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SUMMARY OF THE THESIS

COMBINATION OF ELLIPTICINE CHEMOTHERAPY AND α5β1 INTEGRIN-TARGETED THERAPY IN HUMAN GLIOBLASTOMA

by

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Introduction

Gliomas are the most abundant adult brain tumors nowadays treated with conventional therapies (surgery, radiotherapy and/or chemotherapy). Grade IV astrocytomas (glioblastoma multiform or GBM) account for about half of all astrocytic tumors with a median survival of less than a year whatever the treatment chosen.

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole), an alkaloid isolated from *Apocyanaceae* plants, and several of its more soluble derivatives exhibit significant antitumor and anti-HIV activities and is known to act by multiple mechanisms of action. Recently, ellipticine was also shown to be brain tumor specific, by screening the National Cancer Institute tumor panel (Shi et al., 1998).

This anticancer agent should be considered a pro-drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its cytochrome P450 (CYP) - and/or peroxidase-mediated activation to species forming covalent DNA adducts (Stiborova et al., 2001, Stiborova et al., 2007a). Ellipticine can also act as an inhibitor or inducer of biotransformation enzymes, thereby modulating its own metabolism leading to its genotoxic and pharmacological effects (Aimova et al., 2007).

Recently, we have shown that ellipticine forms covalent DNA adducts in various tissues *in vivo* including rat brain, after being enzymatically activated with cytochromes P450 or peroxidases (Stiborova et al., 2001, Stiborova et al., 2003,. Stiborova et al., 2004, Stiborova et al., 2007a, Stiborova et al., 2007b). Dutheil and co-workers found that twenty-four cytochrome P450 enzymes (CYP1, CYP2 and CYP3 families and CYP46A1) are expressed in human brain tissues (Dutheil et al., 2009). From the cytochromes P450 known to metabolize ellipticine, expression of CYP1B1 and 1A1 were the most important regarding mRNA levels (Dutheil et al. , 2009).

Since the prognosis for the patients diagnosed with glioblastoma remains very poor whatever the conventional therapy chosen, (surgery, radiotherapy and/or chemotherapy), new alternatives are highly needed. Integrins were recently identified as potential therapeutic targets as they take part in various cancer stages such as malignant transformation, tumor growth and progression, invasion and metastasis and

angiogenesis. Particular emphasis has been given to αvβ3/β5 integrins which are overexpressed in neovessels nourishing the tumor. A αvβ3/β5 antagonist, cilengitide, is currently undergoing clinical trials to target neoangiogenesis in malignant glioma (Nabors et al., 2007, Reardon et al., 2008). The α5β1 integrin emerged more recently as a potential anti cancer target as it is overexpressed in both tumoral neovessels (Magnussen et al., 2005, Parsons-Wingerter et al., 2005) and tumoral cells and appears now as an interesting therapeutic target for brain (Kita et al., 2001, Maglott et al., 2006, Martin et al., 2009), lung (Adachi et al., 2000) and ovarian tumors (Sawada et al., 2008). The expression of the α5β1 integrin is related to the tumor stage of gliomas (Kita et al. , 2001) and the integrin signaling pathways were identified as being part of functional networks of GBM (Bredel et al., 2005). Due to the recent interest in α5β1 integrin, design of specific non peptidic ligands has increased these last years (Heckmann et al., 2008, Heckmann et al., 2007, Marinelli et al., 2005). Small non peptidic α5β1 integrin antagonists were already shown to block angiogenesis and lymphangiogenesis. (Kim et al., 2000, Okazaki et al., 2009) (Umeda et al., 2006) Focusing on brain tumors, we have recently shown that perturbing specifically the function of the α5β1 integrin with the prototypical antagonist SJ749 in U87MG glioma cells triggers cell cycle arrest (Maglott et al. , 2006) and decreases cell aggressiveness (Martin et al., 2009). In addition, we demonstrated that the expression of the α5β1 integrin in glioma was under the control of caveolin-1 that acted as a repressor of integrin expression. Our results showed that low caveolin-1/high α5β1 integrin-expressing cells were more sensitive to the anti-proliferative and anti-invasive action of SJ749 (Martin et al. , 2009). Other α5β1 integrin selective ligands were rationally designed by the use of a computer-built 3D model of the integrin (Marinelli et al. , 2005). Among them, K34c appears as a highly active ligand with a good selectivity for α5β1 integrin over αvβ3 integrin and Ke34a as a low affinity non selective ligand (Heckmann et al. , 2007). Attempts to improve the effectiveness of targeted molecular therapies include their use in combination with radio/chemotherapies. Data attributed a role to β1 integrins in mediating the resistance towards therapies in several human cancers including glioma (Cordes and Park, 2007, Park et al., 2008). However as the β1 integrin subunit can dimerize with several α subunits, different heterodimers of β1 integrin may be implicated and no work, to our knowledge, has specifically addressed the role of the α5 integrin subunit

in the response of GBM to chemotherapy. Experiments were thus designed to explore the chemosensitivity of human glioblastoma cell lines, U87MG and U373, in the absence or presence of α5β1 integrin antagonists, SJ749 and K34c. We used ellipticine and temozolomide as chemotherapeutic drugs. Ellipticine has been described as a chemotherapeutic agent exerting specificity towards brain tumors (Jurayj et al., 1994) which intercalates into DNA, inhibits topoisomerase II and forms covalent DNA adducts after its cytochrome P450-dependent or peroxidases-dependent enzymatic activation (Stiborova et al., 2004, Stiborova et al., 2007a). Temozolomide forms methyl adducts in DNA and is currently used as a new standard of care for patients with newly diagnosed GBM (Mrugala and Chamberlain, 2008). In the current study, we show that α5β1 integrin antagonists sensitize glioma cells to chemotherapy by favoring cell apoptosis over premature senescence in cells exhibiting a functional p53 pathway.

Aims of the Study

The principal aims of the first part of the study were as follows:

- to describe ellipticine`s cytotoxicity in model glioblastoma cell lines U87 and U373
- to investigate ellipticine`s metabolism (detoxification and activation) in glioblastoma cell lines U87 and U373

Since ellipticine is currently not used in clinical practice due to its cardiotoxicity, its potential re-utilisation would necessarily be linked with dose-depression or with targeting it directly to tumor tissue. Here, we propose a use of combination therapy of ellipticine and an α5β1 integrin antagonist. Such novel therapeutic approach to glioblastoma treatment could lead to ellipticine dose-depression while final therapy outcome improves.

The principal aim of the second part of the study was to design a combination therapy of integrin α5β1 antagonists and ellipticine for glioblastoma treatment and elucidate its mechanisms.

Results

Part A

Both glioblastoma cell lines, U87MG and U373, are sensitive to ellipticine. However, the mechanisms how ellipticine inhibits their proliferation are different. In U87MG cell line ellipticine induces G0/G1 cell cycle arrest, whereas U373 cells are blocked in S and G2/M cycle phase after ellipticine treatment. Ellipticine induces senescence but not apoptosis in U87MG cells, while U373 cells die from apoptosis and hardly senesce.

The functional p53 pathway is strongly activated by ellipticine treatment. Upon p53 activation, ellipticine induces senescence rather than apoptosis, whereas in the context of non-functional p53, ellipticine induces apoptosis, but not senescence. These data suggest that different responses of U87MG and U373 cells to ellipticine treatment originate from the different p53 status.

Both cell lines express biotransformation enzymes generating ellipticine metabolites known to covalently bind to deoxyguanosine in DNA as well as formation of these ellipticine metabolites: 12-hydroxyellipticine and 13-hydroxyellipticine. U87MG cells formed indispensable amounts of two major ellipticine-DNA adducts. Ellipticine increased its own enzymatic activation by inducing CYP1B1, 3A4 and 1A1 enzymes in U87MG cells. Ellipticine concentration used for U87MG cell treatment is extremely important for its pharmacological effects, as its metabolite profiles differed substantially predicting ellipticine to be either detoxified or activated.

Part B

Our results revealed that SJ749 was more potent to inhibit proliferation when it acts before the adhesion of cells. No floating cells corresponding to unattached cells could be observed during the experiment. Results also show that inhibition of cell proliferation by SJ749 was sensitive to the level of serum in the medium possibly related to the integrin-growth factor receptor interactions. Similarly, ellipticine

efficiency was also dependent on serum level, possibly due to its interactions with serum proteins that are decreasing the free drug concentration. Taken together, both agents, SJ749 and ellipticine, were most efficient when used under reduced serum conditions and on non-attached cells. Therefore, immediate co-treatment with 5 μM SJ749 and 1 μM ellipticine in 2 % serum was evaluated as the optimal combination therapy protocol and was used in all subsequent experiments.

Two integrin antagonists, SJ749 and K34c, arrest U87MG cell cycle in G0/G1 phase. However, such cycle arrest did not persist more than 48 hours. Ellipticine-induced G0/G1 cell cycle arrest was intensified and prolonged by addition of integrin antagonist. Since temozolomide blocked the cell cycle rather in S and G2/M phases, integrin antagonist tended to exhibit inverse effects on cell cycle distribution when used in combination. However, temozolomide effects overrode those of integrin antagonist. This finding underlines the importance of chemotherapeutic drug mechanisms of action when used in combination with integrin antagonist, since their effect on the cell cycle distribution might be either additive, or inverse.

The key results of our study show for the first time that α5β1 antagonists modulate cellular response to chemotherapy. Chemotherapy-induced senescence is depressed by the addition of an α5β1 antagonist, while apoptosis is concomitantly increased. Integrin antagonists are thus favoring apoptotic cell death to senescence after drug-induced stress stimuli.

As confirmed using various models, in p53wt-expressing cells, single chemotherapy induced preferentially senescence than apoptosis. This phenomenon is accompanied by a strong activation of the p53 pathway. Such p53 signaling is down-modulated by addition of integrin α5β1 antagonist and leads to apoptosis favoring against senescence. In contrast, in the system, where p53 is not functional/present, single chemotherapy induces apoptotic cell death, but no or little senescence. Here, the α5β1 integrin antagonist addition brings no further benefits such as further apoptotic population increase.

Expectedly, α5β1 integrin activation by fibronectin resulted in increase of ellipticine-induced senescence. These results are in accordance with the study of combination of ellipticine with integrin antagonists, in which antagonizing integrin led to ellipticine-induced senescence depression.

Although α5 integrin overexpression in U87MG cells did not represent any advantage regarding cell proliferation, high levels of this integrin confer chemoresistance to U87MG cells to ellipticine and temozolomide treatment regarding clonogenicity. The increased senescence induction by ellipticine in F8 (α5-overexpressing) compared to pcDNA cells may at least partially contribute to the explanation of this chemoresistance.

Based on our results with α5 integrin overexpressing U87MG cells (F8) and our experiments with integrin antagonists, we expected higher sensitivity of α5-depleted U87MG cells (D4) to chemotherapeutic agents tested in this study. Surprisingly, D4 cells were more sensitive to temozolomide, but not to ellipticine treatment. Even more surprisingly, D4 cells responded to ellipticine treatment by strong induction of senescence as contrasted to integrin antagonizing.

Interestingly, in the context of non-functional p53, α5 integrin overexpression resulted in multinucleated cell phenotype and senescence induction even in non-treated cells. This senescence is increased by both, single (SJ749 or ellipticine) and combination treatment.

Discussion

Current oncology is still lacking therapies efficient enough to cure highly aggressive tumors such as glioblastomas. Neither of the conventional therapeutic approaches brings crucial survival benefits for patients suffering from this aggressive type of cancer. Therefore, strategies aimed to target specifically altered pathways in tumor cells in combination with conventional approaches represent a great challenge for scientists and clinicians nowadays.

This work is dealing with ellipticine and its combination with α5β1 integrin-targeted therapy of glioblastomas. Ellipticine has already been used in clinical praxis against other aggressive and metastatic cancers. It has been reported to exhibit certain specificity for brain tumors in studies *in vitro* as well. Former results of our laboratory provided evidences that it overcomes the blood-brain barrier in rats and thus is able to reach the tumor of interest. Since it is considered a pro-drug being metabolized by cytochrome P450 and peroxidases, its activity depends on these biotransformation enzymes expression pattern of each patient as well as of the tumoral tissue. Among

the ellipticine-activating enzymatic systems, e.g. CYP1B1 has been reported to be specifically expressed in astrocytic tumours but not in healthy brain tissue (Murray et al., 1997). Due to its selective activation, ellipticine exhibits certain specificity to tumors.

Here we demonstrated sensitivity of two glioblastoma cell lines to ellipticine treatment and pointed out the importance of p53 status in cellular response to ellipticine therapy. Whereas both, p53wt and p53mt expressing, cell lines were sensitive to ellipticine, the mechanisms of their cellular answers were completely different. While p53wt-expressing U87MG cell line answered to ellipticine treatment by senescence induction, in U373 (p53mt) ellipticine provoked apoptotic cell death. Both cell lines were proven to express biotransformation enzymes metabolizing ellipticine. Moreover, in U87MG cells, some of these enzymes were ellipticine-inducible. Thereby, ellipticine is regulating its own metabolism in targeted tissue. Ellipticine concentration and thus degree of selective cytochrome P450 induction plays a key role in metabolic activation-dependent mechanisms of ellipticine`s action such as covalent DNA adducts formation.

Despite these promising findings, possible extensive utilization of ellipticine in clinical praxis remains a bit quastionable. Firstly, it considers a drug that was already withdrawn in the past from the pharmaceutical market due to its cardiotoxicity. Thus it has a kind of negative stigma in view of clinical oncologists. Secondly, its relaunch into clinical practice would not get along without expensive clinical trials. Since ellipticine has already lost its patent protection, it is questionable, whether pharmaceutical industry would be keen on financing its clinical research. Modifying the molecule or patenting a new drug form could overcome this problem. However, ellipticine`s mechanisms of action in the combination therapy may be generalized to other anticancer drugs. Here, all our important results regarding the combination therapy were confirmed with temozolomide, which is currently a reference drug in glioblastoma therapy.

As stated above, glioblastomas are highly aggressive, but also highly resistant tumors, heavily vascularized. As integrins have been reported to possibly confer chemo- or radio-resistance and are simultaneously involved in angiogenesis, integrin-targeted

therapies evoke a lot of interest nowadays. Historically, αvβ3 integrin was the first and most extensively explored therapeutic target among the integrin family. Integrin αvβ3-targeted therapy was predominantly aimed on angiogenesis inhibiton. Integrin α5β1 has recently been identified as even more promising therapeutic target for glioblastomas, as it is implicated in multiple stages of tumorigenesis as well as in processes such as tumoral neoangiogenesis and cell invasiveness. Moreover, its expression positively correlates with tumor grade. Here, α5β1 integrin targeted-therapy does not deal with anti-angiogenesis, but rather explores its signalization modulation potential with its effects on tumoral cells directly.

The key finding of our work is that a specific α5β1 integrin non-peptidic small antagonists (SJ749 or K34c) modulates cellular response of p53wt-expressing glioblastoma to conventional chemotherapy (ellipticine or temozolomide) by triggering apoptotic cell death instead of premature senescence. Having noted that senescent cells may re-enter cell cycle and excrete stimulating signals to the cell in their neighborhood, whereas triggering apoptosis brings unexceptional benefits for tumor repression, this results is of great importance. We demonstrated and confirmed a key role of the p53 signaling in this phenomenon, since integrin antagonists were shown to specifically modulate the p53 pathway resulting in affecting the balance between senescence and apoptosis favoring programmed cell death. Thus we presented for the first time a functional link between p53 and α5β1 integrin. Furthermore, our results suggest an unexpected role for the α5β1 integrin in the phenomenon of senescence. The mechanisms involved remain to be elucidated and are currently under investigation.

We further pointed out that the p53 status is crucial for such combination therapy outcome. In the context of non-functional p53 and high α5 integrin level, ellipticine treatment combined with integrin antagonist resulted not in apoptosis, but senescence induction. Despite combination of chemotherapy with α5β1 integrin antagonist presents certain benefits regardless the p53 status, such combination therapy is supposed to be more suitable for p53wt-expressing tumors due to the controversial benefits of senescence discussed above.

Since all experiments presented in this work were performed *in vitro*, further *in vivo* studies are necessary to be carried out. First and foremost, no studies of SJ749 or K34c integrin antagonist regarding their toxicity in animal models have been realized

till today. Similarly, no indications whether these molecules will be able to penetrate through the blood brain barrier do not exist, although such a difficulty might perhaps be solved by use of drug-containing capsules implanted intracranially, similar to Gleevec®. Work is in progress to answer these questions in xenografted human glioblastoma.

In summary, this work provides novel evidences of profitability of combining conventional chemotherapy with α5β1 integrin-targeted therapy underlying the importance of knowing basic tumor characteristics to may estimate the final therapy outcome. The status of p53 has hardly been demonstrated as a predictor of the chemotherapeutic response in glioblastoma (Leuraud et al., 2004, Weller et al., 1998), but concomitant screening of tumors for α5β1 integrin level and p53 status may be more predictive in patients with brain cancer resistant to chemotherapy.

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