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Original paper

Bortezomib has little *ex vivo* activity in chronic myeloid leukemia: individual tumor response testing comparative study in acute and chronic myeloid leukemia

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Aim of the study: Resistance to imatinib is one of the most important issues in treatment of chronic myeloid leukemia (CML) patients. The objective of the study was to analyze the *ex vivo* drug resistance profile to bortezomib and 22 other antileukemic drugs, including three tyrosine kinase inhibitors (TKIs), in CML in comparison to acute myeloid leukemia (AML).

Material and methods: A total of 82 patients entered the study, including 36 CML and 46 AML adults. Among CML patients, 19 had advanced disease, 16 were resistant to imatinib, and 6 had *ABL*-kinase domain mutations. The *ex vivo* drug resistance profile was studied by the MTT assay.

Results: CML cells were more resistant than AML blasts to the following drugs: prednisolone, vincristine, doxorubicin, etoposide, melphalan, cytarabine, fludarabine, thiotepa, 4-HOO-cyclophosphamide, thioguanine, bortezomib, topotecan, and clofarabine. CML cells were 2-fold more sensitive to busulfan than AML cells. CML patients with clinical imatinib resistance had higher *ex vivo* resistance to vincristine, daunorubicin, etoposide, and busulfan. No significant differences to all tested drugs, including TKIs, were observed between CML patients with non-advanced and advanced disease. CML patients with mutation had higher *ex vivo* resistance to vincristine, idarubicin, thiotepa, and busulfan.

Conclusions: CML cells are *ex vivo* more resistant to most drugs than acute myeloid leukemia blasts. Busulfan is more active in CML than AML cells. In comparison to AML cells, bortezomib has little *ex vivo* activity in CML cells. No differences between CML subgroups in sensitivity to 3 tested TKIs were detected.

Key words: chronic myeloid leukemia, MTT assay, drug resistance, drug sensitivity.

Background

Drug resistance is one of the factors contributing to poor response to therapy. Cellular drug resistance can be defined as a lack of cytotoxic response in cancer cells after administration of a cytotoxic compound. Response of cancer cells to chemotherapy can be tested in *ex vivo* conditions by several assays, such as the methylthiazol tetrazolium (MTT) assay, differential staining cytotoxicity (DiSC) assay, the fluorometric microculture cytotoxicity assay (FMCA) and similar assays. Considerable work based on these assays has been reported during the past 25 years, and recently an ad hoc group of 50 scientists from 10 countries agreed on the term "individualized tumor response (ITRT)" for these tests, describing them as the "effect of anticancer treatments on whole living tumor cells freshly removed from cancer patients" and not including tests with "subcellular fractions, animals or cell lines" [1, 2]. ITRT is regarded as an important risk factor of treatment failure in pediatric acute lymphoblastic leukemia (ALL). It can be demonstrated clinically as a poor steroid response after one-week monotherapy or as a delayed response of bone marrow at day +15 or day +33 of induction therapy. Presence of minimal residual disease also results in drug resistance. In comparison to pediatric ALL, the value of ITRT assays is less established in other types of leukemia, especially in chronic myeloid leukemia (CML). Introduction of tyrosine kinase inhibitors (TKIs) in therapy of CML has contributed to development of *ex vivo* testing in this disease. So far only very limited data on cellular drug resistance in CML cells are available [3–6].

The objective of the study was to analyze the *ex vivo* drug resistance profile to bortezomib and 22 other antileukemic drugs, including three tyrosine kinase inhibitors (TKIs), in CML in comparison to acute myeloid leukemia (AML).

Material and methods

Patients

A total of 82 patients entered the study, including 36 CML and 46 AML adults (age 18–69, median 41 years). However, due to technical reasons, not all drugs were tested for all patients. AML patients were diagnosed for *de novo* (n = 20) or relapsed (n = 26) disease. CML patients were divided into the following subgroups: with advanced (n = 19) or non-advanced (n = 17) disease;

with good (n = 20) or poor clinical response to imatinib (n = 16) [7]; with (n = 6) or without mutation (n = 28). Non-advanced disease was defined as the first chronic CML phase. All other phases were classified as advanced disease. Poor clinical response was defined as clinical resistance to imatinib. All patients with a poor clinical response were tested for *ABL*-kinase domain mutations. Among CML patients, 19 had advanced disease, 16 were resistant to imatinib, and 6 had *ABL*-kinase domain mutations (M244V, E255K, Y253H, M351T and 2 with F317L).

Drugs

The following 20 drugs were used: bortezomib (Velcade, Janssen Pharmaceutica N.V., Beerse, Belgium; concentrations tested: 0.00019–2 μM), prednisolone (Jelfa, Jelenia Góra, 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/mlz, 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), topotecan (Glaxo SmithKline Manu-facturing 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), 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), and clofarabine (Bioenvsion / Genzyme, 0.01–12.5 μM). 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, and further dilution was made in respective medium.

CML patients were also tested for sensitivity to tyrosine kinase inhibitors: imatinib (Novartis Pharmaceuticals; concentrations tested: 0.000977–1 μM), dasatinib (Bristol Meyers Squibb; 0.000977–1 μM) and nilotinib (Novartis Pharmaceuticals; 0.000977–1 μM).

Methylthiazol tetrazolium assay

Ex vivo drug resistance profile (ITRT) was studied by the MTT assay. The procedure of the assay is described elsewhere [2]. The concentration of drug that was lethal to 50% of the cells (LC50) was calculated from the dose response curve and was used as a measure for *ex vivo* drug resistance in each sample. Relative resistance (RR) between analyzed groups for each drug was calculated as the ratio of median values of LC50 for this drug in each group.

Results of AML patients were published previously [8]. Due to similar profiles of drug sensitivity, all AML patients were pooled into one group for further analysis [8].

Statistical analysis

The Mann-Whitney U test was performed to compare differences in drug resistance between groups.

Results

In comparison to adult AML, CML blasts were more resistant to bortezomib (6.2-fold; $p < 0.001$), and to the following other drugs: prednisolone (1.5-fold; $p = 0.037$), vincristine (2.3; $p = 0.004$), doxorubicin (> 6.9; $p < 0.001$), etoposide (7.4; $p < 0.001$), melphalan (5.9; $p = 0.001$), cytarabine (12.5; $p = 0.005$), fludarabine (2.6; $p = 0.008$), thiotepa (5.4; $p = 0.001$), 4-HOO-cyclophosphamide (2.3; $p = 0.015$), thioguanine (> 4; $p < 0.001$), topotecan (20; $p < 0.001$), and clofarabine (50; $p < 0.001$). No differences in sensitivity were found for idarubicin, daunorubicin, mitoxantrone, L-asparaginase, cladribine, and treosulfan, while CML cells were 2-fold more sensitive to busulfan ($p = 0.035$) (Table 1).

CML patients were divided into subgroups (Table 2). No differences in LC50 values for bortezomib were observed between any subgroup of patients. Overall, no significant differences for all tested drugs, including TKIs, were observed between CML patients with non-advanced and advanced disease. CML patients with poor clinical response expressed as clinical resistance to imatinib had higher median LC50 values for vincristine (2.5-fold; $p = 0.016$), daunorubicin (3.1-fold; $p = 0.011$), etoposide (2.2-fold; $p = 0.031$), and busulfan (4.5-fold; $p = 0.032$). No significant differences were observed with respect to other drugs, including all 3 TKIs. CML patients with mutation had higher median LC50 values for vincristine (3.3-fold; $p = 0.044$), idarubicin (> 7.9-fold; $p = 0.031$), thiotepa (13.7-fold; $p = 0.044$), and busulfan (21.6-fold; $p = 0.024$). No significant differences were observed with respect to other drugs, including all 3 TKIs (Table 2).

Discussion

Therapy of CML has been significantly improved with the use of BCR-ABL kinase inhibitors. However, the existence of CML cells that are unaffected by BCR-ABL inhibition represents a major barrier that may prevent curative therapy with the current approaches. To date, it seems that resistance to tyrosine kinase inhibitor-based therapies involving BCR-ABL gene mutations and amplification is the most important mechanism of therapy failure. New evidence suggests that persistence of CML stem cells or acquisition of stem cell-like characteristics may prevent complete elimination of CML by TKIs [9]. New targets should be defined before significant progress in curative therapies is possible. The proteasome inhibitor bortezomib is a potent *in vitro* cytotoxic compound against stem cells in acute and chronic myeloid leukemias [10, 11]. Poor therapy outcome, especially in patients with relapsed and refractory leukemia, might be related to intrinsic drug resistance.

In our previous *ex vivo* analysis we showed the benefit of use of bortezomib in adult patients with relapsed/refractory AML [8]. Differences in *in vitro* sensitivity of leukemic cells to bortezomib are related to variability in the activity profiles of the individual proteasomal subunits between primary leukemia cells. In addition to drug resistance, an aberrant activation

Table 1. Drug resistance in adult AML and adult CML

Drug /Company	Concentration range	Median and quartiles of LC50		RR	*p*
		AML adult (*n* = 46)	CML adult (*n* = 36)		
prednisolone Jelfa, Jelenia Gora, Poland	0.007–250 µg/ml	148 14–250 (*n* = 30)	216 9–>250 (*n* = 25)	1.5	0.037
vincristine Lilly	0.019–20 µg/ml	2.3 0.2–16.9 (*n* = 30)	5.3 0.2–>20 (*n* = 25)	2.3	0.004
idarubicin Farmitalia	0.0019–2 µg/ml	0.32 0.10–>2 (*n* = 33)	0.27 0.02–>2 (*n* = 27)	0.9	0.731
daunorubicin Rhone-Poulenc–Rhorer	0.0019–2 µg/ml	0.61 0.19–>2 (*n* = 30)	0.50 0.02–>2 (*n* = 25)	0.8	0.623
doxorubicin Farmitalia	0.0078–8 µg/ml	1.16 0.43–>8 (*n* = 27)	> 8 0.08–>8 (*n* = 25)	> 6.9	< 0.001
mitoxantrone Jelfa, Jelenia Gora, Poland	0.001 –1 µg/ml	0.43 0.18–>1 (*n* = 31)	0.45 0.001–>1 (*n* = 26)	1.0	0.825
etoposide Bristol – Myers Squibb	0.048–50 µg/ml	4.69 0.7–>50 (*n* = 30)	34.6 0.3–>50 (*n* = 25)	7.4	0.001
L–asparaginase Medac	0.0032–10 IU/ml	1.4 0.2–>10 (*n* = 30)	1.6 0.3–>10 (*n* =25)	1.1	0.635
melphalan Glaxo Wellcome	0.038–40 µg/ml	2.66 0.01–>40 (*n* = 27)	15.8 0.05–>40 (*n* = 25)	5.9	0.001
cytarabine Pharmacia & Upjohn	0.0097–10 µg/ml	0.44 0.14–>10 (*n* = 32)	5.5 0.02–>10 (*n* = 27)	12.5	0.005
fludarabine phosphate Schering	0.019–20 µg/ml	1.16 0.20–>20 (*n* = 33)	2.97 0.6–>20 (*n* = 27)	2.6	0.008
cladribine Bioton, Warsaw, Poland	0.0004–40 µg/ml	0.7 0.01–>40 (*n* = 33)	0.85 0.08–>40 (*n* = 36)	1.2	0.623
thiotepa Lederle	0.032–100 µg/ml	1.84 0.47–13.12 (*n* = 26)	9.98 0.16–>100 (*n* = 26)	5.4	0.001
treosulfan Medac	0.0005–1 µg/ml	0.9 0.0005–>1.0 (*n* = 26)	> 1 0.64–>1.0 (*n* = 25)	> 1.1	0.824
4-HOO-cyclophosphamide Asta Medica	0.096–100 µg/ml	0.8 0.16–>100 (*n* = 24)	1.82 0.09–>100 (*n* = 28)	2.3	0.015
6-thioguanine Sigma, nr A4882	1.56–50 µg/ml	12.1 2.3–>50 (*n* = 26)	> 50 1.56–>50 (*n* = 25)	> 4	< 0.001
bortezomib Janssen Pharmaceutica	0.19–2000 nM	210 1.3–>2000 (*n* = 31)	1302 125–>2000 (*n* = 26)	6.2	< 0.001
topotecan Glaxo SKB	0.097–100 µg/ml	0.78 0.09–>100 (*n* = 30)	16.35 0.097–>100 (*n* = 24)	20.1	< 0.001
clofarabine Bioenvision/Genzyme	0.01–12.5 µM	0.06 0.01–>12.5 (*n* = 10)	3.04 0.02–>12.5 (*n* = 26)	50.6	< 0.001
busulfan Pierre-Fabre Medicament	1.17–1200 µg/ml	31.96 3.9–>1200 (*n* = 27)	16.35 0.09–>1200 (*n* = 24)	0.5	0.035

LC50 – value of in vitro resistance, given in IU/ml for L-asparaginase and in µg/ml for other drugs; RR – relative resistance = median LC50 (CML)/median LC50 (AML); n – the number of patients; p-value – Mann-Whitney U-test

of signal transduction proteins, including the NF-κB pathway, is one of the key mechanisms of treatment failure in AML [12, 13]. Activity of bortezomib in AML and CML, which also acts through the NF-κB pathway, is an important aspect, being investigated in both *in vitro* and in vivo studies [14, 15].

BCR-ABL plays an essential role in the pathogenesis of CML and some cases of ALL. Although ABL kinase inhibitors have shown great promise in the treatment of CML, the persistence of residual disease and the occurrence of resistance have prompted investigations into the molecular effectors of BCR-ABL.

Jagani *et al*. [16] provided a novel insight into the molecular effects of proteasome inhibitor therapy and showed that BCR-ABL stimulated the proteasome-dependent degra-

Table 2. Drug resistance in CML patients with respect to phase of the disease, clinical response to imatinib, and ABL-kinase domain mutation

Drug	Advanced disease				Clinical response to imatinib				ABL-kinase domain mutations			
	No	Yes	RR	*p*	Good	Poor	RR	*p*	No	Yes	RR	*p*
prednisolone	119.22	58.99	0.5	0.363	104.25	116.01	1.1	0.890	117.32	68.36	0.6	0.558
vincristine	5.07	5.54	1.1	0.477	3.29	8.24	2.5	0.016	4.25	14.08	3.3	0.044
idarubicin	0.27	0.28	1.0	0.542	0.17	0.31	1.7	0.113	0.25	> 2.00	> 7.9	0.031
daunorubicin	0.40	0.50	1.3	0.258	0.33	1.03	3.1	0.011	0.47	1.62	3.5	0.072
doxorubicin	5.24	> 8.00	> 1.5	0.171	> 8.00	> 8.00	NE	0.408	> 8.00	> 8.00	NE	0.109
mitoxantrone	0.60	0.44	0.7	0.855	0.60	0.44	0.7	0.547	0.44	> 1.00	> 2.3	0.055
etoposide	30.95	38.62	1.2	0.695	21.57	47.08	2.2	0.031	33.55	46.30	1.4	0.176
L-asparaginase	2.46	0.94	0.4	0.466	0.91	> 10.00	> 11.0	0.064	1.52	5.96	3.9	0.474
cytarabine	3.79	> 10.00	> 2.6	0.918	7.76	2.01	0.3	0.797	3.79	> 10.00	> 2.6	0.494
fludarabine phosphate	4.88	1.55	0.3	0.315	3.21	2.67	0.8	0.960	3.21	> 20.00	> 6.2	0.523
cladribine	1.04	0.66	0.6	0.750	0.59	5.36	9.2	0.745	1.04	> 40.00	> 38.3	0.264
6-thioguanine	> 50.00	> 50.00	NE	0.656	> 50.00	> 50.00	NE	0.949	> 50.00	> 50.00	NE	0.109
treosulfan	> 1.00	> 1.00	NE	0.375	> 1.00	> 1.00	NE	0.375	>1.00	> 1.00	NE	0.655
thiotepa	7.30	14.50	2.0	0.737	7.30	14.50	2.0	0.327	7.30	> 100.00	> 13.7	0.044
melphalan	16.30	15.87	1.0	0.911	10.23	25.58	2.5	0.287	14.74	> 40.00	> 2.7	0.080
4-HOO-cyclophosphamide	2.55	0.84	0.3	0.084	1.66	2.33	1.4	0.774	1.77	39.10	22.1	0.246
bortezomib	1296.84	1615.87	1.2	0.730	1308.4	1225.5	0.9	0.979	1215.3	1807.9	1.5	0.258
topotecan	15.75	21.17	1.3	0.460	15.75	21.17	1.3	0.879	16.9	0.10	0.01	0.116
busulfan	30.30	94.97	3.1	0.133	30.17	134.45	4.5	0.032	32.26	696.82	21.6	0.024
clofarabine	2.30	> 12.50	> 5.4	0.382	2.25	> 12.50	> 5.5	0.223	2.22	> 12.50	> 5.5	0.243
imatinib	0.89	> 1.00	> 1.1	0.910	0.85	> 1.00	> 1.2	0.505	0.60	> 1.00	> 1.6	0.453
dasatinib	0.61	0.24	0.4	0.731	0.61	0.24	0.4	0.827	0.86	0.12	0.1	0.151
nilotinib	0.55	0.84	1.5	0.386	0.42	0.84	2.0	0.216	> 1.00	0.25	< 0.3	0.399

The value of the drug resistance for each group is presented as the median value of all LC50 values in this group. LC50 – value of in vitro resistance, given in IU/ml for L-asparaginase and in μg/ml for other drugs; RR – relative resistance = median LC50 (CML) / median LC50 (AML); NE – not evaluable; p-value (by Mann-Whitney U-test)

dation of members of the forkhead family of tumor suppressors *in vitro*, in an *in vivo* animal model, and in samples from patients with BCR-ABL-positive CML. They showed that inhibition of this pathway, using bortezomib, caused regression of CML disease. Bortezomib treatment led to inhibition of BCR-ABL-induced suppression of FoxO proteins and their proapoptotic targets, and tumor necrosis factor-related apoptosis-inducing ligand. Their study provided evidence that bortezomib induced apoptosis of CML cells *in vitro* and might be a candidate therapeutic in the treatment of BCR-ABL-induced leukemia.

Our study, based on the MTT assay, which is an endpoint type analysis, has shown that in comparison to AML cells, bortezomib alone has little *ex vivo* activity against CML cells. This was observed both for the whole group and for all subsets of patients tested in the study. Recently published results of a pilot study of bortezomib therapy for patients with imatinib-refractory chronic myeloid leukemia in chronic or accelerated phase, performed in the MD Anderson Cancer Center in Houston, have also shown only minimal efficacy, but considerable toxicity in patients with imatinib-refractory CML [14].

The introduction of BCR-ABL1 tyrosine kinase inhibitors during the last decade resulted in long-term disease control in the majority of patients with CML. In those who fail to respond and/or develop intolerance to these agents, still transplantation remains the only effective therapeutic solution [17]. Possibly, combined use of a tyrosine kinase inhibitor and proteasome inhibitor might be helpful for optimizing treatment of refractory/resistant CML [18]. New possibilities can arise with new modalities, related to immunotherapy or other targeted therapy [19, 20]. Further studies should focus on alternative approaches in using proteasome inhibitors in the treatment of CML, such as in combination with TKIs or as a strategy to eradicate leukemic stem cells [18, 21].

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