Drug-resistance Profile in Multiple-relapsed Childhood Acute Lymphoblastic Leukemia

MONIKA POGORZALA*, MALGORZATA KUBICKA*, BEATA RAFINSKA, MARIUSZ WYSOCKI and JAN STYCZYNSKI

Department of Pediatric Hematology and Oncology, Collegium Medicum, Nicolaus Copernicus University Torun, Bydgoszcz, Poland

Abstract. *Aim: To analyze the drug-resistance profile at first and subsequent relapse in children with acute lymphoblastic leukemia (ALL). Patients and Methods: A total of 154 pediatric ALL samples were tested for ex vivo chemosensitivity for up to 19 drugs. Their combined drug resistance profile (PVA score) was analyzed. Results: The median relative resistance scores between patients with multiple relapse and those with first relapse considering all drugs was 2.0. The median PVA score at subsequent relapses was 8 vs. 6 at first relapse (p=0.004). Samples from multiple-relapsed ALL were more drug resistant to: prednisolone (>1.9-fold), dexamethasone (>1.5-fold), vincristine (3.1-fold), L-asparaginase (5-fold), mitoxantrone (2.4-fold), cytarabine (4.3-fold), mercaptopurine (2.2-fold), thioguanine (4.8-fold), etoposide (2.6-fold) and melphalan (2.7-fold). Lymphoblasts at multiple relapse were comparably resistant to: daunorubicin, doxorubicin, cyclophosphamide, ifosfamide, busulfan, treosulfan, fludarabine, clofarabine and bortezomib. Conclusion: In comparison to first relapse, subsequent relapsed childhood ALL is more ex vivo-resistant to most tested drugs.*

Children with acute lymphoblastic leukemia (ALL) currently have more than 80% of chances for long-term survival (1). However, despite continuous progress in diagnostics and therapy, relapses still occur frequently, and relapsed ALL can be regarded as the fourth most frequent childhood cancer (2). The results of therapy in relapse of pediatric ALL are poor for the majority of patients, thus most of them require high-dose therapy and hematopoietic stem cell transplantation (HSCT) (3). On failure of HSCT and subsequent relapse, the outcome is usually unfavorable due to previous cytotoxic treatment, frequent organ dysfunction, pharmacokinetic resistance, cellular drug resistance and residual disease.

Cellular drug resistance can be defined as cellular insensitivity to drugs reaching the cell. Leukemic cells from children with relapsed ALL have higher *ex vivo* resistance to most drugs when compared to cells of patients with *de novo* ALL, particularly to glucocorticoids, vincristine and L-asparaginase (4, 5). The knowledge on cellular drug resistance in relapse of ALL in children is still unsatisfactory, and no data are available on cellular drug resistance in subsequent relapses of ALL. The objective of the present study was to compare the *ex vivo* drug-resistance profile at first and subsequent relapses in children with ALL.

Patients and Methods

Study design. A total of 154 leukemia samples were included in the study of *ex vivo* drug resistance, including 113 samples obtained from children at first relapse of leukemia, and 41 obtained at subsequent leukemic relapse. Detailed patient characteristics with respect to the phase of the disease are presented in Table I. The distribution of patients in both groups was comparable. The study was approved by the Local Bioethical Committee (Nr 135/2003; 384/2006; 550/2010).

Chemosensitivity testing. Ex vivo drug resistance was tested by 3-4,5-dimethylthiazol-2-yl-2,5-difenyl tetrazolium bromide (MTT) assay, as described previously (5). The drug concentration that was inhibitory to 50% of the cells (IC_{50}) was calculated from the dose–response curve and was used as a measure of *ex vivo* drug resistance for each sample. The relative resistance (RR) between groups for each drug was calculated as the ratio of the mean IC_{50} of the respective groups for this drug. Only patients who had a successful MTT assay at diagnosis were included in the study.

Drugs. The following 19 drugs and concentrations were used: prednisolone (Fenicort; Jelfa, Jelenia Gora, Poland), tested

*These Authors contributed equally to this study.

Correspondence to: Jan Styczynski, MD, Ph.D., Department of Pediatric Hematology and Oncology, Collegium Medicum, Nicolaus Copernicus University, ul. Curie-Sklodowskiej 9, 85-094 Bydgoszcz, Poland. Tel: +48 525854860, Fax: +48 525854867, e-mail: jstyczynski@cm.umk.pl

Key Words: Ex vivo drug resistance, individualized tumor response testing, acute lymphoblastic leukemia, children, relapse, minimal residual disease.

concentration range 0.007-250 μg/ml; dexamethasone (Dexamethasone; Jelfa), 0.0002-6 μg/ml; vincristine (Vincristine; Eli-Lilly, Indianapolis, IN, USA), 0.019-20 μg/ml; L-asparaginase (Medac; Medac, Hamburg, Germany), 0.0032-10 IU/ml; daunorubicin (Daunorubicin; Rhone-Poulenc-Rhorer, Paris, France), 0.0019-2 μg/ml; doxorubicin (Doxorubicin; Farmitalia, Milan, Italy), 0.0078-8 μg/ml; mitoxantrone (Mitoxantrone; Jelfa), 0.001-1 μg/ml; cytarabine (Cytosar; Pharmacia & Upjohn, Kalamazoo, MI, USA), 0.0097-10 μg/ml; 6-mercaptopurine (Sigma, St. Louis, MO, USA), 15.6-500 μg/ml; 6-Thioguanine, 1.56-50 μg/ml (Sigma); 4-HOO-cyclophosphamide (Asta Medica, Hamburg, Germany), 0.096-100 μg/ml; 4-HOO-ifosfamide (Asta Medica), 0.096-100 μg/ml; etoposide (Vepeside; Bristol–Myers Squibb, Princeton, NJ, USA), 0.048-50 μg/ml; busulfan (Busilvex; Pierre-Fabre Medicament, Boulogne, France), 1.17-1200 μg/ml; treosulfan (Ovastat; Medac, Hamburg, Germany), 0.0005-1 μg/ml; melphalan (Alkeran; Glaxo, Parma, Italy), 0.038-40 μg/ml; fludarabine (Fludara; Schering, Berlin, Germany), 0.019-20 μg/ml; clofarabine (Evoltra; Bioenvision, Edinburgh, UK), 0.0122-12.5 μM; and bortezomib (Velcade; Janssen Pharmaceutica N.V., Beerse, Belgium), 19-2000 nM.

Combined ex vivo drug-resistance profile. Results obtained for ALL samples were also calculated according to the percentile values of cytotoxicity for *ex vivo* concentrations for each drug. Based on previous experience, we determined the combined *ex vivo* resistance to prednisolone, vincristine and L-asparaginase (PVA) score (6-9). Reference values for PVA were determined based on the division of IC_{50} values into three equal groups; the cut-off values were designated by the 33rd and 67th percentiles. All patients for whom successful MTT assay was performed were re-assessed according to the PVA score based on the results obtained in a group of children with *de novo* ALL. Patients whose samples underwent spontaneous apoptosis were not analyzed for PVA score.

Statistical methods. Observed differences in proportions were tested for statistical significance using the appropriate chi-square statistic. For small sample sizes, the Fisher exact test was used. Differences in the distribution of the IC_{50} values between the two groups were analyzed using the Mann–Whitney *U*-test. Using the two-tailed test, a value of $p<0.05$ was considered statistically significant.

Results

In comparison to first relapse, subsequent relapsed childhood ALL was more resistant to most tested drugs (Table II). The median PVA score in patients with multiple relapse was 8 *vs*. 6 for patients at first relapse ($p=0.004$). The median RR considering all 19 tested drugs was 2.0, indicating higher drug-resistance on subsequent relapse. Samples from patients with multiple relapses of ALL were more drug resistant to: prednisolone (>1.9-fold), dexamethasone (>1.5-fold), vincristine (3.1-fold), L-asparaginase (5-fold), mitoxantrone (2.4-fold), cytarabine (4.3-fold), mercaptopurine (2.2-fold), thioguanine (4.8-fold), etoposide (2.6-fold) and melphalan (2.7-fold). On the other hand, lymphoblasts at subsequent relapse were comparably resistant to: daunorubicin, doxorubicin, 4-HOO-cyclophosphamide, 4-HOO-ifosfamide, busulfan, treosulfan, fludarabine, clofarabine and bortezomib. No drug showed a trend towards better cellular cytotoxicity at subsequent relapse.

Table I. *Patients' characteristics*.

	First ALL relapse	Subsequent ALL relapse
Number of patients	113	41
Gender (male/female)	14/8	6/4
Median age, years (range)	9.8 (0.4-18)	9.1 (0.5-18)
Immunophenotype		
Pre-pre-B	17	10
Pre-B/common	73	24
T	13	7
Median WBC count (range), 10^9/l	10.0 (0.5-430.0)	7.6 (1.4-208.1)

ALL: Acute lymphoblastic leukemia; WBC: white blood cell count.

Discussion

Relapse remains a significant problem for patients with ALL. Despite significant progress, the introduction of molecularly-targeted therapies and innovative immunotherapeutic approaches that include specialized monoclonal antibodies and cellular therapies that hold promise of enhanced leukemia-cell killing with non-overlapping toxicities, the outcome in relapsed pediatric ALL is not satisfactory. Improved biological understanding of mechanisms of relapse and drug resistance, the identification of actionable molecular targets by studying leukemia cell and host genetics, precise risk stratification with minimum residual disease measurement, and the development of new therapeutic drugs and approaches are needed to improve outcomes of patients with relapse (10).

Without HSCT, relapsed ALL has a dismal prognosis both in children and adults, mainly related to the time interval between initial diagnosis and relapse, and cellular drug resistance can play a key role in therapy failure. Since HSCT is inevitably burdened with an unacceptable risk of toxicity, conventional and innovative chemo- and immunotherapies are important therapeutic options. Most children worldwide with relapse of ALL are still treated with conventional chemotherapy (10).

It is commonly assumed that patients with relapse are more drug resistant than those diagnosed *de novo*. We previously showed that patients with relapse were resistant to busulfan, which is a key compound used in conditioning of patients with acute leukemia before HSCT. On the other hand, no significant differences were found between *de novo* and relapsed cases for cyclophosphamide and treosulfan (5). In currently used clinical therapeutic regimens, based on reduced-intensity conditioning, these drugs play an important role in the HSCT setting.

Table II. *Comparison of the ex vivo drug-resistance profile (median and range of 50% inhibitory concentrations) between first and subsequent relapse of acute lymphoblastic leukemia.*

Drug	First relapse			Subsequent relapse			RR	*p*-Value
	N	Median	Range	N	Median	Range		
PVA	108	6	3-9	39	8	5-9	1.3	0.004
Prednisolone (μg/ml)	109	134.46	0.32->250	39	>250.00	26.18->250	1.9	<0.001
Dexamethasone (μg/ml)	51	3.92	0.01->6	16	>6.00	0.89->6	1.5	0.038
Vincristine (μg/ml)	108	2.18	0.06->20	39	6.78	0.10->20	3.1	0.016
L-Asparaginase (IU/ml)	108	1.73	0.03->10	39	8.66	0.10->10	5.0	<0.001
Daunorubicin (μg/ml)	98	0.81	0.02->2	36	1.40	0.12->2	1.7	0.091
Doxorubicin (μg/ml)	64	1.50	0.08->8	28	2.28	0.09->8	1.5	0.164
Mitoxantrone (μg/ml)	61	0.12	0.01->1	27	0.29	0.02->1	2.4	0.008
Cytarabine (μg/ml)	71	1.42	0.04->10	29	6.15	0.44->10	4.3	0.003
6-Mercaptopurine (μg/ml)	42	86.54	15.63->500	19	189.29	32.24->500	2.2	0.047
6-Thioguanine (μg/ml)	64	6.25	1.56->50	25	29.92	2.21->50	4.8	0.003
4-HOO-Cyclophosphamide (μg/ml)	73	1.69	0.10->100	19	3.39	0.14->100	2.0	0.064
4-HOO-Ifosfamide (μg/ml)	44	13.65	1.03->100	13	28.94	4.69->100	2.1	0.180
Etoposide (μg/ml)	92	2.63	0.05->50	34	6.82	0.08->50	2.6	0.010
Busulfan (μg/ml)	42	26.10	3.13->1200	18	36.98	1.17->1200	1.4	0.102
Treosulfan (μg/ml)	51	0.37	0.02->1	22	0.54	0.01->1	1.5	0.357
Melphalan (μg/ml)	46	1.89	0.06->40	22	5.09	0.35->40	2.7	0.043
Fludarabine (μg/ml)	63	0.61	0.09->20	27	0.98	0.11->20	1.6	0.256
Clofarabine (μg/ml)	22	0.11	0.01->12	13	0.12	0.01->12	1.1	0.845
Bortezomib (nM)	25	269.04	1.95->2000	12	295.23	33.24->2000	1.1	0.922

N: Number of patients; RR: relative resistance=median IC_{50} (subsequent relapse)/median IC_{50} (first relapse).

The role of the drug-resistance profile in pediatric newly-diagnosed ALL is relatively well known. Since it is correlated with outcome, the PVA score was incorporated into the therapeutic program CoALL-06-97, showing the possibility of reducing treatment intensity for patients with a sensitive *ex vivo* profile without jeopardizing treatment outcome. However, it only moderately correlates with assessment of minimal residual disease in B-precursor ALL (8). We demonstrated significantly higher drug resistance at first relapse of pediatric ALL to prednisolone, vincristine, L-asparaginase and daunorubicin, and the trend towards higher resistance for most other tested drugs (5). Recently, new compounds were shown to have good anti-leukemic activity in childhood ALL, such as clofarabine and bortezomib (5, 11). Both compounds also showed relatively good activity in multiple relapses in the present study. In contrast, patients with multiple relapses showed increased resistance to glucocorticoids, vincristine and L-asparaginase, the key compounds in therapy of *de novo* ALL. Glucocorticoid resistance may be a fundamental biological aspect that explains the difference in prognosis. It seems that in vitro resistance to prednisolone might be a continuous variable in patients with ALL, with respect to progression of the disease. This may be due to induction of various defense mechanisms, such as an activation of variety of resistance mechanisms, which develop throughout life and protect humans from xenobiotics (12).

It is important to note that chemosensitivity of leukemia cells at multiple relapses did not significantly decrease for most of the drugs which are often used in high-dose therapy before HSCT. Similar findings in the drug-resistance profile were also observed in relapsed pediatric acute myeloid leukemia (13). Even though this finding did not apply to etoposide and melfalan in relapsed ALL, advances in preparative regimens, donor selection, and supportive care should improve the success of HSCT for patients with relapse.

In conclusion, the IC_{50}s for the *ex vivo* drug-resistance profile in multiple relapsed childhood ALL are higher in comparison to those at first relapse, which also indicates the higher drug resistance than at initial diagnosis. It seems that poor outcome in subsequent relapses in pediatric ALL can partially be explained by cellular drug resistance.

References

1 Pui CH and Evans WE: A 50-year journey to cure childhood acute lymphoblastic leukemia. Semin Hematol *50*: 185-196, 2013.

2 Gaynon PS, Qu RP, Chappell RJ, Willoughby ML, Tubergen DG, Steinherz PG and Trigg ME: Survival after relapse in childhood acute lymphoblastic leukemia: Impact of site and time to first relapse–the children's cancer group experience. Cancer *82*: 1387-1395, 1998.

3 Peters C, Schrappe M, von Stackelberg A, Schrauder A, Bader P, Ebell W, Lang P, Sykora KW, Schrum J, Kremens B, Ehlert K, Albert MH, Meisel R, Matthes-Martin S, Gungor T, Holter W, Strahm B, Gruhn B, Schulz A, Woessmann W, Poetschger U, Zimmermann M and Klingebiel T: Stem-cell transplantation in children with acute lymphoblastic leukemia: A prospective international multicenter trial comparing sibling donors with matched unrelated donors - The ALL-SCT-BFM-2003 trial. J Clin Oncol *33*: 1265-1274, 2015.

4 Pieters R, Klumper E, Kaspers GJ and Veerman AJ: Everything you always wanted to know about cellular drug resistance in childhood acute lymphoblastic leukemia. Crit Rev Oncol Hematol *25*: 11-26, 1997.

5 Styczynski J, Gil L, Derwich K, Wachowiak J, Balwierz W, Badowska W, Krawczuk-Rybak M, Matysiak M, Wieczorek M, Balcerska A, Sonta-Jakimczyk D, Stefaniak J, Kowalczyk J, Urasinski T, Sobol G, Komarnicki M and Wysocki M: Comparison of clofarabine activity in childhood and adult acute leukemia: Individual tumor response study. Anticancer Res *29*: 1643-1650, 2009.

6 Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Korholz D, Graubner U, Haas RJ, Jorch N, Spaar HJ, Kaspers GJ, Kamps WA, Van der Does-Van den Berg A, Van Wering ER, Veerman AJ and Janka-Schaub GE: Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol *21*: 3262-3268, 2003.

7 Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJ, Kazemier KM, Pei D, Cheng C, Pui CH, Relling MV, Janka-Schaub GE, Pieters R and Evans WE: Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med *351*: 533-542, 2004.

8 Escherich G, Troger A, Gobel U, Graubner U, Pekrun A, Jorch N, Kaspers G, Zimmermann M, zur Stadt U, Kazemier K, Pieters R, Den Boer ML, Horstmann M and Janka GE: The long-term impact of in vitro drug sensitivity on risk stratification and treatment outcome in acute lymphoblastic leukemia of childhood (CoALL 06-97). Haematologica *96*: 854-862, 2011.

9 Piatkowska M, Styczynski J, Kolodziej B, Kurylo-Rafinska B, Kubicka M, Pogorzala M, Czyzewski K, Debski R, Matysiak M, Malinowska I, Balwierz W, Juraszewska E, Wachowiak J, Konatkowska B, Wieczorek M, Olejnik I, Krawczuk-Rybak M, Kuzmicz M, Kowalczyk J, Stefaniak MJ, Badowska W, Szczepanski T, Tomaszewska R, Adamkiewicz-Drozynska E, Maciejka-Kapuscinska L, Sobol G, Mizia-Malarz A and Wysocki M: Individualized tumor response testing profile has a prognostic value in childhood acute leukemias: Multicenter non-interventional long-term follow-up study. Leuk Lymphoma *54*: 1256-1262, 2013.

10 Bhojwani D and Pui CH: Relapsed childhood acute lymphoblastic leukaemia. Lancet Oncol *14*: e205-217, 2013.

11 Szczepanek J, Pogorzala M, Konatkowska B, Juraszewska E, Badowska W, Olejnik I, Kuzmicz M, Stanczak E, Malinowska I, Stefaniak J, Sobol G, Szczepanski T, Czyzewski K, Wysocki M and Styczynski J: Differential *ex vivo* activity of bortezomib in newly diagnosed paediatric acute lymphoblastic and myeloblastic leukaemia. Anticancer Res *30*: 2119-2124, 2010.

12 Styczynski J and Wysocki M: *In vitro* drug resistance profiles of adult acute lymphoblastic leukemia: Possible explanation for difference in outcome to similar therapeutic regimens. Leuk Lymphoma *43*: 301-307, 2002.

13 Styczynski J, Piatkowska M, Czyzewski K, Pogorzala M and Wysocki M: Individual tumor response testing in multiple relapsed acute myeloid leukemia in children. Anticancer Res *33*: 1189-1193, 2013.

Received May 31, 2015
Revised July 4, 2015
Accepted July 7, 2015