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IN VITRO DRUG RESISTANCE IN CHILDHOOD MATURE B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

OPORNOŚĆ *IN VITRO* NA CYTOSTATYKI W OSTREJ BIAŁACZCE LIMFOBLASTYCZNEJ B-KOMÓRKOWEJ U DZIECI

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Summary

Background. Mature B-cell acute lymphoblastic leukemia (B-ALL) is a rare type of leukemia in children and counts for 1-2% of all leukemia cases. Pediatric B-ALL is treated according to protocols for non-Hodgkin lymphomas.

Objective. The aim of the study was to analyze the *in vitro* drug resistance in children with B-ALL compared to ALL with other phenotypes.

Material and methods. A total number of 15 children with B-ALL (6 girls and 9 boys, median age 8 years, range 1.9-15 years) were included into the analysis. The *in vitro* drug resistance tests on leukemic cells were performed by means of the MTT assay. The results of B-ALL patients were compared to those obtained in patients with pre-B/common ALL (479 cases), pro-B-ALL (31cases) and T-ALL (87 cases). Results: B-ALL cells were more resistant than pre-B/common-ALL cells to dexamethasone and idarubicin. In comparison to pro-B-ALL phenotype, B-ALL blasts were more resistant to idarubicin and more sensitive to treosulfan. No significant differences were found in the *in vitro* drug resistance between B-ALL and T-ALL. Blasts of T-ALL were more resistant than pre-B/common-ALL cells to most of tested drugs. Conclusion: From the clinical point of view, B-ALL cells have similar *in vitro* drug sensitivity when compared to T-ALL, and higher drug resistance to dexamethasone than pre-B/common-ALL.

Streszczenie

Pomimo znacznej liczby publikacji poświęconych oporności in vitro na cytostatyki u dzieci z ostrymi białaczkami, brakuje jakichkolwiek danych o komórkach ostrej białaczki limfoblastycznej z dojrzałych limfocytów B. Jest to najrzadsza postać ostrej białaczki limfoblastycznej u dzieci i obejmuje ok. 1-2% wszystkich rozpoznań. Białaczkę tę jest leczy się według odrębnych protokołów, wspólnych dla B-ALL i B-NHL.

Celem pracy była analiza oporności in vitro na cytostatyki w B-ALL w porównaniu z komórkami ALL o innych fenotypach: pro-B ALL, pre-B/common ALL i T-ALL. Do badania włączono 15 pacjentów (6 dziewczynek i 9 chłopców, mediana wieku 8 lat, zakres od 1,9 do 15 lat) z ALL z dojrzałych limfocytów B. Wyniki oporności in vitro porównywano z komórkami chorych z pre-B/common ALL (479 chorych), pro-B ALL (31 chorych) i T-ALL (87 pacjentów). Badania prowadzono w warunkach *in vitro* z wykorzystaniem komórek białaczkowych pobranych i wyizolowanych od pacjenta ze szpiku kostnego i/lub krwi obwodowej w momencie rozpoznania białaczki. Badania wrażliwości i oporności na leki przeprowadzono za pomocą testu MTT. Blasty B-ALL były bardziej oporne niż blasty pre-B/common ALL na deksametazon i idarubicynę. Komórki B-ALL w porównaniu z blastami pro-B ALL bardziej oporne na idarubicynę i bardziej wrażliwe na treosulfan. Pomiędzy komórkami B-ALL i T-ALL nie stwierdzono różnic w zakresie oporności na badane leki. Dzięki przeprowadzonym badaniom poszerza się wiedza o dziecięcej B-ALL. Być może w protokołach terapeutycznych znajdą zastosowanie leki, dla których wykazano największą wrażliwość *in vitro* komórek B-ALL, na przykład treosulfan.

Key words: B-ALL, acute B-cell leukemia, *in vitro* drug resistance, children
Słowa kluczowe: **:** B-ALL, ostra białaczka B-komórkowa, oporność *in vitro* na cytostatyki, dzieci

INTRODUCTION

Acute leukemias are malignancies characterized by clonal growth of immature cells of hematopoietic origin not subject to regulatory processes and therefore resulting in impairment of regular bone marrow activity and expulsion of normal cells [1]. Acute leukemias are divided into two main types: lymphoblastic and non-lymphoblastic (myeloblastic). Each type includes several subtypes according to morphologic, cytochemical, immunological, genetic and molecular features of leukemic clone [2]. The division of acute leukemias is based on ontogenetical development of hematopoietic system. Leukemias are classified according to the dominant cell line [2,3]. Immunological classification of acute lymphoblastic leukemias (ALL) is presented in Table 1.

Table 1. *Immunological classification of acute lymphoblastic leukemias [2]*

Subtype	Tdt	HLA-DR	Cytoplasmatic Ig	Cell membrane Ig	Cellular differentiation antigens
Null	+	+/-	-	-	CD34
Pre-pre-B	+	+	-	-	CD34, cyCD79a, CD19, CD22
Common	+	+	-	-	cyCD79a, CD19, CD22, CD10
Pre-B	+	+	+	-	cyCD79a, CD19, CD22, CD10
B-cell	-	-	-	+	cyCD79a, CD19, CD22 (CD10), CD37, CD20
Pro/pre-T	+	+/-	-	-	CD7, cyCD3, (CD2, CD5)
Tymocyte-T	+	-	-	-	CD7, cyCD3, CD2, CD5, CD1, (TCR-CD3, CD10, CD4/8)
T-cell	+	-	-	-	CD7, CYCD3, CD2, CD5, TCR-CD3, (CD4/CD8)

cy – cytoplasmatic, Ig – immunoglobulines, TdT – terminal deoxynucleotidyl transferase

ALL is treated by chemotherapy and radiotherapy. One of the most important factors limiting the progress of efficacy of chemotherapy is development of drug resistant malignant cells. Problem of drug resistance appeared with the introduction of the first cytostatic drug – aminopterin [4].

Despite many publications dedicated to *in vitro* drug resistance in childhood acute leukemias, resistance of leukemic cells in B-cell acute lymphoblastic leukemia (B-ALL) still remains to be explored. B-ALL is a rare type of leukemia in children and counts for 1-2% of all leukemia cases. Pediatric B-ALL is being treated according to protocols for B-cell non-Hodgkin lymphomas. The similar approach to these two malignancies results from the presence of identical type of malignant cells. No study are available which determine *in vitro* drug resistance in mature B-cell ALL.

The objective of the study was to analyze the *in vitro* drug resistance in mature childhood B-ALL, in comparison to the *in vitro* drug resistance in other phenotypes of pediatric ALL cells.

MATERIAL AND METHODS

A total number of 15 patients with diagnosis of B-ALL (6 girls and 9 boys, median age 8 years, range 1.9-15 years) were included into the study. In each case diagnosis was made with the use of flow cytometry, by expression of surface immunoglobulines, kappa or lambda chains, HLA-DR, CD19, CD20, or cytoplasmatic expression of CD22 . The results of *in vitro* drug resistance were compared to the results of *in vitro* drug resistance obtained from patients with pre-B/common ALL (479 patients), pro-B ALL (31patients) i T-ALL (87 patients).

The study was performed in the *in vitro* environment on leukemia cells isolated from bone marrow and/or peripheral blood at the time of diagnosis. Analysis of the *in vitro* drug resistance was performed by means of the MTT assay [5]. Bone marrow aspirates were collected into tubes containing heparin in concentration 15-20 U/ml of bone marrow. Leukemia cells were isolated from bone marrow aspirates by density gradient (Gradisol L, Aqua Medica, Łódź) and then washed twice with RPMI-1640 (Sigma, St Louis, USA). The cells were then subjected to red cell lysis using ammonium chloride lysis buffer. Isolated leukemia cells were suspended in culture medium in concentration $2.0x10^6$ cells/ml. Drugs used in the tests were the number of cytotoxic drugs including those used in treatment of *de novo* and relapsed acute lymphoblastic and nonlymphoblastic leukemias. In this study, the term „cytotoxic drugs" also refers to prednisolone and dexamethasone used in treatment of acute lymphoblastic leukemia. Active forms of cyclophosphamide and ifosfamide were used, i.e. 4-hydroperoxy-cyclophosphamide and 4-hydro-peroxy-ifosfamide. The list of cytotoxic drugs used in the study along with their concentrations is presented in Table 2. The study was performed with the agreement of the Local Bioethical Committee.

Table 2. *Drugs and their concentrations used in the testing of in vitro drug resistance profile*

Drug	Concentrations
Prednisolone (Jelfa, Jelenia Góra)	0.076-250 µg/ml
Dexamethasone (Jelfa, Jelenia Góra)	0.0002-6 µg/ml
Idarubicin (Farmitalia, Milan)	0.002-2 µg/ml
Daunorubicin (Rhone-Poulenc-Rhorer)	0.002-2 µg/ml
Doxorubicin (Farmitalia, Milan)	0.045-8 µg/ml
Mitoxantrone (Jelfa, Jelenia Góra)	0.01-1 µg/ml
Vincristine (Eli-Lilly, Indianapolis)	0.0195-20 µg/ml
Etoposide (Vepesid, Bristol-Myers Squibb, Princeton)	0.048-50 µg/ml
L-asparaginase (Medac, Hamburg)	0.0032-10 IU/ml
Cytarabine (Cytosar, Pharmacia & Upjohn)	0.0098-10 µg/ml
Fludarabine (Fludara, Schering AG, Berlin)	0.019-20 µg/ml
Cladribine (Biodribin, Bioton, Warszawa)	0.001-40 µg/ml
6-Thioguanine (Sigma, nr A4882)	1.5625-50 µg/ml
Melphalan (Alkeran, Glaxo Wellcome, Parma)	0.039-40 µg/ml
Cyclophosphamide (4-HOO-Cyclophosphamide, Asta Medica, Hamburg)	0.097-100 µg/ml
Ifosfamide (4-HOO-Ifosfamide, Asta Medica, Hamburg)	0.0977-100 µg/ml
Treosulfan (Ovastat, Medac, Hamburg)	0.00001-1.0 µg/ml
Thiotepa (Lederle, Riemser, Greifswald)	0.032-100 µg/ml

For the MTT assay the following validity criteria were used: (a) samples only with at least of 90% of blasts were tested and (b) samples only with at least 70% of blasts at the end of incubation were included in further analysis. Optical density testing in control sample had to exceed value 0.050.

Statistical analysis: *In vitro* drug resistance in analyzed groups was presented as median and range of the LC50 index value for each drug. In order to compare relative *in vitro* drug resistance between two analyzed groups, parameter of relative resistance (RR) was used, as a ratio of LC50 median value for tested drug in one group to LC50 median value for this drug in the other group. The value RR<1 indicates better sensitivity to the tested drug and RR>1 indicates higher drug resistance. The Mann-Whitney U test was performed to compare differences between groups. In all analyses p-values <0.05 were regarded significant and p-value was determined based on two-sided test. Statistical analysis was performed with SPPS21 software.

RESULTS

Significant differences in the *in vitro* drug resistance in B-ALL compared to other ALL phenotypes have been determined (Table 3). The B-ALL blasts were more resistant than pre-B/common-ALL cells to dexamethasone (RR=19.3, p=0.038) and idarubicin (RR=2.2, p=0.025). No significant differences were found in the *in vitro* drug resistance between B-ALL and T-ALL cells. B-ALL cells, as compared to pro-B-ALL cells were more resistant to idarubicin (RR=2.5, p=0.037) and more sensitive to treosulfan (RR=0.15, p=0.05). Compared to pre-B/common-ALL cells, blasts of pro-B-ALL were significantly more *in vitro* resistant to treosulfan (RR=10, p=0.011) and cyclophosphamide (RR=1.9, p=0.047). The test showed significant differences in *in vitro* drug resistance between T-ALL and pro-B-ALL cells for following drugs: (RR= 1.6, p=0.025), etoposide (RR=3.4, p=0.037), fludarabine (RR=7, p=0.004) and cladribine (RR= 43, p=0.003) and borderline significance to vincristine (RR=1.99, p=0.051). Blasts of T-ALL were more resistant than pre-B/common-ALL cells to most of tested drugs. Statistical significance was shown for prednisolone (RR=5.2, p=0.002), dexamethasone (RR=12.8, p=0.036), daunorubicin (RR=1.2, p=0.018), epirubicin (RR=2.3, p=0.009), vincristine (RR=2.4, p<0.001), etoposide (RR=1.7, p=0.001), L-asparaginase (RR=2.7, p<0.001), cytarabine (RR=1.8, p=0.028), fludarabine (RR=5.7, p<0.001), cladribine (RR=17.2, p=0.003), nelarabine (RR=5.2, p=0.036), clofarabine (RR=13, p=0.009) and thiotepa (RR=2.1, p=0.006).

Table 3. *Comparison of values of in vitro drug resistance to tested drugs in different ALL phenotypes*

Drug	Median and quartiles of LC50			
	B-ALL (n=15)	pre-B/common ALL (n=479)	pro-B ALL (n=31)	T-ALL (n=87)
Prednisolone	90.98 (n=10) 45.10-160.92	12.26 (n=353) 0.69-109.91	15.63 (n=27) 2.96-111.90	64.62 (n=75) 3.40-138.01
Dexamethasone	6.0 (n=7) 6.0-6.0	0.31 (n=170) 0.02-6.0	3.17 (n=10) 0.20-6.0	3.98 (n=29) 0.11-6.0
Idarubicin	0.35 (n=9) 0.23-2.0	0.16 (n=274) 0.06-0.43	0.14 (n=23) 0.02-0.37	0.23 (n=59) 0.07-0.50
Daunorubicin	0.34 (n=10) 0.22-1.87	0.24 (n=351) 0.09-0.46	0.19 (n=27) 0.07-0.37	0.30 (n=75) 0.15-1.10
Doxorubicin	1.25 (n=5) 0.90-8.0	1.22 (n=230) 0.58-8.0	1.33 (n=19) 0.54-8.0	1.29 (n=60) 0.63-8.0
Mitoxantrone	0.37 (n=5) 0.12-0.76	0.08 (n=224) 0.03-0.36	0.03 (n=19) 0.02-0.31	0.13 (n=51) 0.03-0.45
Vincristine	5.15 (n=10) 1.50-13.06	1.19 (n=356) 0.26-4.28	1.46 (n=27) 0.14-3.73	2.91 (n=76) 0.80-7.29
Etoposide	13.75 (n=10) 0.88-25.44	1.67 (n=337) 0.62-9.40	0.84 (n=25) 0.59-11.57	2.90 (n=70) 1.09-50.0
L-asparaginase	1.17 (n=11) 0.38-10.0	0.33 (n=351) 0.06-1.47	0.66 (n=25) 0.13-1.14	0.90 (n=76) 0.23-10.0
Cytarabine	0.73 (n=10) 0.25-10.0	0.66 (n=271) 0.29-1.82	0.47 (n=23) 0.21-2.50	1.20 (n=53) 0.40-6.03
Fludarabine	0.84 (n=5) 0.38-15.23	0.39 (n=225) 0.18-1.63	0.32 (n=18) 0.16-5.77	2.24 (n=54) 0.75-9.45
Cladribine	0.08 (n=8) 0.03-30.04	0.05 (n=217) 0.02-1.28	0.02 (n=18) 0.01-0.69	0.86 (n=46) 0.03-16.53
6-Thioguanine	8.46 (n=6) 2.18-29.75	5.72 (n=221) 3.08-15.46	7.47 (n=19) 5.20-50.0	5.39 (n=53) 2.70-18.36
Melphalan	8.99 (n=5) 2.37-25.63	3.02 (n=151) 1.10-8.20	2.27 (n=14) 0.83-15.60	5.78 (n=45) 1.16-17.97
Cyclophosphamide	0.39 (n=5) 0.31-0.92	0.60 (n=156) 0.26-1.23	1.15 (n=19) 0.39-19.84	0.78 (n=42) 0.41-2.15
Ifosfamide	6.07 (n=5) 1.31-58.19	4.91 (n=82) 1.55-16.59	9.38 (n=5) 2.98-61.50	5.82 (n=21) 3.04-16.45
Treosulfan	0.15 (n=5) 0.0002-0.66	0.10 (n=185) 0.01-1.0	1.00 (n=16) 0.23-1.0	1.00 (n=53) 0.03-1.0
Thiotepa	1.49 (n=5) 1.08-50.76	0.97 (n=185) 0.32-3.25	0.71 (n=17) 0.28-4.78	2.06 (n=52) 0.81-8.78

The values are presented in following units: IU/ml for L-asparaginase, µg/ml for the rest of the drugs. n – the number of patients

DISCUSSION

In this study we analyzed the *in vitro* drug resistance in children with B-ALL, rare type of leukemia in childhood and compared it to other ALL phenotypes: pro-B ALL, pre-B/common ALL and T-ALL. We found that blasts of mature B-ALL were more resistant to dexamethasone and idarubicin than pre-B/common ALL cells. We found also that B-ALL cells compared to pro-B ALL blasts were more resistant to idarubicin and more sensitive to treosulfan. No significant statistical differences were discovered in the *in vitro* drug resistance between B-ALL and T-ALL cells. Significant differences were shown in the resistance to treosulfan and cyclophosphamide between pro-B and pre-B/common ALL blasts. Significant differences were found between T-ALL and pro-B ALL in *in vitro* resistance to daunorubicin, etoposide, fludarabine, cladribine, while borderline statistical significance was determined in case of vincristine. Blasts of T-ALL were more resistant than pre-B/common ALL cells to most of tested drugs.

In the study of Pieters et al., it was shown that T-ALL blasts are more resistant than pre-B ALL cells to prednisolone, daunorubicin, L-asparaginase, cytarabine and 6-thioguanine [6]. In our previous analyses, it was shown that common-ALL cells were more sensitive than T-ALL cells to most tested drugs, probably except from 6-thioguanine and ifosfamide [7], and that pre-B/common ALL were more sensitive to L-asparaginase, vincristine, alkylating agents and prednisolone, when compared to T-ALL cells [8]. In another study, no significant differences were discovered in the *in vitro* drug resistance to daunorubicin, mitoxantrone and 6-thioguanine between T-ALL and pre-B/common ALL cells, but it was shown that T-ALL cells were more sensitive to bortezomib [9].

In our study, B-ALL blasts had similar *in vitro* drug resistance to tested drugs as T-ALL cells. T-ALL blasts were more resistant to most tested drugs than pre-B/common-ALL. This presumably has significant impact on results of the treatment since B-ALL, as well as T-ALL have worse prognosis when compared to pre-B/common-ALL in children.

Cellular drug resistance is one of the main causes of the frequent ultimate failure of chemotherapy in childhood acute leukemias [10], especially on relapse [11-12]. Current concepts suggest that drug resistance is related to the presence of leukemic stem cells [13-14].

From the clinical point of view, results of our study show that B-ALL is the subtype of leukemia characterized by *in vitro* drug resistance similar to T-ALL and more resistant to dexamethasone than pre-B/common-ALL. The other conclusion from our study is that B-ALL is relatively sensitive to treosulfan in the *in vitro* conditions; however, this drug is not used in therapy of acute leukemias.

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