Abstract. Background: Chemotherapy is the commonly accepted standard therapy for most types of brain tumor, especially in medulloblastoma, primitive neuroectodermal tumor and astrocytoma. However, no efficient therapy has been established to date for glioblastoma multiforme. The aim of the present study was to analyze the activity of bortezomib in glioblastoma cell lines in comparison with that in a pediatric acute lymphoblastic leukemia cell line. Materials and Methods: Glioblastoma multiforme T98G, glioblastoma-astrocytoma U373M and T-lineage acute lymphoblastic leukemia CCRF-CEM cell lines were used. Proteasome inhibitor, bortezomib and 14 other anticancer drugs were tested using the MTT assay. Results: Compared to the acute lymphoblastic cell line, both glioblastoma cell lines showed relatively good sensitivity to bortezomib, as well as to cisplatin, carboplatin, etoposide and actinomycin-D. The lines showed intermediate sensitivity to thiotepa and daunorubicin, but were highly resistant to first-line drugs used in the therapy of acute lymphoblastic leukemia, such as prednisolone, L-asparaginase, vincristine, doxorubicin and cytarabine. Bortezomib, which is not a substrate for PGP and MRP1, did not show cross resistance to drugs transported by these proteins. Conclusion: Our results support the necessity for further research on the role of bortezomib in the therapy of glioblastoma.

The proteasome is an ubiquitous enzyme complex that plays a critical role in the degradation of many proteins involved in cell cycle regulation, apoptosis and angiogenesis (1). Inhibition of the 26S proteasome permits accumulation of substrate polyubiquinated proteins, while normal activity rapidly clears them from the cell. Alteration of the levels of these cellular proteins leads to inhibition of proliferation, migration and angiogenesis and to the promotion of apoptosis of cancer cells.

Bortezomib (formerly PS-341, Figure 1) is an extremely potent and selective proteasome inhibitor that showed strong activity in in vitro and in vivo laboratory studies against many solid and hematologic tumor types. Moreover, bortezomib, mainly by inhibition of the NF-kappaB pathway, had a chemosensitizing effect when administered together with other antitumoral drugs (2). Bortezomib is a biologically active agent, producing predictable, dose-related and reversible proteasome inhibition; it has shown antitumor activity in various malignancies and was the first proteasome inhibitor to be used in clinical practice. Several trials demonstrated that bortezomib is relatively well-tolerated, causing manageable non-hematological and hematological toxicity. Clinical phase I, II and III studies, showed good tolerance of bortezomib and high response rates in refractory multiple myeloma patients (3, 4). It was used as a single agent and in combination with chemotherapeutic drugs, showing potentiation of the effect. In variety of other hematological malignancies and solid tumors, phase I and II studies with bortezomib alone or in combination with other drugs have produced encouraging results, both in children and adults (5-8), for carcinomas of the breast, lung (9, 10), colon (11), bladder (3), ovary (12), pancreas (13) and prostate (12), melanoma (14), thyroid carcinoma (15) and metastatic neuroendocrine tumors (16).

The objective of this study was the evaluation of the activity of bortezomib and 14 other anticancer drugs in glioblastoma cell lines, in comparison with that of childhood acute lymphoblastic leukemia CCRF-CEM cell line.

Materials and Methods

Cell lines. Glioblastoma (T98G, U373MG) and T-lineage acute lymphoblastic leukemia (CCRF-CEM) cell lines were analyzed. Both brain tumor cell lines were maintained in EMEM (EBSS,
Sigma, Munich, Germany) medium supplemented with 2 mM glutamine (Sigma), 1 mM non-essential amino acids (NEAA, Sigma), 1% sodium pyruvate (NaP, Sigma) and 10% fetal bovine serum (FBS, Gibco BRL, Paisley, UK). For seeding, 0.25% trypsin/EDTA was used. The culture was carried out under conditions of 5% CO2, 37°C and 95% humidity. The culture medium for CCRF-CEM cell line contained RPMI 1640 medium (Sigma), supplemented with 2 mM glutamine and 20% FBS. Both culture media were supplemented with 100 U/ml penicilin (Polfa Tarchomin, Poland), 100 ìg/ml streptomycin (Polfa Tarchomin), 200 ìg/ml gentamycin (Krka, Nove mesto, Slovenia) and 0.125 ìg/ml amphotericine B (Fungizone, Brisol-Myers Squibb, Ruel-Malmaison, France).

**Drugs.** The following 15 drugs were used: bortezomib (Velcade, Janssen Pharmaceutica N.V., Beerse, Belgium, concentrations tested: 0.19-200 nM), prednisolone (Jelfa, Jelenia Gora, Poland, 0.0076-250 ìg/ml), vincristine (Gedeon Richter, Budapest, Hungary, 0.019-20 ìg/ml), L-asparaginase (Medac, Hamburg, Germany, 0.0032-10 IU/ml), daunorubicin (Rhone-Poulenc Rorer, Montrouge, France, 0.0019-2 ìg/ml), doxorubicin (Pharmacia Italia S.p.A., Milan, Italy, 0.031-40 ìg/ml), actinomycin-D (Lyovac, MSD, Viena, Austria, 0.0048-5 ìg/ml), cytarabine (Bioton, Warsaw, Poland, 0.0004-40 ìg/ml), cisplatin (Pliva-Lachema, Brno, Czech Republic, 0.97-100 ìg/ml), carboplatin (Pliva-Lachema, 0.48-500 ìg/ml), etoposide (Bristol-Myers Squibb, Sermoneta, Italy, 0.048-50 ìg/ml), thiotepa (Lederle, Wolfratshausen, Germany, 0.032-100 ìg/ml), arsenic trioxide (Sigma, 0.019-20 ìM), topotecan (Glaxo Smith Kline, Brentford, UK, 0.097-100 ìg/ml). Before the assay was performed, most drug stock solutions were stored frozen in small aliquots at −20°C, except cladribine, cisplatin and carboplatin, which were stored at +4°C. Stock solutions were prepared in water for injection; further dilution was made in respective media.

**The MTT assay.** Cellular drug resistance was tested by means of the MTT assay. The procedure of the assay was described previously (17). The drug concentration that was inhibitory to 50% of the cells (IC50) was calculated from the dose-response curve and was used as a measure for in vitro drug resistance in each sample. Results were compared between respective cell lines. At least four independent experiments were performed for each cell line. The relative resistance (RR) between cell lines for each drug was calculated as a ratio of mean value of IC50 for this drug in tested cell lines.

**Statistical analysis.** The t-test for independent samples was used to compare differences in drug resistance between groups. The correlation between cytotoxicity of drugs was determined by Spearman’s rho coefficient.

**Table I. Drug sensitivity and resistance of cell lines.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (mean±SD)</th>
<th>CCRF-CEM</th>
<th>T98G</th>
<th>U373MG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>18.5±4.3</td>
<td>28.9±4.5</td>
<td>48.2±12.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR=1.56</td>
<td>RR=2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>0.1±0.1</td>
<td>0.69±0.25</td>
<td>0.39±0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR=6.9</td>
<td>RR=3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladribine</td>
<td>0.02±0.01</td>
<td>&gt;40</td>
<td>0.9±0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR=2000</td>
<td>RR=45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arsenic trioxide</td>
<td>6.8±3.2</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td>Topotecan</td>
<td>&lt;0.097</td>
<td>0.2±0.01</td>
<td>21.36±18.93</td>
<td></td>
</tr>
<tr>
<td>Prednisolone</td>
<td>1.01±0.98</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td>Vincristine</td>
<td>0.09±0.03</td>
<td>7.19±2.36</td>
<td>8.23±2.28</td>
<td></td>
</tr>
<tr>
<td>L-asparaginase</td>
<td>0.1±0.08</td>
<td>0.97±0.71</td>
<td>2.67±2.12</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.08±0.02</td>
<td>5.2±1.2</td>
<td>6.9±1.6</td>
<td></td>
</tr>
<tr>
<td>Cytarabine</td>
<td>&lt;0.24</td>
<td>67.8±46.7</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR=282</td>
<td>RR=1041</td>
<td></td>
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</tr>
</tbody>
</table>

RR (relative resistance)-ratio of mean values of IC50 of brain tumor cell line and leukemic CCRF-CEM cell line. P-value was calculated using the Student’s t-test. Concentration is given in nM for bortezomib, ìM for arsenic trioxide, IU/ml for L-asparaginase and in ìg/ml for the rest of the drugs.
Results

Bortezomib was only 1.56-2.6-fold less active in the glioblastoma cell lines when compared to the CCRF-CEM cell line. The leukemic cell line was the most drug-sensitive cell line for all tested drugs, with possible exception to cisplatin and carboplatin (Table I). These two drugs showed good activity against both brain tumor cell lines. Actinomycin-D and etoposide also showed good activity and were only 1.2- to 2.5-fold less cytotoxic against malignant brain tumor cells, in comparison with the leukemic cell line.

First-line drugs used in the therapy of acute lymphoblastic leukemia, such as prednisolone, L-asparaginase, vincristine, doxorubicin and cytarabine proved to be inactive in both glioblastoma cell lines. Both cell lines were highly resistant to arsenic trioxide and cladribine, which are used in the treatment of acute myeloid leukemia M3 and chronic lymphocytic leukemia, respectively. Thiotepa and daunorubicin showed relatively weak activity against glioblastoma cell lines. Non-conclusive results were obtained for topotecan, mainly due to large variability in IC50 in the respective cell lines.

Analysis of the correlation of resistance among the anticancer drugs indicated high cross-resistance of drugs belonging to the same class (such as cisplatin and carboplatin or daunorubicin and doxorubicin) or being the substrate for the multidrug resistance protein PGP. Bortezomib did not show cross-resistance to most tested drugs, with the exception of arsenic trioxide and cytarabine (Table II). However, the relative activity of bortezomib in the glioblastoma cell lines was much higher than the relative activity of arsenic trioxide and cytarabine, in comparison with the CCRF-CEM cell line.

Discussion

Glioblastoma multiforme is the most malignant, invasive, difficult to treat primary brain tumor. This form of cancer has a rapid growth rate and is capable of doubling in size within 10-20 days. Successful treatment of glioblastoma is rare; standard therapies result in a mean survival of only about 10-12 months (18). In this study, the anti-glioma activity of bortezomib was examined using two human glioblastoma cell lines and was compared with that on an acute lymphoblastic leukemia cell line.

A rationale for the use of bortezomib in glioblastoma is related to a hyperactive NF-κB pathway in glioblastoma multiforme cells; the rate of growth of these cells paralleled the activity of this pathway in these tumors (19, 20). Therefore, this pathway becomes an important therapeutic target for these tumors. NF-κB activation depends on the signal-induced phosphorylation and ubiquitination of the inhibitory protein IκBα. PS-341 can stabilize IκBα by...
inhibiting the chymotryptic activity of the 26S proteasome. Yin et al. have demonstrated that bortezomib could significantly decrease the nuclear activity of NF-κB and induce cell death and apoptosis in glioblastoma cells (21). In animal models, bortezomib did not enter the brain, spinal cord, testes, or the eye, thus, sparing these tissues from the adverse effects of proteasome inhibition (22). It might, however, be active either when the blood-brain or blood-testes barrier has been disrupted by the malignancy, or in any form of local therapy.

We have shown that in comparison to leukemic cells, glioblastoma cells were sensitive to bortezomib. This compound did not present cross-resistance with most of the other drugs tested, including substrates for multidrug resistance proteins. The intrinsic multidrug resistance in human gliomas, including T98G and U373MG cell lines, as well as in tumor specimens, may be related to PGP and MRP expression (23, 24). Thus, the efficacy of bortezomib does not seem to be influenced by the presence of known drug resistance factors. A systematic check of multidrug resistance transporters indicated that bortezomib is a poor substrate for this class of proteins (25-27). Bortezomib has the potential to circumvent multilcellular drug resistance and, therefore, may show promising activity against solid tumors with low growth fractions in vivo, which are frequently intrinsically resistant to conventional cytotoxic anticancer drugs (12). Homologues of the drug resistance protein MDR1/ MRPI were used by Steiner et al. to test the ability of these proteins to confer bortezomib resistance on otherwise sensitive cells (28). Bortezomib was equally effective in cells that overexpressed either MRP3 or MRP5, which can confer resistance to methotrexate and etoposide or mitomycin C, etoposide, cisplatin, 5-fluorouracil and methotrexate, thus, demonstrating that these proteins were insufficient to confer resistance to this proteasome inhibitor (28).

Apart from bortezomib, the good activity of cisplatin, carboplatin and etoposide, which are used in chemotherapy protocols for sensitive brain tumors was also demonstrated. In meta-analysis on drug resistance in brain tumor cells, including 18 glioma cell lines, Wolff et al., have concluded that SNB56, A172, SNB75, SF188 and U373MG cell lines were the most drug resistant while C6, PS60, U251MG were relatively drug sensitive. The best overall sensitivity was observed for actinomycin-D, vincristine, mitoxantrone, vinblastine, doxorubicin, cisplatin, methotrexate, cytarabine, 5-fluorouracil and resistance was observed to bleomycine, carboplatin, Carmustine, nimustine and lomustine (29).

The activity of arsenic trioxide, topotecan and cladribine was also tested. There are scanty data available regarding the use of these compounds with respect to brain tumors. Kanzawa et al. have shown that arsenic trioxide inhibited proliferation of cells in all tested glioma cell lines and induced apoptosis (30). Topotecan seemed to be a promising drug for the therapy of brain tumors, due to high penetration of the blood-brain barrier, good activity against solid tumors and because of its radiosensitizing properties. Fisher et al. showed growth inhibition in ependymoma, high-grade glioma and medulloblastoma cells caused by topotecan (31). The antiproliferative potential of topotecan was increased after combination with vincristine in brain tumor cell lines (32). Our study is the first to provide analysis of the in vitro activity of cladribine in brain tumor cells. However, data obtained do not support further studying of this compound with respect to glioblastoma cells.

Bortezomib seems to be an effective agent against glioblastoma cells in vitro and may have a future role in the therapy of this disease. Bortezomib induced growth arrest and apoptosis in glioma cell lines and explants (21). A phase I/II study in malignant gliomas is ongoing (18). This study also confirmed the good activity of cisplatin and carboplatin against glioblastoma cell lines, and supported the use of multi-agent chemotherapy based on platinum compounds in this subtype of brain tumors. The presented data provide insights both into the anti-tumor activity of proteasome inhibitor and the rationale for future clinical trials of bortezomib in conventional chemotherapy for glioblastoma in order to improve patient outcome.

References
Styczynski et al: Activity of Bortezomib in Glioblastoma


