Title page

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Title: Imatinib and Docetaxel in combination can effectively inhibit glioma invasion in an *in vitro* 3D invasion assay.

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Abstract

The main problem in the treatment of malignant astrocytomas is their invasive behaviour. Successful resection of the main tumour mass cannot prevent recurrence due to single cells invading the surrounding brain parenchyma at the time of diagnosis. The classical combination therapy, PCV (Procarbazine, CCNU and Vincristine) used for over 30 years; has shown its clinical effectiveness in the treatment of malignant astrocytomas and glioblastomas is still doubtful. Using an *in vitro* three dimensional invasion model, we tested the effect of the tyrosine kinase inhibitor imatinib and the microtubule inhibitor docetaxel on the invasion activity of a panel of astrocytic tumour cell lines, including two established glioma cell lines, IPSB-18 and SNB-19, and two primary cell lines, originating from glioblastomas, CLOM002 and UPHHJA, and in normal astrocytes. A dose response curve for each drug alone and in combination was determined. The half maximal inhibitory concentration (IC_{50}) concentration of imatinib was between 15.7 µM and 18.7 µM, which did not affect invasion activity of the cell lines. The IC_{50} concentration of docetaxel was between 0.7 nM and 19.8 nM, and at 14.9 nM docetaxel had a slight transient inhibitory effect on invasion activity of all tested cells. The combination of imatinib at 13.5 µM and docetaxel at 14.9 nM, however, synergistically inhibited cell growth and invasion activity and could not be reversed by drug removal. A combination treatment with tyrosine kinase inhibitors and cytotoxic drugs shows promise in tackling both glioma proliferation and invasion, and could present a new treatment regimen for malignant astrocytomas.

Keywords : Imatinib, Docetaxel, Invasion, Proliferation, Glioma.

Introduction

High-grade gliomas are the most common malignant brain tumours in adults and are characterized by their highly invasive nature. They are frequently resistant to current therapies, which include surgery, radiotherapy and chemotherapy. Although there is some improvement in overall survival with daily radiotherapy and concomitant temozolomide in patients with newly diagnosed glioblastomas [1], the average 1-year survival is 17.7% and the 2-year survival is 3.3% [2]. Identification of new targets and treatment strategies is urgently needed.

Tyrosine kinases had been a focus for the development of new targeted inhibitors. Tyrosine kinases regulate a large range of proteins involved in processes including growth, metabolism and differentiation. They are divided into receptor and non-receptor tyrosine kinases, which initiate signalling processes by phosphorylation and dephosphorylation of a variety of downstream proteins including e.g. Akt, phosphoinositol- 3-kinase (PI3k) and members of the ras/raf/Map-kinase pathway [3].

Glioblastomas (GBMs) are divided into primary, which appear *de novo* and secondary GBMs, which progress from lower grade gliomas and are more frequent in younger patients [4]. One characteristic of primary GBMs is the amplification of epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, which is often mutated (EGFRvIII). Secondary GBMs are associated with overexpression and co-expression of platelet derived growth factor (PDGF) and its receptor tyrosine kinase PDGFR forming an autocrine loop, which contributes to tumour growth and probably to the transformation process to a more malignant phenotype [5, 6].

A number of small molecule inhibitors were developed to targeted tyrosine kinases; some of these tyrosine kinase inhibitors (TKIs) are highly effective in the treatment of various cancer types, e.g. imatinib (Glivec, Gleevec, STI571; Novartis). Imatinib had been designed to target Bcr-Abl, a mutated form of the non-receptor kinase C-Abl found in Philadelphia chromosome positive chronic myeloid leukaemia (CML) [7]. The success of this compound in the treatment of CML triggered the hope, that imatinib could also be effective against other tumour types. Besides Bcr-Abl, imatinib also inhibits PDGFR, stem cell factor C-Kit and C-Abl, causing cell cycle arrest and/or apoptosis [8, 9]. However, clinical trials using imatinib as a single agent for the treatment of recurrent GBM resulted in increased progression free survival after 6 months for only a small number of patients [10, 11]. A similar result was seen in primary cultures of glioma specimen; out of 15 specimens Hägerstrand

and his colleagues identified a small subgroup of imatinib-sensitive high-grade human glioma cultures [12]. In these cultures the PDGFR expression and phosphorylation status were significantly correlated with imatinib sensitivity, indicating the growth dependency of the PDGFR signalling.

A possibility for increasing the amount of responders and the effectiveness of TKIs could be a combination treatment consisting of a TKI and a cytotoxic drug, e.g. docetaxel (Taxotere®; Aventis). Docetaxel is a hemisynthetic product derived from the European yew tree and promotes the assembly and inhibits the depolymerisation of microtubules in the cells [13]. This drug is widely used in the treatment of various forms of cancer and is particularly effective in the treatment of non-small cell lung cancer [14]. The use of docetaxel alone for the treatment of GBMs has shown little or no significant response [15, 16]. Docetaxel combined with radiotherapy has shown partial response [17], however local delivery of docetaxel *in vivo* in an animal model resulted in a significant improvement in survival [18], local delivery of docetaxel may be a promising treatment for glioma.

We tested the combined effect of imatinib and docetaxel alone and in combination on proliferation and invasion of a panel of glioma cell lines and in normal human astrocytes (NHAs).

Materials and Methods

Cell Lines

We used the following cell lines CLOM002, UPHHJA, SNB-19, which were established from glioblastoma multiforme and IPSB-18 developed from an anaplastic astrocytoma. CLOM002, UPHHJA and IPSB-18 were developed in Geoff Pilkington's laboratory, University of Portsmouth, UK. SNB-19 was purchased from the German cell bank Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany (German Collection of Microorganisms and Cell Cultures). All cell lines were cultured in complete medium (CM) consisting of Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 5% fetal calf serum (Harlan 5-0001AE). The NHAs were purchased from Lonza (CC-2565) and cultured in an astrocyte bullet kit (CC-3186).

Toxicity assay

This was performed according to Heenan *et al* [19]. Briefly, cytotoxic drug dilutions ranging from 0.85 µM to 27 µM for imatinib and 0.37 nM to 24.8 nM for docetaxel were prepared at 2X of their final concentration in CM for single drug assays (drug dilutions for combination assays were prepared at 4X concentrations). Cells were seeded in 96 well plates at a concentration of 500/1000 µl cells/well and incubated overnight at 37°C and 5% CO_2. On the next day diluted drug (100 µl for single drug and 50 µl for combination drug) were added to each well and cells were incubated for a further 6-7 days at 37°C and 5% CO_2, until control wells had reached 80-90% confluency. Cell survival was measured using the acid phosphatase assay [19]. The concentration of drug which caused 50% cell kill relative to the control (no drug) represented the IC_{50} value of the tested drug. Each concentration was tested in 8 parallel wells and in 3 biological repeats. NHAs were tested in triplicates.

Analysis of drug combination effects

The effect of the drug combination on cell kill was performed and analysed according to a protocol by Chou and Talalay [20]. For each cell line the IC_{10}, IC_{20}, and IC_{30} of imatinib was combined with the IC_{10}, IC_{20}, and IC_{30} of docetaxel, resulting in a total of nine value points per combination index (CI) plot. A CI value smaller than 1

indicates a synergistic action of the two drugs; a CI value equal 1 an additive effect and a CI value greater than 1 indicates an antagonistic effect.

Apoptosis Assay

Cells were seeded in a 24-well plate at a concentration of $1x10^4$cells/well. After 72 hrs cells were incubated with or without drug at 37^{o}C and 5% CO_2. After 24 or 48 hrs cells were trypsinized and counted. Cells were assessed for early and total apoptosis, and cell viability using the Guava Nexin® Assay and the Guava® EasyCyte™ Flow Cytometer according to manufacturer's recommendations.

Protein Extraction

Cells were grown to 80-90% confluency in CM in 100 mm Petri dishes (Nunc). Media was removed and cells were washed once with cold phosphate buffered saline. The following procedures were performed on ice. 150 µl of lysis buffer (100 ml containing 0.6055g Tris, 0.8766g NaCL, 500 µl Igepal, pH 7.5), 15 µl protease inhibitor (P2714, Sigma), 1.5 µl PMSF (Sigma), and 1.5 µl DTT (Sigma) were added to each Petri dish. After 20 minutes incubation on ice, cells were scrapped off and transferred to sterile eppendorfs. The cell lysate was passed through a 21-gauge needle attached to a 1ml syringe and centrifuged at 14,000 rpm for 5 minutes at 4^{o}C. The supernatant was stored in 100 µl aliquots at -80^{o}C. Protein levels of the cell lysates were measured using the Bio-Rad protein assay kit (Bio-Rad, 5000006) according to the manufacturer's recommendations.

SDS-Page and Western Blot Analysis

Proteins were separated on 7.5% Tris-Glycine PAGEr®duramide®Precast Gels (Lonza, 59601) and transferred to PVDF membranes (GE Healthcare, RPN3032D). Transfer and antibody staining were performed as described by Towbin *et al.* [21] using the following antibodies: platelet derived growth factor receptor-β (PDGFR-β) molecular weight (MW) 189, 170 kilo Dalton (kDa) (Calbiochem, Ab-4, PR7212 at 1:50), platelet derived growth factor receptor-α (PDGFR-α) Mw 180 kDa (Abcam, ab35765 at 1:500), C-Kit MW 110 kDa (Abcam, ab32363 at 1:1000), C-Abl Mw 140 kDa (Cell Signalling, Tyr245 at 1:500), P-glycoprotein (Pgp) MW 170 kDa (Santa Cruz, sc-13131 at 1:200), breast cancer resistant protein (BCRP/ABCG2) MW 72.3 kDa (Abcam, ab3380 at 1:40), glial fibrillary acidic protein (GFAP) MW 50 kDa

(Abcam, ab7260 at 1:10,000), Bcr-Abl MW 210 kDa (Cell Signalling, 3902 at 1:1000) and β-actin MW 45 kDa (Sigma, A5441 at 1:10,000).

Enhanced Chemiluminescence (ECL) detection

To facilitate the detection of peroxidase-conjugated secondary antibodies immunoblots analysing were developed using an Enhanced Chemiluminescence kit (Amersham, RPN2109) for BCRP, Pgp, and β-actin; and ECL Advance™ (Amersham, RPN2135) according to manufacturer's recommendations (for all other proteins).

3D Collagen Invasion Assay

Cell spheroids were formed using the hanging drop method described by Del Duca *et al* [22]. Briefly, after trypsinisation the cells were diluted with CM reaching a concentration of $1x10^6$ cells/ml. Drops (20 µl) of cell suspensions were placed onto the lids of 100 mm Petri dishes, which were inverted over dishes containing 10 ml sterile water. Hanging drop cultures were incubated for 24-48 hrs until cell aggregates were formed, which were transferred to a 100 mm dish coated with 4% agar and filled with 10 ml CM. The aggregates/spheroids were incubated for another 2 days, while they round up, and then implanted into collagen gel. Cold PureCol™ (INAMED, USA) was mixed with cold 10-fold concentrated minimal essential medium (Sigma) and cold 0.1 M sodium hydroxide at a ratio of 8:1:1 reaching a final concentration of 2.4 mg/ml collagen type I. The pH was neutralised by adding 1M NaOH (Sigma). The collagen solution was distributed into 24-well plates (0.5 ml/well) and one spheroid was placed into each well. The plates were kept at 37°C for about 30-60 minutes. After solidification the gels were overlaid with 0.5 ml CM and kept at 37°C under 5% CO_2. On day 4, the medium was replaced with fresh CM (control) or drug-containing CM. Cell migration out of the spheroid was measured before drug addition (day 0) and on days 1, 3, 5, 8, 10 and 12 after drug addition.

Results

Effect of imatinib and docetaxel on proliferation activity

The IC_{50} values were established for imatinib and docetaxel on four different glioma cell lines; CLOM002 and UPHHJA are primary cell lines, which were used at low passage numbers, and SNB-19 and IPSB-18 are established cell lines. The IC_{50} for imatinib was similar for all four cell lines, ranging between 15.7 µM and 18.7 µM (Table 1). Docetaxel inhibited cell proliferation at much lower concentrations. SNB-19 was the most sensitive cell line with an IC_{50} of 0.7 nM docetaxel, and UPHHJA was the least sensitive cell line with a 28-times higher IC_{50} value of 19.8 nM. In addition, we tested NHAs, where we achieved an IC_{50} with imatinib of 17 µM, which is very similar to the value found with the glioma cell lines. With the NHAs we could not achieve an IC_{50} concentration with docetaxel.

Combined treatment with imatinib and docetaxel synergistically inhibited cell proliferation in 3 out of 4 cell lines.

To investigate the drug combination effect three low kill concentrations of docetaxel and one low kill concentration for imatinib were chosen, which reduced cell proliferation of CLOM002, UPHHJA and IPSB-18 cells only up to 37% (Fig. 1). The combination of both drugs at low concentration resulted in up to 87% inhibition in these three cell lines compared to imatinib or docetaxel alone. On the other hand, the combined treatment of imatinib and docetaxel had no significant effect on SNB-19 (Fig. 1).

For each cell line we examined the combined effect of the IC_{10}, IC_{20} and IC_{30} of imatinib and docetaxel with the analysis of the combination plot according to Chou and Talalay [20] this revealed a synergistic effect of imatinib together with docetaxel for 3 out of 4 tested cell lines. The highest synergism was seen in IPSB-18 cells with a combination index (CI) between 0.05 and 0.25; in UPHHJA the CI was between 0.1 and 0.4 and the weakest synergisms was in CLOM002 cells with a CI between 0.1 and 0.7 as expected. The CI in SNB-19 cells was between 0.7 and 1.0 representing an additive effect.

Imatinib combined with docetaxel induces apoptosis in primary glioma cell lines.
We analysed total apoptosis in the presence of single drug and drug combinations. Over 24 to 48 hours we measured between 2 and 3.4% apoptotic cells in the controls (no drug) (Fig. 2(a)). Drug effects were low after 24 hours, but were much more pronounced after 48 hours: imatinib alone induced apoptosis in 4.7-7% cells and docetaxel alone in 4.3-10% cells, while the combination of imatinib and docetaxel caused apoptosis in 13.8-40.1% cells, with CLOM002 cells being the most sensitive. In SNB-19, however, docetaxel alone caused 10% apoptotic cells and the drug combination increased this effect to 13.8% apoptotic cells (Fig. 2 (a)). There was no major difference in early and late apoptosis (not shown). Neither drug alone had a significant effect on cell viability of CLOM002, IPSB-18, and UPHHJA cells; however the combination significantly reduced cell viability by over 30% in all three cell lines (Fig. 2 (b)). In SNB-19 cells, docetaxel alone caused 10% reduction in cell viability, which was similar to the effect of the combination (Fig. 2 (b)).

The combination of imatinib and docetaxel synergistically inhibited cell invasion in 3 out of 4 cell lines.
In the absence of drug the cell lines UPHHJA, IPSB-18 and CLOM002 show similar invasion activity reaching a distance between 1700 and 2000 μm from the spheroid within 11/12 days; SNB-19 cells invaded much less and reached only a distance of 760 μm within the specified time (Fig. 3). We also examined the invasion activity of NHAs (Fig. 3) and found an invasion distance of 1193 μm on day 10. To investigate the effect of imatinib and docetaxel on actively invading cells, the drug was added 4 days after implanting the spheroids, when invasion had started already. Imatinib alone had little inhibitory effect (CLOM002, UPHHJA, and IPSB-18) or even a slightly increasing effect on invasion activity (SNB-19) compared to invasion in the absence of drug (Fig. 4). Docetaxel alone reduced the invasion distance reached after 11/12 days up to 40% in CLOM002, IPSB-18 and UPHHJA and 50% in SNB-19. The combination of the two compounds did not add to the inhibitory effect on SNB-19 invasion compared to docetaxel alone; however, substantially decreased invasion of IPSB-18 (87.7%), CLOM002 (63%), and UPHHJA (59%) compared to single drug treatment and control (Fig. 4 and 5). NHAs showed significant invasion activity,

which was only inhibited up to 60% although treated with much higher concentrations of drug than glioma cell lines (imatinib 40.7 µM, docetaxel 29 nM). The combination of imatinib and docetaxel did not significantly increase the effect of docetaxel alone, a similar result to that obtained with SNB-19 (Fig. 8).

The protein expression profile of SNB-19 cells differs from that of the other three cell lines.

Using Western Blot we analysed the protein expression of tyrosine kinases (PDGFR-β, PDGFR-α, C-Kit, C-Abl), multidrug resistance pumps (Pgp, BCRP) and the glial marker protein glial fibrillary acidic protein (GFAP). All four cell lines were positive for GFAP proving their glial origin (Fig. 6). Low expression of PDGFR-β was seen in CLOM002, UPHHJA, and SNB-19, and higher expression was found in IPSB-18 (Fig. 6). All cell lines expressed low levels of PDGFR-α, but no C-Kit. C-Abl was expressed in all cell lines (Fig. 6). In SNB-19 cells the C-Abl antibody recognized an additional band around 210 KDa which is similar to the pattern expressed in the positive control K562, a chronic myelogenous leukaemia cell line expressing both C-Abl and the mutated form Bcr-Abl (Fig. 6). A Bcr-Abl-specific antibody recognized the same band in SNB-19 cells suggesting the expression of a mutated form of C-Abl in these cells (Fig. 7).

The multidrug resistance protein BCRP (ABCG2, MXP) was found in all four cell lines (Fig. 6); however, SNB-19 and IPSB-18 expressed much higher levels compared to CLOM002 and UPHHJA cells. Pgp (MDR-1, ABCB1) was only expressed in SNB-19 cells (Fig. 6). In summary SNB-19 protein expression differed from the other cell lines in the strong expression of BCRP, the expression of Pgp, and the expression of a protein similar to the mutated tyrosine kinase Bcr-Abl.

Discussion

In our experiments we examined the effect of imatinib and docetaxel alone and in combination on proliferation and invasion of a panel of glioma cell lines; as a possibility for increasing the effectiveness of imatinib treatment with gliomas. Three (CLOM002, UPHHJA, SNB-19) out of four cell lines originated from a glioblastoma multiforme and one cell line was developed from an anaplastic astrocytoma (IPSB-

18). CLOM002 and UPHHJA were newly developed cell lines (by G. Pilkington, Portsmouth) and used at low passage numbers. IPSB-18 and SNB-19 represent established cell lines and are commercially available. All cell lines had fast proliferation rates (not shown) and were highly invasive. The IC_{50} values for imatinib were in the micromolar range (15.7 µM to 18.7 µM) which is consistant with published data [23], and for docetaxel in the nanomolar range (0.7 nM and 19.8 nM) with SNB-19 being the most sensitive cell line (Table 1). Surprisingly, we were not able to achieve an IC_{50} value for docetaxel in NHAs, which might be due to the fact that docetaxel specifically interferes with cell division by targeting the microtubles of cells; cancer cells have a higher proliferation rate, and therefore, would be more likely to be affected by docetaxel than normal cells. The combination of both drugs caused strong synergistic toxicity in 3 out of 4 glioma cell lines, while the effect on SNB-19 proliferation was antagonistic (Fig. 1). The combination of both drugs at low concentration resulted in up to 87% inhibition of proliferation in these three cell lines. The combination of imatinib and docetaxel had no effect on SNB-19 (Fig. 1). In summary imatinib and docetaxel in combination effectively inhibited proliferation in 3 out of 4 glioma cell lines. The combined treatment of imatinib and docetaxel has also decreased cellular proliferation in chronic myeloid leukaemia cell lines [24].

A similar effect was seen on invasion activity. Imatinib alone had little or no effect on invasion activity while docetaxel slightly inhibited invasion in 3 out of 4 cell lines. The drug combination, however strongly inhibited the invasion activity of CLOM002, UPHHJA and IPSB-18, and was much more effective compared to single drugs. In SNB-19, however in the presence of docetaxel alone invasion was strongly inhibited, and the drug combination did not significantly increase this effect. In summary a combination of imatinib and docetaxel strongly inhibited invasion in CLOM002, UPHHJA and IPSB-18; whereas docetaxel alone strongly inhibited invasion in SNB-19, an effect which could not be increased by the drug combination, a similar result was found with the NHAs (Fig.8).

The high invasion activity of NHAs could possibly due to the presence of a mixture of astrocytes and astrocyte precursor cells (oligodendrocyte-type 2 astrocyte progenitor cells (O-2A)), which had been shown to be highly motile [25].

We examined the apoptotic effect of both drugs on each cell line. Imatinib and docetaxel individually induced very little apoptosis in glioma cell lines; whereas the combination of imatinib and docetaxel caused up to 40% apoptosis in 3 out of 4 cell

lines. The least amount of apoptosis was found in SNB-19 (13.8%) and was most likely due to docetaxel alone (Fig. 3). Docetaxel causes G2/M cell cycle arrest [24] and imatinib induces cell cycle arrest at the G0-G1 or G2/M phase in glioma cells [26]. The combination of imatinib and docetaxel has also been found to increase apoptosis in human K562 chronic myeloid leukaemia cells, which was induced through caspase-3 enzyme activity [24].

We examined a range of combination concentrations for imatinib and docetaxel to find the lowest effective concentrations for the inhibition of proliferation and invasion. We wanted the concentrations to be as clinically significant as possible since only about 10% of the drug concentration in the plasma can cross the blood-brain barrier (BBB). Effective concentrations in the proliferation assays with SNB-19 and CLOM002 were within the reported peak plasma concentration for docetaxel (0.5 nM) [1], and only slightly higher for UPHHJA and IPSB-18 (Table 2).

It was shown that in mice only 20% of imatinib present in the plasma is crossing the BBB, while 80% is effluxed by BCRP and Pgp [27]. Le Coutre *et al* have reported a mean plasma concentration of 4-5 µg/ml of a 600 mg dose and 2-3 µg/ml for a 400 mg dose 4 hours after oral administration for imatinib and 531 ng/ml for the metabolite N-desmethyl-imatinib. The average CSF concentration for imatinib was 38 ng/ml, and less than 10 ng/ml for N-desmethyl-imatinib showing that only a small amount of drug crosses the BBB [28]. High-grade glioma patients have a disrupted BBB, which would allow a higher concentration of imatinib in the CNS; however, it is not clear if an effective concentration can be reached.

A similar problem exists with docetaxel, which also shows little penetration of the BBB, which might explain the poor response seen in recurrent GBM when used as second line treatment in phase II trials [16, 29, 30]. Peak plasma concentrations for docetaxel have been reported at 0.5 nM, while concentrations in the CSF were ten times smaller (0.05 nM) [31]. This issue can be addressed by using convection-enhanced delivery (CED), which is currently tested using paclitaxel (another taxane derivative) in an ongoing clinical trial in recurrent gliomas with some promising results. CED is a drug application method in which the drug is injected directly into the tumour avoiding the blockage through the BBB [32].

Analysis of protein expression revealed that all four cell lines were positive for GFAP proving their glial origin. Imatinib targets PDGFR-α, PDGFR-β, C-Abl,

and C-Kit; therefore we analysed the expression of these tyrosine kinases in the four test cell lines. All cell lines expressed C-Abl; however, the C-Abl antibody recognized an additional band around 210 kDa in SNB-19 cells which is similar to the Bcr-Abl band expressed in the positive control K562, a CML cell line, which expresses both C-Abl and the fusion protein Bcr-Abl (Fig. 6). A different Bcr-Abl-specific antibody recognized the same band in SNB-19 cells suggesting the expression of a mutated form of C-Abl in these cells (Fig. 7). As imatinib specifically targets Bcr-Abl, we would have expected SNB-19 to be sensitive to imatinib; however, this was not the case, the IC_{50} for imatinib was similar to the other cell lines. Suggesting that SNB-19 may have a mutation in the Bcr-Abl protein, which prevents imatinib binding. In CML acquired imatinib resistance is common and has been explained by Bcr-Abl kinase domain mutations, which leads to decreased imatinib sensitivity [33, 34]. PDGFR-β and PDGFR-α were expressed at low levels in all cell lines, and none expressed C-Kit. These gliomas may be more dependent on other growth factor receptors such as EGFR which signals through the PI3K/Akt pathway [35] and is commonly overexpressed in gliomas [36]. The low levels of PDGFR-α and β, and absence of C-Kit expression in the cell lines, as well as the low expression of C-Abl could explain the low sensitivity to imatinib, as these tyrosine kinases are specific targets for imatinib. C-Kit and C-Abl are frequently mutated or overexpressed in GBMs [37], and are associated with glioma progression [38, 39].

The multidrug resistance protein BCRP was found in all four cell lines with the highest expression in SNB-19 and IPSB-18. Bcr-Abl expression is associated with high PI3K activity [40, 41], and BCRP function is regulated by PI3K-Akt signalling [42]. The high BCRP expression in SNB-19 may have been a result of Bcr-Abl expression. Pgp was expressed only in SNB-19 cells, and not in the other cell lines. Since docetaxel is a substrate for Pgp we would have expected SNB-19 to be more resistant to docetaxel, however, we found it to be the most sensitive cell line to docetaxel. Imatinib is a substrate for both BCRP and Pgp [43], which might lead to a higher efflux of imatinib in SNB-19 cells, since they express both membrane efflux pumps. This might explain why the drug combination was not as effective on SNB-19 as it was on the other cell lines. Co-expression of Pgp and BCRP has also been found in an imatinib resistant subgroup of acute myeloid leukaemia patients [44].

Conclusion

The combined treatment of imatinib and docetaxel had a strong apoptotic effect on 3 glioma cell lines, with the least amount of apoptosis found in SNB-19. In SNB-19, however, in the presence of docetaxel alone proliferation and invasion were strongly inhibited, and the drug combination did not significantly increase this effect. We suggest that SNB-19 may have a mutation in the Bcr-Abl protein, which prevents imatinib binding. Of the 4 cell lines only SNB-19 expressed both BCRP and Pgp, and as imatinib has been reported to be a substrate for these, this may have led to a higher efflux of imatinib in SNB-19 cells. This might also explain why the drug combination was not as effective on SNB-19 as it was on the other cell lines.

Imatinib combined with docetaxel strongly inhibited both proliferation and invasion, and had a pro-apoptotic effect in 3 out of 4 glioma cell lines. Therefore the combination of docetaxel and imatinib may be a promising treatment for glioma.

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Table captions

Table 1: IC_{50} values for imatinib and docetaxel on cell proliferation of four glioma cell lines and NHAs.

Standard deviations are on triplicate assays

Table 2 Docetaxel and imatinib concentrations used in the proliferation assays in Fig. 1

Figure captions

Fig. 1 Combination effect of imatinib (Im) and docetaxel (Do) on cell proliferation. *: p <0.05. Error bars represent deviations from triplicate assays.

Fig. 2(a) Percentage of total apoptotic cells measured after 24 and 48 hrs in the absence and presence of drug; imatinib 13.5 μM; docetaxel 14.9 nM. Im & Doc: imatinib and docetaxel. * : P < 5E-3. Error bars represent deviations from triplicate assays.

Fig. 2(b) Percentage cell viability after 24 and 48 hrs in the absence and presence of drug; imatinib 13.5 μM; docetaxel 14.9 nM. Im & Doc: imatinib and docetaxel. * : P < 5E-3. Error bars represent deviations from triplicate assays.

Fig. 3 Invasion activity of four cell lines and NHAs in the absence of drug; UPHHJA (○), IPSB-18 (□), CLOM002 (▲), SNB-19 (×), NHA (---♦---). Error bars represent deviations from triplicate assays. NHAs were tested in duplicate in one experiment.

Fig. 4 Percentage invasion distance on day 12 (*day 11) in the absence (control) and presence of imatinib 13.5 μM and docetaxel 14.9 nM alone and in combination. Error bars represent deviations from triplicate assays.

Fig. 5 CLOM002 cell invasion on day 24 in the absence (a) and presence of the combination of imatinib 13.5 μM and docetaxel 14.9 nM (b).

Fig. 6 Western blot analysis of tyrosine kinases PDGFR-β, PDGFR-α, Bcr-Abl, and C-Abl, multidrug resistance pumps BCRP, Pgp and the astrocyte marker GFAP. CLOM002 (1), UPHHJA (2), SNB-19 (3) IPSB-18 (4).

Fig. 7 Western blot analysis with an additional Bcr-Abl antibody. Positive control (1), SNB-19 (2), IPSB-18 (3).

Fig. 8 percentage of average invasion over 13 days with normal human astrocytes with imatinib (Imt) 40.7 µM and docetaxel (Doc) 29 nM in comparison to the control (Ctr). Error bars represent a duplicate of one experiment.