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Krzysztof Czyżewski[1*], Katarzyna Skonieczka[2], Patryk Różycki[2], Beata Kołodziej[1], Beata Kuryło-Rafińska[1], Małgorzata Kubicka[1], Karolina Matiakowska[2], Barbara Mucha[2], Olga Haus[2,3], Mariusz Wysocki[1], Jan Styczyński[1]

BCR-ABL AMPLIFICATION PLAYS A MAJOR ROLE IN RESISTANCE TO TYROSINE KINASE INHIBITORS IN K-562 CELL LINE

AMPLIFIKACJA *BCR-ABL* MA ISTOTNE ZNACZENIE W OPORNOŚCI NA INHIBITORY KINAZY TYROZYNOWJ W LINII KOMÓRKOWEJ K-562

[1] Department of Pediatric Hematology and Oncology Nicolaus Copernicus University Collegium Medicum in Bydgoszcz
[2] Department of Clinical Genetics Nicolaus Copernicus University Collegium Medicum in Bydgoszcz
[3] Department of Hematology, Medical University Wroclaw

Summary

An emerging problem in patients with chronic myeloid leukemia (CML) is increasing resistance to tyrosine kinase inhibitors (TKIs). To determine genetic and cellular mechanisms involved in the development of resistance to TKIs, nine imatinib-resistant cell lines were derived from K-562 cell line followed by testing of drug sensitivity, multidrug resistance proteins and cytogenetic studies. In imatinib-resistant cell lines cross-resistance to daunorubicin, etoposide and cytarabine were observed whereas sensitivity to dasatinib, nilotinib, cyclophpsphamide, bortezomib and busulfan was preserved. Treatment with imatinib decreased PGP and LRP expression, however it did not significantly influence MRP1 expression. Amount of signals in FISH analysis from ABL, BCR and from fusion genes (BCR-ABL or ABL-BCR) was mostly higher in imatinib-resistant cell lines in comparison to parental K-562 cell line. We concluded that BCR-ABL amplification but not cellular sensitivity is the major mechanisms of resistance in K-562 cell line.

Streszczenie

Narastająca oporność na inhibitory kinazy tyrozynowej (TKIs) jest niepokojącym problemem u pacjentów z przewlekłą białaczką szpikową (CML). Aby określić genetyczne i komórkowe mechanizmy oporności na TKIs z linii K-562 wyhodowano 9 opornych na imatynib linii komórkowych, w których przeprowadzono badania: oporności na leki, ekspresji białek oporności komórkowej oraz badania cytogenetyczne. W opornych na imatinib liniach komórkowych stwierdzono krzyżową oporność na daunorubicynę, etopozyd i cytarabinę, podczas gdy wrażliwość na dasatinib, nilotinib, cyklofosfamid, bortezomib i busulfan była zachowana. Hodowla z imatinibem zmniejszała ekspresję białka PGP i LRP ale nie wypływała na ekspresję białka MRP1. W badaniu metodą FISH w liniach opornych na imatinib w porównaniu do macierzystej linii K-562 obserwowano większą ilość sygnałów pochodzących od genów ABL, BCR oraz od genów fuzyjnych (BCR-ABL i ABL-BCR). Przeprowadzone badania wskazują, że nie mechanizmy oporności komórkowej ale amplifikacja sekwencji BCR-ABL, jest głównym mechanizmem oporności na TKIs w linii K-562.

Key words: *BCR-ABL* amplification; chronic myeloid leukemia; drug resistance; imatinib; tyrosine kinase inhibitors; K-562 cell line
Słowa kluczowe: amplifikacja *BCR-ABL*, przewlekła białaczka szpikowa, oporność na cytostatyki, imatynib, inhibitory kinazy tyrozynowej, linia K-562

INTRODUCTION

A distinctive feature of chronic myeloid leukemia (CML) is the abnormal activity of p210(Bcr-Abl) kinase (1). Selective tyrosine kinase inhibitors, such as imatinib or nilotinib have been established successfully for the treatment of CML (1). Resistance to imatinib monotherapy frequently develops in advanced stages of chronic myelogenous leukemia, thus other tyrosine kinase inhibitors (TKIs) like dasatinib and nilotinib are recommended (2). Resistance to TKIs is an emerging problem in CML patients and can be divided into primary and secondary resistance (3-4). *BCR-ABL*-gene-dependent mechanisms of resistance to imatinib include mutations in the *BCR-ABL* sequences and duplication/amplification of *BCR-ABL* sequences (1, 3). *BCR-ABL*-gene-independent mechanisms of resistance to TKIs include pharmacokinetic, mechanisms related to import of imatinib by human organic cation transporter 1 (hOCT1) or export by the P-glycoprotein (3). The other *BCR-ABL*-gene-independent mechanisms are: activation of alternative signaling cascades leading to *BCR-ABL* independent growth or alterations in the epigenetic regulation of the expression of *BCR-ABL* sequence (3) as well as clonal evolution caused by additional cytogenetic abnormalities (4-5). Clonal evolution and mutations are recognize as the most important factors, and are related to each other (4) whereas the role of resistance proteins is still not established.

To determine if genetic and/or cellular mechanisms are involved in mechanism of resistance to imatinib, nine cell lines were derived from K-562 cell line and tested for drug sensitivity, multidrug resistance proteins expression and by cytogenetic studies.

MATERIAL AND METHODS

Cell lines. Original K-562 cell line from ATCC (ATCC No CCL-243) was used in this study. The cells were suspended in culture medium with imatinib in three different concentrations: 0.001 μM (small), 0.01 μM (medium) and 0.1 μM (high). After 10 days derived cell lines were established as imatinib-resistant cell lines and named K-562R-0.001, K-562R-0.01 and K-562R-0.1 respectively. Each cell line was further cultured in increasing imatinib concentration for a period of 50 days up to final concentration of imatinib 0.005 μM, 0.05 μM 0.5 μM (named K-562R-0.005, K-562R-0.05 and K-562R-0.5, respectively) or for period of 100 days up to final concentration of imatinib 0.01 μM, 0.1 μM and 1.0 μM of imatinib (named K-562R-0.01K, K-562R-0.1K, and K-562R-1.0K, respectively). All cell lines were maintained as previously described (6-7).

Drugs. Imatinib (Novartis Europharm, West Sussex, UK) was tested in concentrations range 0.000977 - 1 μM. Other drugs used in the study included dasatinib (Bristol Meyers Squibb, Uxbridge, UK, 0.000977 - 1 μM), nilotinib (Novartis Europharm, West Ussex, UK, 0.000977 - 1 μM), daunorubicin (Rhone-Poulenc Rhorer, Köln, Germany, 0.0019 - 2 μg/ml), etoposide (Bristol Meyers Squibb, Princeton, USA, 0.048 - 50 μg/ml) cytarabine (Pharmacia Limited, Sandwich, UK, 0.0097 - 10 μg/ml) 4-HOO-cyclophosphamide (Asta Medica AG, Frankfurt, Germany, 0.096 - 1 μg/ml), bortezomib (Janssen Pharmaceuticals, Toronto, Canada, 0.19 - 2000 nM), busulfan (Pierre Fabre Medicament, Boulogne, France, 1.17 - 1200 μg/ml).

The MTT viability assay. The MTT viability assay was used to asses cytotoxicity of tested drugs, as it was previously described (6-7). All experiments were performed in triplicate. The data were confirmed to be reproducible. The cytotoxicity was expressed as IC50 - inhibitory concentration for 50% of cells.

Multidrug resistance proteins. Multidrug resistance proteins: P-glycoprotein (PGP), Lung-Resistance-Protein (LRP) and Multidrug-Resistance Related Protein-1 (MRP1) expressions were measured for each sample by flow cytometry. Human anti-PGP (clone JSB-1, and isotype control of mice IgG1), anti-LRP (clone LRP 56, and isotype control of mice IgG2a) and anti-MRP1 (clone MRPr1, and isotype control of rat IgG2a) antibodies were used to determine the expression of respective proteins (all: Alexis Biochmicals, Lausanne, Switzerland). PGP, LRP and MRP1 proteins expressions were measured using mean fluorescence intensity (MFI) corrected by isotype control. The MFI value of tested proteins was expressed in flow cytometry arbitrary units [AU].

Rhodamine retention. Rhodamine (Rh123, Sigma-Aldrich, Dorset, UK) in concentration 200 ng/ml was used. Rh123 retention in the presence of cyclosporine (CsA, Novartis Pharma, Basel, Switzerland) at concentration of 2 μg/ml, was tested simultaneously. The incubation time was 30 minutes. (Rh123+CsA)/Rh123 retention ratio was calculated for each tested sample.

Table I. *IC50 values for tested drugs and rhodamine retention in K-562 and resistant derivative cell lines*
Tabela I. *Wartości IC50 testowanych leków oraz retencja rodaminy w linii K-562 i opornych liniach komórkowych*

	K-562	K-562R-0.001	K-562R-0.005	K-562R-0.01K	K-562R-0.01	K-562R-0.05	K-562R-0.1K	K-562R-0.1	K-562R-0.5	K-562R-1.0K
IC50 values Wartości IC50										
Imatinib [µM]	**0.13**±0.03	**0.43**±0.14*	**0.13**±0.02	**0.27**±0.02*	**0.30**±0.03*	**0.33**±0.12*	**0.30**±0.20	**0.22**±0.01*	**0.45**±0.13*	**0.87**±0.18*
Dasatinib [µM]	**<0.000977**	**<0.000977**	**<0.000977**	**<0.000977**	**<0.000977**	**0.004**±0.003	**<0.000977**	**<0.000977**	**0.03**±0.04	**>1.00****
Nilotinib [µM]	**0.066**±0.03	**0.14**±0.10	**0.05**±0.005	**0.07**±0.01	**0.06**±0.03	**0.11**±0.07	**0.08**±0.06	**0.06**±0.02	**0.19**±0.04	**>1.00****
Daunorubicin [µg/ml]	**0.10**±0.05	**0.54**±0.38#	**0.56**±0.18#	**0.25**±0.01#	**0.34**±0.02	**0.18**±0.02	**0.44**±0.24#	**0.26**±0.20#	**0.43**±0.38#	**0.25**±0.07#
Etoposide [µg/ml]	**0.19**±0.18	**35.42**±25.26*	**7.88**±5.11*	**6.08**±2.77*	**13.57**±13.36	**14.11**±11.35*	**2.12**±1.53*	**9.16**±9.02	**1.29**±0.87*	**9.50**±7.68*
Cytarabine [µg/ml]	**1.07**±0.92	**>10.00****	**6.88**±5.41	**>10.00****	**>10.00****	**6.87**±5.39	**>10.00****	**>10.00****	**6.81**±5.52	**9.27**±1.46**
Cyclophosphamide[µg/ml]	**1.34**±0.47	**2.06**±0.10#	**1.51**±0.47#	**0.56**±0.24#	**2.04**±0.36#	**3.93**±1.96	**1.60**±0.39	**2.29**±0.29#	**1.55**±0.60	**1.90**±0.20#
Bortezomib [nM]	**445.65**±217.78	**644.27**±349.27	**258.55**±9.06	**266.44**±5.05#	**665.31**±279.40	**514.28**±226.89	**174.30**±138.51	**696.64**±267.4	**221.67**±30.08	**258.89**±13.89*
Busulfan [µg/ml]	**25.20**±8.60	**28.62**±7.81	**14.03**±2.08@	**18.86**±9.09	**16.85**±6.84	**11.11**±1.44**	**29.61**±2.98	**13.07**±5.67@	**10.01**±4.17*	**19.98**±1.21
MFI										
PGP [AU]	**27.05**±1.20	**5.11**±1.59**	**3.46**±0.46**	**5.39**±0.74**	**4.67**±0.88**	**5.97**±0.33**	**8.66**±2.31**	**4.28**±3.59**	**4.93**±0.69**	**4.96**±0.40**
MRP1 [AU]	**12.59**±6.37	**4.88**±1.27#	**3.10**±0.06@	**6.41**±1.31	**3.83**±1.02#	**6.61**±1.05	**7.55**±0.94	**4.18**±0.87#	**5.46**±0.01#	**5.51**±0.01#
LRP [AU]	**20.30**±10.18	**5.10**±1.41	**3.10**±0.13#	**7.57**±0.55	**5.12**±2.18	**6.93**±2.07	**9.08**±0.34	**4.88**±1.75	**5.32**±0.01	**5.40**±1.16
Rhodamine retantion Retencja rodaminy										
Rh123 [AU]	37.9±7.5	48.1±17.4	37.6±6.3	40.1±8.9	38.5±4.3	58.3±32.5	52.7±3.5	34.3±1.5	31.2±0.6	44.5±8.8
Rh123+CsA [AU]	90.9±18.5	107.3±37.8	88.6±7.1	87.6±0.6	89.3±23.6	142.5±41.7	112.0±14.1	82.3±10.9	87.5±17.0	81.6±3.5
RR	2.4	2.2**	2.4	2.2**	2.3	2.5	2.1	2.4	2.8	1.8*

[AU] – arbitrary flow cytometry units, **MFI** – mean fluorescence index of multidrug resistance proteins, **Rh123** – rhodamine retention, **Rh123+CsA** – rhodamine retention in the presence of cyclosporin, **RR** – retention ratio = [(Rh123+CsA)/Rh123]. Statistical significance in comparison to K-562: * -p<0.01, @ - p<0.02, # - p<0.05, ** - p<0.001. Values are given as arithmetic mean ± SD.
[AU] – jednostki cytometryczne, **MFI** – średnia wartość fluorescencji białek oporności komórkowej, **Rh123** – retencja rodaminy, **Rh123+CsA** – retencja rodaminy w obecności cyklosporyny, **RR** – współczynnik retencji= [(Rh123+CsA)/Rh123]. Znamienność statystyczna w porównaniu do linii K-562: * -p<0.01, @ - p<0.02, # - p<0.05, ** - p<0.001. Wartości przedstawiono jako średnia arytmetyczna ± odchylenie standardowe.

Flow cytometry studies. Multidrug resistance proteins expressions and rhodamine retention were analyzed using Cytomics FC500 flow cytometer (Beckman Coulter, Miami, USA) with CXP Software. All experiments were performed in triplicates.

Cytogenetic studies. Fluorescence in situ hybridization (FISH). For each cell line quantity analysis of *BCR, ABL,* and fusion genes (*BCR-ABL, ABL-BCR)* was performed with use of specific D-FISH BCR/ABL, ABL/BCR FISH probe (Abbot, Vysis, USA). Specific probe hybridization and DNA staining with DAPI I (Abbott) were performed according to producer protocol. For each cell line 100 metaphases and/or interphasal nuclei were analyzed in light microscope (Nikkon Eclipse 80i) at 1000x magnification, with Applied Spectral Imaging Software (Migdal Haemek, Israel) use.

Statistical analysis. Differences in drug resistance between samples tested for cytotoxicity and differences in multidrug proteins expression were analyzed by Student's t-test. Correlations between MFI of multidrug resistance proteins were given with Pearson's correlation. All reported p-values were 2-sided; p<0.05 was considered statistically significant.

Table II. *Number of signals from normal ABL or BCR genes and BCR-ABL and ABL-BCR fusion genes in K-562 cell line and its imatinib-resistant derivative cell lines*
Tabela II. *Liczba sygnałów pochodząca od genów ABL, BCR oraz od genów fuzyjnych BCR-ABL i ABL-BCR w linii K-562 i w pochodnych, opornych na imatinib liniach komórkowych*

Cell line Linia komórkowa	Clone number/% of cells in cell line Numer klonu/% komórek w linii komórkowej	Number of copies of *ABL* gene Ilość kopii genu *ABL*	Number of copies of *BCR* gene Ilość kopii genu *BCR*	Median number of copies of fusion genes (range) Mediana ilości kopii genów fuzyjnych (zakres)
K-562	1/100%	3	2	17 (11-26)
K-562R-0.001	1/56%	3	2	21 (14-29)
	2/32%	2	2	19 (15-22)
	3/12%	2	1	15 (12-19)
K-562R-0.005	1/100%	3	2	16 (10-28)
K-562R-0.01K	1/70%	3	2	15 (10-20)
	2/30%	2	2	15 (11-27)
K-562R-0.01	1/100%	3	2	25 (19-34)
K-562R-0.05	1/100%	4	4	24 (12-39)
K-562R-0.1K	1/100%	3	2	18 (10-23)
K-562R-0.1	1/55%	2	2	27 (21-35)
	2/17%	4	3	18 (16-20)
	3/17%	3	2	24 (19-28)
	4/11%	3	3	20 (17-20)
K-562R-0.5	1/100%	2	2	23 (14-36)
K-562R-1.0K	1/100%	3	2	26 (18-36)

Results were obtained from the analysis of 100 methaphases/interphase nuclei
Wyniki przedstawiono na podstawie analizy 100 metafaz/jąder interfazalnych

RESULTS

The MTT viability assay. In all except 2 cell lines (K-562R-0.005 and K-562R-0.1K) resistance to imatinib was induced in comparison to K-562 cell line ($p<0.01$). Cross-resistance to daunorubicin, etoposide and cytarabine was also observed in all cell lines. Resistance to dasatinib and nilotinib was observed only in K-562R-1.0K cell line, while the rest of tested derivative imatinib-resistant cell lines were highly sensitive to dasatinib and nilotinib. Most of tested imatinib-resistant cell lines presented good sensitivity to cyclophosphamide. All tested cell lines, particularly K-562R-0.1K, K-562R-0.5 and K-562R-1.0K cell lines, were highly sensitive to bortezomib. Resistance to imatinib had no impact on sensitivity to busulfan, and IC50 values for this drug were comparable or lower than for K-562 cell line (Table I).

Multidrug resistance proteins. Long-term *in vitro* treatment with imatinib caused PGP and LRP decrease of expression in all tested derivative cell lines, however did not significantly influence MRP1 expression (Table I). All tested multidrug resistance proteins were highly correlated in tested cell lines (PGP vs. MRP1 $r=0.875$, $p<0.01$; PGP vs. LRP, $r=0.919$, $p<0.01$; MRP1 vs. LRP, $r=0.939$, $p<0.01$).

Rhodamine retention. In the most resistant to imatinib K-562R-1.0K cell line, the lowest rhodamine retention ratio was observed ($p<0.01$). There were no significant differences in rhodamine retention between other resistant cell lines and K-562 cell line (Table I).

Cytogenetic studies. Amount of signals in FISH analysis from *ABL*, *BCR* and from fusion genes (*BCR-ABL* or *ABL-BCR*) was mostly higher in imatinib-resistant cell lines in comparison to K-562 cell line. One dominant clone was observed in each cell line. This resistant clone had 3 copy of *ABL* gene and 2 copy of *BCR* gene in majority of imatinib-resistant cell lines, whereas in K-562R-0.1 and K-562R-0.05 dominant clone had only 2 copy of *ABL* gene and 2 copy of *BCR* gene. Amount of signals from *ABL*, *BCR* and fusion genes in particular cell lines are presented in Table II.

DISCUSSION

In recent years, TKIs have been recognized as central players and regulators of cancer cell proliferation, apoptosis, and angiogenesis, and are therefore considered suitable potential targets for anti-cancer therapies. However, increasing evidence of acquired resistance to these drugs has been documented, and extensive preclinical studies are ongoing to try to understand the molecular mechanisms by which cancer cells are able to bypass their inhibitory activity (8). Common mechanisms of resistance include, but are not limited to: point mutations, deletions and amplifications of genomic areas (3, 8). Point mutations are the most common mechanism of resistance to TKIs and the most frequent types of mutations are those that decrease the affinity of the drug for the target kinase domain, while maintaining its catalytic activity (8). Other mechanisms of resistance to TKIs include, drug influx/efflux, alternative pathways activation, modifications of proteins expression (1, 3, 8).

In this study we analyzed cellular response to tyrosine kinase inhibitors in imatinib-resistant cell lines. Potential mechanisms of drug resistance were also tested by means of cytotoxicity assays, flow cytometry and cytogenetic studies. IC50 value for imatinib was higher in almost all derivative cell lines in comparison to imatinib-sensitive K-562 cell line. In the study of Suzuki et al. (9), a 6-fold increase of resistance to imatinib was obtained in Philadelphia positive acute lymphoblastic leukemia cell line established at relapse in comparison to cell line established from this same patient at diagnosis. Analogical observation was done in patients, who acquired resistance to imatinib after standard 400 mg daily dose whereas second remission was observed after daily imatinib dose was increased to 800 mg (10).

We observed that incubation in increasing imatinib concentrations for 100 days in concentration range 0.1-1.0 μM leads to induction of cross-resistance to dasatinib and nilotinib, whereas it was not observed in other cell lines, resistant to imatinib in range 0.001-0.05 μM. O'Hare et al. also observed that in case of increasing resistance to imatinib, good sensitivity to dasatinib and nilotinib were still observed (11-12). In K-562 cell line and its derivatives, high effectiveness of cyclophosphamide and busulfan was observed what supports their use in conditioning regimen in CML before hematopoietic stem cell transplantation (13-14). In all tested cell lines high bortezomib effectiveness was observed. Servida et al. (15) also observed good activity of bortezomib in various cancer cell lines, including K-562. Noteworthy, 50 and 100 days incubation with imatinib significantly increased sensitivity to bortezomib (K-562R-0.1K, K-562R-0.5

and K-562R-1.0K cell lines), while simultaneously resistance to imatinib was observed in the MTT assay. In FISH technique more signals form *BCR-ABL* or *ABL-BCR* fusion genes were observed in resistant to imatinib cell lines in comparison to K-562 cell line. These results correspond with Albero et al. who observed better sensitivity to bortezomib in cell lines with *BCR-ABL* gene expression than in control cells (16). These data suggest a potential value of bortezomib in imatinib-resistant CML cases, especially in those with T351I mutation, resistant to imatinib (16). We observed good cytarabine and etoposide activity to K-562 cell line, what was also found by other authors (17-19), whereas high resistance to cytarabine and etoposide in imatinib-resistant cell lines was observed. In imatinib-resistant cell lines but not in K-562 cell line resistance to daunorubicin was observed as in other studies (20-21), what suggest other independent to *BCR-ABL* gene mechanism of resistance.

PGP protein expression is a classical mechanism of resistance in cancer (22). We observed that in comparison to K-562 cell line, exposure to imatinib caused significant decrease of PGP and LRP but not MRP1 expression in imatinib-resistant cell lines. This effect was independent on concentration of imatinib and time of cell treatment. No data are available so far on imatinib resistance and LRP expression, whereas contradictory data about PGP and MRP1 expression are presented. Zong et al. found that K-562 derivative cell lines have higher PGP expression, however no correlation with sensitivity to imatnib was observed (23). No correlation between PGP expression and resistance to imatinib was found in study of Ferrao et al. (24) and Mahon et al. (25), while in other PGP over-expression was the only mechanism of resistance to imatinib, and *BCR-ABL* over-expression was not observed (26). Davies at al. found no evidence for either active uptake of nilotinib through hOCT1 or efflux through MDR1, MRP1 or ABCG2 what suggest that these transporters have no effect on the clinical response to this drug (27). It is suggested that although dasatinib is a substrate for hOCT1, its uptake is less dependent on hOCT1 than for imatinib, and in vivo, simple diffusion may be much more important than active uptake (28).

In FISH analysis, with D-FISH BCR-ABL1/ABL1-BCR probe, we found that K-562 cell line presented with multiple copies of *BCR-ABL* and *ABL-BCR* fusion genes. Exposure to 0.01 – 1.0 μM of imatinib lead to selection and domination of resistant clones with higher number of fusion genes. Our data indicate that exposure to imatinib, especially in concentrations 0.1 - 1.0 μM has led to selection of resistant clones. It could be caused by genetic instability of fusion genes. This was a dose dependent effect, an increase of number of fusion genes was observed mainly in cells incubated with 0.01-1.0 μM imatinib. Increase copy number of *BCR-ABL* fusion gene is one of the most important mechanisms of resistance to imatinib. Much more fusion gene copies and the same number of novel *BCR* and *ABL* gene copies were observed in the K-562R-1.0 cell line. This cell line was the most resistant to imatinib and highly resistant to dasatinib and nilotinib cell line when compared to parental K-562 cell line. Le Coutre et al. (29) and Weisberg et al. (30) after serial passages with imatinib generated cells with higher Bcr-Abl kinase activity, what was caused by *BCR-ABL* amplification. Mahon et al. have found that different cell line which had different number of copies of *BCR-ABL* gene (25). They also observed in resistant cell lines higher number of *BCR-ABL* gene copies in comparison to sensitive cell lines (25). These data suggest an increased genetic instability in this cell line. *BCR-ABL* sequences amplification is probably one of the most important mechanisms of resistance in CML patients (31-32).

In conclusion, we have demonstrated that CML cells exposition to imatinib induced resistance to TKIs and most of other anticancer drugs, except busulfan and bortezomib. We observed lower expression of multidrug resistance proteins in imatinib-resistant CML cells but the role of these proteins in development of resistance to TKI's is ambiguous. Finally, we showed that exposition of K-562 cell line to imatinib had induced higher number of *BCR-ABL* and *ABL-BCR* fusion gene copies and promoted resistant clones. It seems that *BCR-ABL* amplification is significant mechanism of resistance in K-562 cell line.

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Address for correspondence:
dr n. med. Krzysztof Czyżewski
Department of Pediatric Hematology and Oncology
Collegium Medicum
Nicolaus Copernicus University
ul. M. Sklodowskiej-Curie 9
85-094 Bydgoszcz, Poland
tel.: +48 52 585 48 60
fax: +48 52-585 4867,
e-mail: krzysztofczyzewski@wp.pl

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