Activity of Bortezomib in Adult *De Novo* and Relapsed Acute Myeloid Leukemia

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Abstract. *Background: Bortezomib is an inhibitor of proteasome and NF-κB, with activity in various solid tumors and hematological malignancies. Aim: The aim of the study was the analysis of in vitro drug resistance to bortezomib and other anticancer drugs in de novo and relapsed adult acute myeloid leukemia (AML). Patients and Methods: The leukemic cells of 46 adult patients with AML were tested for the in vitro drug resistance profile. The group included 20 de novo and 26 relapsed AML patients, among whom, 12 relapsed after allogeneic hematopoietic stem cell transplantation (HSCT) and 4 after autologous HSCT. The MTT assay was performed for 21 drugs. Expression of P-glycoprotein (PGP), multidrug resistance-associated protein-1 (MRP1) and lung resistance protein (LRP) proteins was measured by flow cytometry. Results: No significant differences in drug resistance were found for all tested drugs between de novo and relapsed AML samples, while expression of PGP, MRP1 and LRP was higher in relapsed patients. Patients with refractory or relapsed disease, had higher resistance of myeloblasts to cyclophosphamide (RR=2.4, p=0.050), and better sensitivity to busulfan (RR=0.4, p=0.054) and topotecan (RR=0.4, p=0.031). Those who have died due to refractory/relapsed disease (n=16) had better sensitivity to bortezomib (RR=0.6, p=0.046) and treosulfan (RR=0.1, p=0.018). Conclusion: In vitro drug resistance in relapsed adult AML is comparable to that in de novo disease. Activity in vitro of bortezomib might be a rationale for its use in refractory/relapsed AML adult patients.*

The ubiquitin-proteasome pathway is one of the most important systems for protein degradation, involved in cell cycle regulation, transcription factor activation, apoptosis and cell migration (1). Key regulatory proteins degraded by this system include p53, cyclins, cyclin-dependent kinase inhibitors $p27^{KIPI}$ and p21, and inhibitor protein IκBα of nuclear factor (NF)-κB (2-4). NF-κB has tumor-promoting activity, antiapoptotic activity and regulates cell adhesion molecules involved in tumor metastasis and angiogenesis (5-7).

Constitutive activation of NF-κB was reported for various solid tumors and hematological malignancies (5, 6). Aberrant expression of NF-κB family proteins has been described for primitive acute myeloid leukemia (AML) cells leading to expression of antiapoptotic proteins (8). It suggests that therapies inhibiting NF-κB have the potential to target AML cells.

Bortezomib is the first selective proteasome inhibitor approved for use in humans (9). Its efficacy and safety have been documented in multiple myeloma (10, 11). *In vitro* and *in vivo* studies have demonstrated the activity of bortezomib in prostate (12), breast, lung (13, 14), colon (15), bladder (16), ovary (12) and pancreatic cancer (17), squamous cell carcinoma (18) and acute leukemia (19). Activity of bortezomib was shown in human melanoma (20), human neuroendocrine tumor cell lines (21) and in glioblastoma (22). Preclinical studies have demonstrated the ability of bortezomib in chemosensitization and overcoming of chemotherapy resistance (23, 24). In *in vitro* studies, NF-κB inhibition specifically induced apoptosis in AML cells and potentiated the apoptotic response to anthracyclines and cytarabine, while sparing normal CD34+ hematopoietic precursors (25). Phase I studies with bortezomib, used as single agent or in combination in patients with refractory or relapsed acute leukemias, have shown promising antitumor activity, supporting further testing of this drug (26, 27).

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The aim of this study was the analysis of *in vitro* drug resistance to bortezomib and another 20 anticancer drugs in *de novo* and relapsed adult AML.

Patients and Methods

Patients and leukemic cells. The leukemic cells of 46 patients (21 male, 25 female; median age 41 years; range 18-69 years) with AML were tested for their *in vitro* drug resistance profile. The group included 20 *de novo* and 26 relapsed AML patients. Among relapsed patients, 12 had relapsed after allogeneic and 4 after autologous hematopoietic stem cell transplant. Patients were followed-up for a median period of 9 months (range: 2-18 months).

Samples of bone marrow (BM) were collected in a heparinised tube (or 15-20 U heparine was used for 1 ml BM). Before testing, the samples were diluted 1:1 or more with RPMI-1640 (Sigma, St. Louis, MO, USA). In case of the presence of small clots, the sample of BM was first filtered through a cell strainer (70 μm nylon filter; Falcon, Franklin Lakes, NJ, USA) using RPMI-1640 to rinse off the strainer. Leukemic cells were separated on Ficoll gradient at 540 g for 20 minutes at room temperature. After centrifugation, cells were washed twice with RPMI-1640. The viability and recovery of the cells was tested by Trypan blue exclusion assay. Cell morphology and percentage of blasts was analysed on cytospine slides stained using the May-Grunwald-Giemsa (MGG) method. Only samples with at least 70% of myeloblasts, both in the beginning and at the end of the assay, were included in the study. The study was approved by local Ethics Committee and written informed consent was obtained from all patients.

Cell line. The AML, HL-60, cell line was analyzed. Cells were maintained in RPMI 1640 medium (Sigma), supplemented with 2 mM glutamine and 20% fetal bovine serum (FBS). The culture medium was supplemented with 100 U/ml penicillin (Polfa, Tarchomin, Poland), 100 μg/ml streptomycin (Polfa), 200 μg/ml gentamycin (Krka, Slovenia) and 0.125 μg/ml amphotericine B. The culture was carried out in conditions of 5% CO_2, 37˚C, and 95% humidity. All experiments on the cell line were performed at least 6 times.

Drugs. The following 21 drugs were used: bortezomib (Velcade, Janssen Pharmaceutica N.V., Beerse, Belgium; concentrations tested: 0.00019-2 μM), prednisolone (Jelfa, Jelenia Gora, Poland; 0.0076-250 μg/ml), vincristine (Gedeon Richter, Budapest, Hungary; 0.019-20 μg/ml), L-asparaginase (Medac, Hamburg, Germany; 0.0032-10 IU/ml), daunorubicin (Rhone-Poulenc Rorer, Paris, France; 0.0019-2 μg/ml), doxorubicin (Pharmacia Italia S.p.A., Milan, Italy; 0.031-40 μg/ml), cytarabine (Upjohn, Puurs, Belgium; 0.24-250 μg/ml), cladribine (Bioton, Warsaw, Poland; 0.0004-40 μg/ml), etoposide (Bristol-Myers Squibb, Sermoneta, Italy; 0.048-50 μg/ml), thiotepa (Lederle, Wolfratshausen, Germany; 0.032-100 μg/ml), arsenic trioxide (Sigma; 0.019-20 μM), topotecan (Glaxo SmithKline Manufacturing S.p.A., Parma, Italy; 0.097-100 μg/ml), busulfan (Busilvex, Pierre-Fabre-Medicament, Castres, France; 1.17-1200 μg/ml), 4-HOO-cyclophosphamide (Asta Medica, Hamburg, Germany; 0.096-100 μg/ml), 4-HOO-ifosfamide (Asta Medica; 0.096-100 μg/ml), fludarabine phosphate (Schering AG, Berlin, Germany; 0.019-20 μg/ml), idarubicin (Pharmacia, Milan, Italy; 0.0019-2 μg/ml), melphalan (Glaxo Wellcome, Parma, Italy; 0.038-40 μg/ml), mitoxantrone (Jelfa; 0.001-1 μg/ml), 6-thioguanine (Sigma, nr A4882; 1.56-50 μg/ml), treosulfan (Medac; 0.0005-1 μg/ml). Before the assay was carried out, most drug stock solutions were stored frozen in small aliquots at –20˚C, except cladribine, which was stored at +4˚C. Stock solutions were prepared in water for injection, further dilution was made in respective medium.

Multidrug resistance proteins. Expression of intracellular epitopes of three multidrug resistance proteins, P-glycoprotein (PGP) (clone JSB-1), multidrug resistance-associated protein (MRP1) (clone MRPr1) and lung resistance protein (LRP) (clone LRP-56) (all: Alexis Biochemicals, Lausanne, Switzerland), were analyzed by flow cytometry on diagnosis. Protein expression is presented as mean fluorescence intensity, corrected by expression of the respective isotype controls. The negative control for multidrug resistance proteins was the CCRF-CEM cell line, while the positive control was the adenocarcinoma LoVo-Dx cell line.

MTT assay. Cellular drug resistance was tested by means of the MTT assay. The procedure of the assay is described elsewhere (28). The concentration of drug that was inhibitory to 50% of the cells (IC_{50}) was calculated from the dose response curve and was used as a measure for *in vitro* drug resistance in each sample. Relative resistance (RR) between analyzed groups for each drug was calculated as a ratio of mean value of IC_{50} for this drug.

Statistical analysis. The Mann-Whitney *U*-test was performed to compare differences in drug resistance between groups. Correlations in resistance between groups were determined by Spearman's rho coefficient.

Results

No significant differences in drug resistance were found for the 21 tested drugs between *de novo* and relapsed AML samples (Table I), while expression of PGP, MRP1 and LRP was higher in relapsed patients. Refractory disease developed in 9 *de novo* and 17 relapsed AML patients. Patients with refractory/relapsed disease during follow-up had higher resistance of myeloblasts to cyclophosphamide (RR=2.4, p=0.050), and better sensitivity to busulfan (RR=0.4, p=0.054) and topotecan (RR=0.4, p=0.031). Those patients who have died due to refractory/relapsed disease (n=16) had better sensitivity to bortezomib (RR=0.6, p=0.046) and treosulfan (RR=0.1, p=0.018).

Discussion

Current treatment strategies of AML based on conventional cytotoxic agents provide complete remission in 60%-80% of younger patients, however, only 30%-40% survive 5 years. For elderly patients and patients with relapsed and refractory disease, significant improvements in survival have not emerged for years. Most recently, insights into the molecular pathogenesis of AML and the mechanism of treatment failure have led to the development of specific targeted therapy (29, 30).

Table I. *Drug resistance in HL-60 cell line and in AML samples.*

Drug / MDR protein	HL-60 cells	*De novo* AML	Relapsed AML
Cytarabine	0.32 (0.16-0.42)	1.16 (0.22->10)	1.10 (0.05->10)
	n=6	n=16	n=16, p=0.777
Arsenic trioxide	4.3 (2.1-6.2)	>20 (3.15->20)	>20 (2.45->20)
	n=6	n=15	n=15, p=0.190
L-asparaginase	0.43 (0.28-0.55)	0.54 (0.01->10)	0.48 (0.01->10)
	n=6	n=16	n=15, p=0.812
Bortezomib	0.068 (0.045-0.082)	0.201 (0.013-0.486)	0.285 (0.107->2.00)
	n=6	n=15	n=16, p=0.176
Busulfan	22.3 (15.8-29.5)	31.9 (11.4-68.4)	34.2 (3.9->1200)
	n=6	n=5	n=10, p=0.770
Cladribine	0.02 (0.01-0.04)	0.024 (0.0076->40)	0.069 (0.0006->40)
	n=6	n=16	n=17, p=0.540
Cyclophosphamide	1.62 (1.11-1.97)	1.73 (0.61-4.63)	1.78 (0.19-4.01)
	n=6	n=14	n=10, p=0.380
Daunorubicin	0.17 (0.09-0.32)	0.21 (0.02->2)	0.19 (0.01->2)
	n=6	n=16	n=15, p=0.937
Doxorubicin	0.92 (0.82-1.04)	1.25 (0.16->8)	1.18 (0.34->8)
	n=6	n=15	n=12, p=0.494
Fludarabine	0.71 (0.58-0.84)	0.74 (0.19->20)	0.68 (0.0094->20)
	n=6	n=16	n=17, p=0.385
Idarubicin	0.15 (0.08-0.25)	0.21 (0.08->2)	0.18 (0.02->2)
	n=6	n=16	n=17, p=0.759
Ifosfamide	12.5 (9.4-14.7)	16.7 (1.2-52.1)	13.6 (4.8-20.1)
	n=6	n=14	n=10, p=0.292
Melphalan	2.4 (1.0-5.1)	3.6 (0.05->40)	2.6 (0.11->40)
	n=6	n=15	n=12, p=0.494
Mitoxantrone	0.15 (0.09-0.22)	0.22 (0.038-13.28)	0.19 (0.021->1)
	n=6	n=16	n=15, p=0.736
Prednisolone	58.2 (43.8-69.8)	78.9 (1.43-203)	86.3 (1.48->250)
	n=6	n=16	n=15, p=0.664
Thioguanine	2.8 (0.9-5.3)	10.9 (0.08->50)	9.2 (1.56->50)
	n=6	n=14	n=10, p=0.883
Thiotepa	4.2 (2.2-6.5)	4.5 (0.032->100)	4.1 (0.09->100)
	n=6	n=15	n=11, p=0.337
Topotecan	0.58 (0.34-0.76)	0.78 (0.0976-23.5)	0.79 (0.0967->100)
	n=6	n=15	n=15, p=0.507
Treosulfan	0.48 (0.23-0.71)	0.85 (0.0001->1)	0.66 (0.0001->1)
	n=6	n=15	n=11, p=0.821
Vincristine	0.8 (0.62-0.98)	1.8 (0.11-10.85)	3.9 (0.2-16.9)
	n=6	n=16	n=15, p=0.304
Etoposide	2.8 (1.7-3.8)	5.7 (0.1-166.87)	4.2 (0.2->50)
	n=6	n=16	n=15, p=0.693
PGP expression	2.2 (1.1-2.8)	7.6 (4.5-19.2)	12.5 (1.2-25.6)
	n=6	n=12	n=18, p=0.083
MRP expression	3.1 (1.2-4.6)	10.2 (4.1-17.2)	13.7 (1.1-31.4)
	n=6	n=12	n=18, p=0.057
LRP expression	2.8 (1.9-4.0)	9.3 (4.3-15.2)	14.2 (0.88-27.6)
	n=6	n=12	n=18, p=0.014

The values represent median and range of IC_{50} values; n – number of tested samples; p-value was calculated using Mann-Whitney U-test (between relapsed and *de novo* AML samples). Concentration is given in μM for arsenic trioxide, IU/ml for L-asparaginase and in μg/ml for other drugs.

Poor prognosis, especially in older patients, might be related either to intrinsic drug resistance or the inability to withstand intensive therapy. In comparison to childhood AML, no significant differences were reported in the *in vitro* drug resistance in adult AML. Since no differences were reported between *de novo* and relapsed pediatric AML (31), we hypothesized that there were no differences in cellular drug resistance between *de novo* and relapsed adult AML. In spite of the small size of the group, the presented results seem to confirm the lack of such differences.

No differences were found in *in vitro* drug resistance between *de novo* and relapsed adult AML patients for all tested drugs, in spite of the higher expression of multidrug resistance proteins in relapsed AML. Nevertheless, patients who developed refractory disease had, at diagnosis, better *in vitro* sensitivity to busulfan and topotecan. Moreover, patients who later died, due to progression or relapsed AML, had better sensitivity to bortezomib and treosulfan. The results of our study might suggest the benefit of the use of bortezomib and topotecan in adult patients with relapsed/refractory AML, as busulfan and treosulfan are used in high-dose therapy before stem cell transplantation

We have shown that AML cells were sensitive to bortezomib and the *in vitro* activity of this drug was not influenced by the presence of drug resistance proteins, which was also noted by other authors (32). It suggests insensitivity of bortezomib to these proteins and this might have an important value in relapsed/refractory AML patients with overexpression of multidrug resistance proteins. Relatively good sensitivity to bortezomib was seen in our study in patients with clinically progressive and resistant disease, supporting this theory. Differences in the *in vitro* sensitivity of AML cells to bortezomib are related to variability in the activity profiles of the individual proteasomal subunits between primary leukemia cells (33).

In addition to drug resistance, an aberrant activation of signal transduction proteins, including NF-κB, is one of the key mechanisms of treatment failure in AML (8). Activity of bortezomib in AML, which also acts through the NF-κB pathway, is an important aspect currently being investigated not only in *in vitro*, but also in *in vivo* studies (26, 34). It is confirmed in clinical studies that bortezomib has antileukemic activity and effective drug concentrations are readily attainable in patients (26, 27, 32). So far however, the clinical benefit has usually been minor with modest and transient decrease of peripheral blood and bone marrow blasts. Significant interpatient heterogeneity in intrinsic drug sensitivity was seen. Some observations suggest a use of bortezomib in more indolent disease, such as myelodysplastic syndrome or as postremission therapy in acute leukemia (26). Another tested option is to combine bortezomib with standard chemotherapy or modern targeted therapy (25-27, 35).

In conclusion, the *in vitro* drug resistance in relapsed adult AML is comparable to that on *de novo* disease. Activity *in vitro* of bortezomib might be a rationale for its use in refractory relapse in AML adult patients.

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