrast to the de Boüard et al, [7] study both agents were toxic** for U87 and M059K cells when applied at 10 μ M. Differences in the nature of cells cultured could account for this discrepancy between results. We investigated the effect of sunitinib and/or lapatinib particular on U87 cells, whereas a different culture medium of U87MG cells was used in the de Boüard et al, study [7]. Additionally, although U87 cells are commercially available, it is possible to give dissimilar clones when cultured in different laboratories.

Although to a smaller extent compared to sunitinib, lapatinib affected U87 and M059K cells in a dose-dependent manner, due to its ability to target EGFR and HER2. However, human glioblastoma cells may be less sensitive to this molecule compared to sunitinib, because lapatinib seems less able to downregulate the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway, which is a critical pathway for glioma growth and invasion [22]. In any case, it should be mentioned that lapatinib is the first HER2-directed drug, which was validated in an in vivo preclinical model for activity against brain metastases of breast cancer [19].

The activation state of PI3K and mitogen-activated protein kinase pathways may be determined by the sum of multiple inputs, and multiple RTKs may be at the same time or sequentially employed by MGs cells to maintain signal flux. In such a case, effective MG therapy may require combination of drugs against different activated RTKs [23]. In our study and in line with the latter view, the combination of sunitinib and lapatinib exhibited a dual effect on cell proliferation, apoptosis and migration of U87 cells, which should be attributed to multiple targeting against EGFRs, HER2, VEGFRs and PDGFRs, resulting in the inhibition of angiogenesis and the downregulation of EGFR and HER-2 protein levels. In a similar manner, combination therapy of inhibitors of EGFR/VEGFR-2 (AEE788) and the mammalian target of rapamycin (RAD001) offered in another in vitro study improved glioblastoma tumor growth inhibition [24]. In all likelihood, our results advocate in favor of the view that the multiple-targeting treatment approaches using combined use of two different agents could be more effective than application of each agent alone.

In the current setting, we have examined the activity of sunitinib and/or lapatinib on a single cell line and one could mention that this represents a significant methodological limitation. We might acknowledge this limitation; however, the cell line that we have used (U87) ranks among the most widely used and well studied human glioblastoma cell lines.

In the clinical setting, although the role of sunitinib in advanced RCC has been evaluated through large prospective trials, the efficacy of the drug in patients with primary or secondary brain tumors has not been explored, since patients with cerebral lesions were excluded in those studies [25,26]. Interestingly, in line with the results of the current study, on the potent antitumor activity of sunitinib in glioma cells, is a clinical case report recently published by our group. In this report, monotherapy with sunitinib resulted in a

considerable shrinkage of a solitary brain metastasis from RCC without manifestation of any serious adverse event or central nervous system toxicities [27]. On the other hand, the regimen consisting of lapatinib plus capecitabine in patients with HER-2 positive refractory advanced breast cancer has been found in a randomized trial superior than capecitabine alone in terms of less CNS progression [28].

To summarize, the application of sunitinib and/or lapatinib appears to exhibit significant effects on proliferation, apoptosis and migration of human glioblastoma cell lines. When applied alone, sunitinib has a more potent antitumor activity than lapatinib in cells. Multiple targeting as a result of the combined application of sunitinib and lapatinib improved the antitumor effect. Further in vitro studies are warranted to address this important issue.

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Figure 1: A, B) Different doses of sunitinib or lapatinib were applied on U87 and M059K cells, respectively. **B)** The combination of sunitinib and lapatinib inhibited U87 proliferation. Results are expressed as mean $\pm$ SEM of the number of cells. Asterisks denote a statistically significant difference (unpaired *t*-test) compared to untreated cells. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 2: The effect of sunitinib and lapatinib on U87 (**A**) and M059K (**B**) apoptosis 24 and 48 h after treatment of cells with the tested agents. Results are expressed as the percentage of Annexin⁺ cells $\pm$ SEM. Asterisks denote a statistically significant difference (unpaired *t*-test) compared to untreated cells. **Control:** untreated U87/M059K cells, **Sun 1 μ M:** U87/M059K cells treated with 1 μ M sunitinib, **Lap 1 μ M:** U87/M059K cells treated with 1 μ M lapatinib, **S+L 1 μ M:** U87/M059K cells treated with 1 μ M sunitinib and 1 μ M lapatinib. ** $P < 0.01$ and *** $P < 0.001$.

Figure 3: The effect of sunitinib and lapatinib on U87 (A) and M059K (B) invasion capacity. Results are expressed as mean $\pm$ SEM of the % change compared to the untreated cells. Asterisks denote a statistically significant difference (unpaired *t*-test) compared to untreated U87/M059K cells. **C:** untreated U87/M059K cells, **Sun 1 μ M:** U87/M059K cells treated with 1 μ M sunitinib, **Lap 1 μ M:** U87/M059K cells treated with 1 μ M lapatinib, **Sun+Lap 1 μ M:** U87/M059K cells treated with 1 μ M sunitinib and 1 μ M lapatinib.* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 4: Effect of sunitinib and lapatinib on MMP-9 and MMP-2 secretion from U87 and M059K cells. This picture is a representative gel of three independent experiments.