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Imatinib is a substrate for various multidrug resistance proteins

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An increasing resistance to imatinib is an emerging problem in patients with chronic myeloid leukemia (CML). The aim of the study was to asses mechanisms related to cellular drug resistance in imatinib-resistant derivates of chronic myeloid leukemia K-562 cell line. A parental K-562 and its imatinib-resistant derivate cell lines were used. Cell lines were tested for cytotoxicity of imatinib, cytarabine, busulfan and etoposide by the MTT assay. The cytotoxicity was expressed as IC50, inhibitory concentration for 50% of cells. Multidrug resistance proteins expression, rhodamine retention and daunorubicin accumulation were measured for each cell line. Continuous exposition of K-562 cell line to 0.01-0.02 mM imatinib resulted in development of resistance, while exposition to 0.1 μM imatinib increased cell sensitivity to this drug. There was a high correlation between PGP, MRP1 and LRP expression and IC50 values for imatinib and etoposide. All tested cell lines were highly resistant to cytarabine. Rhodamine retention alone and in the presence of cyclosporine was the lowest in imatinib-resistant K-562R-0.1 cell line, what suggest high PGP activity in this cell line. The highest daunorubicin accumulation was observed in parental K-562 cell line, while it was lower in imatinib-resistant cell lines. These data suggest that imatinib is a substrate for multidrug resistance proteins, and an increased expression of PGP, MRP1 and LRP play a role in resistance to imatinib in CML.

Key words: imatinib, multidrug resistance proteins, chronic myeloid leukemia, PGP, MRP1, LRP

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder that arises in the stem cell compartment. CML is a biphasic disease, occurring in all age groups, characterized by an initial chronic phase that progresses through accelerated phase to the terminal blast crisis phase [1]. The molecular cause of the disease is the *BCR-ABL* gene rearrangement, which occurs as the result of a reciprocal translocation between chromosomes 9 and 22 [1]. This translocation produces the oncogenic fusion BCR-ABL protein, with a constitutive tyrosine kinase activity, with maintained autophosphorilation and substrate activation [2]. The subsequent dysregulation of the ABL protein leads to enhanced proliferation, resistance to apoptosis, and altered adhesion, which are the key characteristics of CML cells [3].

The first drug which inhibits ABL tyrosine kinases is imatinib mesylate. This 2-phenylaminopyrimidine derivative potently inhibits cellular ABL, BCR-ABL kinases and two receptor tyrosine kinases: c-KIT and PDGFR. Imatinib is the first molecular targeted therapy drug, that has shown clinical success in patients with CML [2,4-5]. The introduction of imatinib has revolutionized management of the disease, as it induced complete cytogenetic response up to 90% of chronic phase CML patients, including those resistant or refractory to interferon-α [6]. While most of these responses are stable, resistance to treatment after an initial response is common in more advanced phases of the disease.

Response to imatinib therapy can be defined at hematologic, cytogenetic or molecular level [3]. Lack of response was classified by an international consensus panel as a failure, suboptimal response and warning signs [7]. Currently, the issue of resistance is more complex than simply lack or loss of some predefined response. This absence of response is known as primary resistance or, more appropriately, primary refractoriness. Patients that achieve a certain level of response – i.e. haematological, cytogenetic, or molecular – and that subsequently lose that response, can be described as having acquired resistance [3].

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A number of mechanisms of resistance to imatinib is known, including: clonal evolution with the development of novel chromosome abnormalities; overexpression of BCR-ABL associated with amplification of *BCR-ABL*; mutations in *ABL*-kinase domain; overexpression of the multidrug-resistant P-glycoprotein (MDR-1, multidrug resistance) [5,8-13]. Important factors involved in the process of resistance are also gastrointestinal absorption, plasma protein binding, drug efflux and influx, enzymatic inactivation, defects in apoptosis or repair mechanisms.

The aim of the study was to asses mechanisms related to cellular drug resistance of imatinib-resistant derivates in chronic myeloid leukemia K-562 cell line.

Materials and methods

Cell line. Parental, imatinib-sensitive K-562 cell line and imatinib-resistant K-562-derivative cell lines (K-562R) were used in this study. K-562R originated from K-562 cell line by continuous incubation with low doses of imatinib: in concentration of 0.01 μM (K-562R-0.01), 0.02 μM (K-562R-0.02) and 0.1 μM (K-562R-0.1) imatinib. Resistant cell lines were obtained within 2-4 weeks, after stable culture was obtained. All cell lines were maintained in RPMI 1640 (Sigma Chemical Co. St. Louis, MO, USA) supplemented with heat-inactivated 20% fetal calf serum (FCS, Gibco BRL Life Technologies, Paysley, UK), 100 IU/ml penicillin (Polfa Tarchomin, Tarchomin, Poland), 100 μg/ml streptomycin (Polfa Tarchomin, Tarchomin, Poland), 0.125 μg/ml amphotericin B (Bristol-Meyers Squibb, La Defense, France), 200 μg/ml gentamicin (Krka, Novo Mesto, Slovenia), 2 mM L-glutamine, 5 μg/ml insulin, 5 μg/ml transferine and 5 ng/ml sodium selenite (all Sigma).

Drugs. Imatinib (Glivec, Novartis Pharma GmbH, Nurnberg, Germany) was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mM and stored in aliquots in –20°C. Further imatinib dilutions was made with RPMI 1640. Imatinib was tested in concentrations range 0.0625-2 μM. Others drugs used in the study included: cytarabine (EBEWE Pharma GmbH, Unterach, Austria, concentration range 0.0097-10 μg/ml), busulfan (Pierre Fabre Medicament, Boulogne, France, 0.0125-0.4 μg/ml) and etoposide (Bristol Myers Squibb, Sermoneta, Italy, 1.56 – 50 μg/ml).

The MTT viability assay. The MTT viability assay was used to asses cytotoxicity of tested drugs, as it was previously described [14]. All experiments were performed in triplicate, and 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), Multidrug-Resistance Related Protein-1 (MRP1) and Lung-Resistance-Protein (LRP) were measured for each sample by flow cytometry (Epics XL, Coulter Miami, FL, USA). Human: anti-PGP (clone JSB-1, and isotype control of mice IgG1), anti-MRP1 (clone MRPr1, and isotype control of rat IgG2a) and anti-LRP (clone LRP 56, and isotype control of mice IgG2a) antibodies were used to determine an expression of respective proteins (all: Alexis Biochmicals, Lausanne, Switzerland). PGP, MRP1 and LRP 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]. All assay were performed in at least three independent experiments.

Rhodamine retention. For retention studies, rhodamine (Rh123, Sigma) in concentration 200 ng/ml was used. Rh123 retention in the presence of cyclosporine (CsA) 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.

Daunorubicin accumulation. Tested cells at a concentration of 0.5 x 10^6 cells/ml were incubated with daunorubicin (DNR, Rhone Poulenc Rorer, Amstelveen, The Netherlands). Accumulation of DNR was determined during continuous exposure to DNR. To study the retention, cells were loaded with 10 μg/ml DNR in a pre-warmed medium. Intracellular DNR fluorescence (FL) was measured by flow cytometry using the fluorescent properties of the drugs. DNR was excited by an argon laser at 488 nm and the fluorescence signal was collected through a 515–535 nm (FL1 height), 565–585 nm (FL2 height) and 610–630 nm (FL3 height) bandpass filter set. The strongest emission was detected by FL3 filter. The instrument settings for FL3 heights, forward scatter (FSC) and sideward scatter (SSC) were kept constant between each two runs of the flow cytometer. The data were analyzed using the SYSTEM II v. 3.0 software (Beckman Coulter, Miami, FL) [15]. Daunorubicin accumulation index (DAI) was calculated as a intracellular drug fluorescence in time.

Statistical methods. Results were given as mean values ±SD. Differences in drug resistance between samples tested for cytotoxicity and differences in multidrug proteins expression were analyzed by the ANOVA test for unpaired comparisons and by the Paired Sample Test for paired comparisons. Correlations between IC50 mean value and multidrug resistance proteins expression were given with Spearman's rho followed by p-values. All reported p-values are 2-sided; $p<0.05$ was considered statistically significant.

Results

Continuous exposure to low concentrations of imatinib (0.01-0.02 μM) caused the development of resistance to imatinib and etoposide, but not to busulfan in K-562 cell line, while continuous exposure to higher concentrations of imatinib (0.1 μM) caused the development of sensitivity to imatinib in identical cell line. Mean IC50 value for K-562 cell line was 1.14 μM, while it was higher in K-562R-0.01 and K-562R-0.02 with the values of 1.31 μM and 1.65 μM, re-

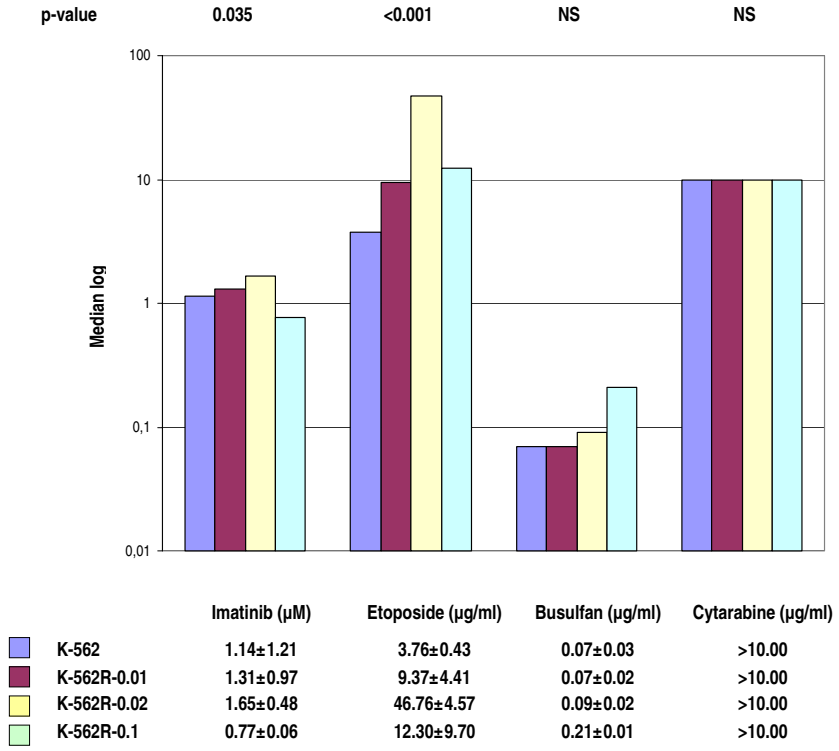

Figure 1. IC50 mean values for tested drugs.
Continuous exposure to low concentrations of imatinib caused development of resistance to imatinib and etoposide in K-562 cell line, while continuous exposure to higher concentrations of imatinib caused development of sensitivity to imatinib in K-562 cell line. All tested cell lines were highly resistant to cytarabine. p-value was calculated by ANOVA test.

spectively. The lowest value of IC50 at 0.77 µM was found in K-562R-0.1 cell line (Fig. 1). The same trend was observed for IC50 values for etoposide, whereas resistance to busulfan was highest in K-562R-0.1 cell line. All tested cell lines were highly resistant to cytarabine.

With respect to multidrug resistance proteins, continuous concentration of imatinib 0.01-0.02 µM resulted in induction of PGP, MRP1 and LRP expression in respective cell lines, however downregulation of tested multidrug resistance proteins in K-562R-0.1 was observed (Table 1, Fig. 2). There was a high

Table 1. Expression of PGP, MRP1 and LRP.

	K-562	K-562R-0.01	K-562R-0.02	K562R-0.1	p-value
PGP	8.10±0.59	12.50±0.25	12.30±2.40	6.68±0.12	0.001
MRP1	10.86±1.89	14.3±0.24	16.45±1.20	8.15±0.51	<0.001
LRP	7.63±0.02	12.7±0.53	13.25±0.49	9.73±0.94	0.001

Comparing to parental K-562 cell line multidrug resistance proteins expression increase in K-562R0.01 and K-562R-0.02 cell line, and then decrease in K-562R-0.1. Continuous exposition to low imatinib concentrations induct expression of tested multidrug resistance proteins. The expression of PGP, MRP1 and LRP is given in arbitrary units of flow cytometry. p-value was calculated by ANOVA test.

Table 2. Rhodamine retention in tested cell lines.

	K-562	K-562R-0.01	K-562R-0.02	K562R-0.1	p-value
Rh123	115.95±1.06	59.70±0.89	77.55±5.16	60.95±2.61	0.005
Rh123+CsA	244.40±0.98	108.40±1.18	164.00±7.07	91.10±4.10	<0.001
p	0.003	0.010	0.003	0.035	
(Rh123+CsA)/Rh123 retention ratio	2.10	1.81	2.11	1.49	

p-value was calculated by ANOVA test.

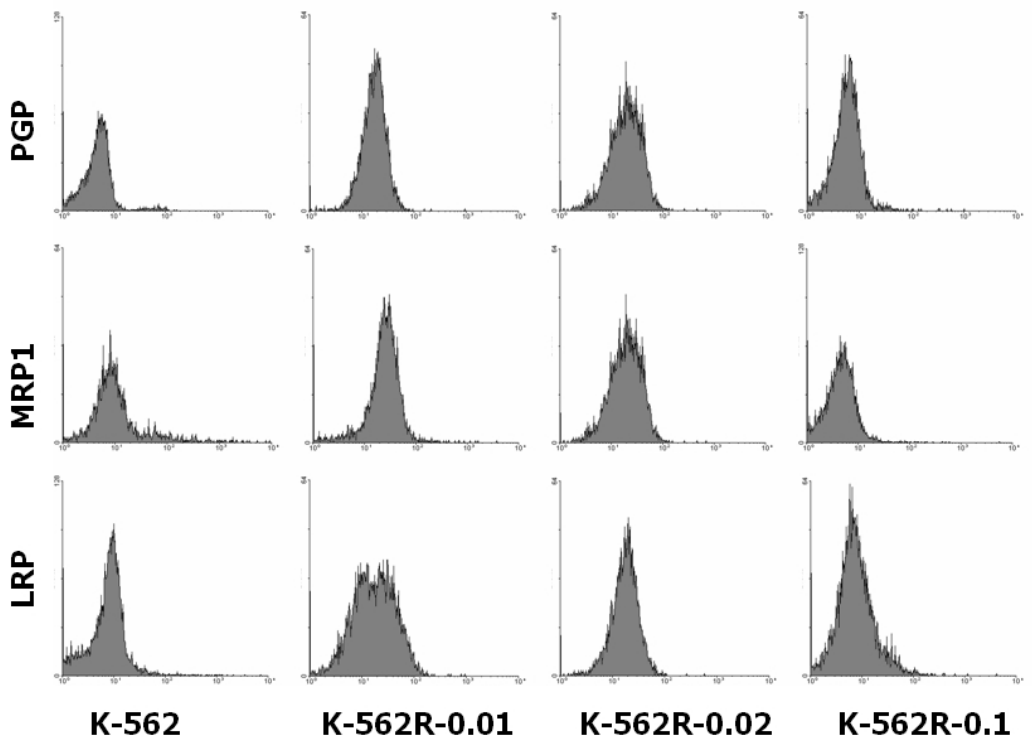

Figure 2. Expression of multidrug resistance proteins in K562 and its derivative cell lines.

correlation between multidrug resistance proteins expressions and IC50 mean value for imatinib and etoposide (Fig. 3).

The highest Rh123 accumulation was observed in parental K-562 cell line (Table 2) and the lowest one in K-562R-0.1 cell line. Rh123 accumulation in each tested cell line has increased after CsA was added. Rh123 retention in the presence of CsA was the lowest in K-562R-0.1 cell line, similar as (Rh123+CsA)/Rh123 retention ratio.

Daunorubicin accumulation index (DAI) was the highest in parental K-562 cell line, and the lowest in resistant to imatinib K-562R-0.02 cell line (Fig. 4).

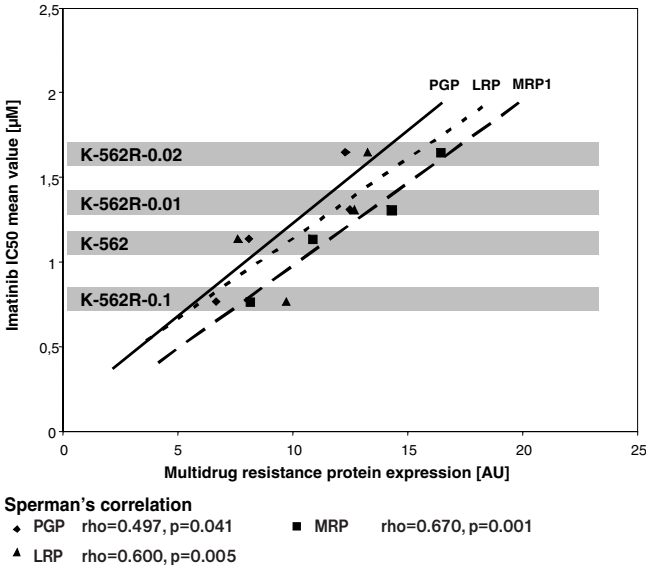

Figure 3. Correlation between IC50 mean value of imatinib for cell lines and expression of PGP, MRP1, LRP.
There was a high correlation between multidrug resistance proteins expressions and IC50 mean value for imatinib.

Discussion

Imatinib mesylate is a selective inhibitor of BCR-ABL kinases. An emerging problem is the development of resistance to imatinib and novel inhibitors of tyrosine kinases [8]. In the most cases, resistance against imatinib results from kinase domain mutations and/or over-expression of the *BCR-ABL* gene. There are also some cases of resistance that occur through mechanisms independent on *BCR-ABL* [16]. Besides mutations, other mechanisms seems to play an important role in imatinib resistance.

We have observed that continuous exposition of K-562 cell line to imatinib at concentration 0.01-0.02 μM caused development of resistance, presenting as an increase of IC50 value. Exposition of K-562 cell line to 0.1μM imatinib induced sensitivity to this drug by decreasing IC50 value. A high correlation between PGP, MRP1 and LRP expression and IC50 for imatinib and etoposide was also found. Imatinib is known to

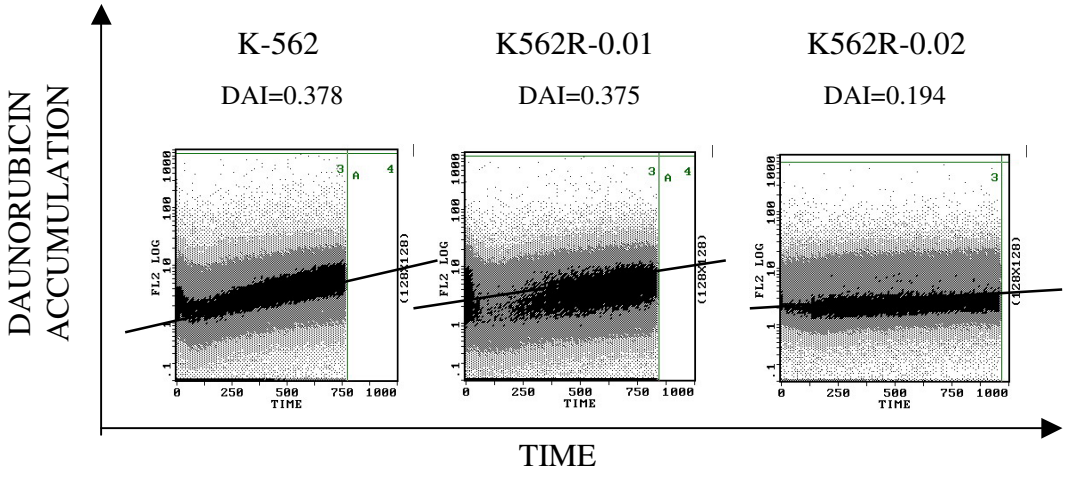

Figure 4. Time-dependent daunorubicin accumulation.

be a substrate for PGP [13,17], however there are contradictory data about PGP expression in CML and its induction of resistance to imatinib [13, 18-21]. We have found a correlation between both PGP and MRP1 expression and resistance to imatinib in K-562-derivative cell lines. Low level of MRP1 is known to be an independent positive predictor to imatinib treatment in patients with myeloid blast crisis [22], however some authors suggest that imatinib is not a substrate for MRP1 [17,18].

Currently, no data are available if imatinib is a substrate for LRP. We have shown that LRP expression correlates with imatinib resistance, thus indicating imatinib as a substrate for LRP. To our knowledge, this is the first study to show this relationship. Since PGP, MRP1 and LRP proteins play an important role in multidrug resistance of leukemic cells through decreasing the intracellular drug concentration, overcoming this mechanism may have an important role in circumvention of resistance [23].

Rh123 is a substrate, being actively effluxed of the cell by PGP, and is known as an indicator of functional activity of this transmembrane protein. CsA is an inhibitor of PGP, MRP1, LRP, and is supposed to be inhibitor of other multidrug resistance proteins [24]. The lowest Rh123 retention was observed in imatinib-resistant K-562R-0.1 cell line. Rh123 retention in the presence of CsA as well as (Rh123+CsA)/Rh123 retention ratio reached also the lowest values in this cell line. Altogether, in the most imatinib-resistant cell line (K-562R-0.1), the highest PGP activity and the lowest CsA modulating activity was observed. The same results were observed for MRP1 and LRP proteins, which suggests that imatinib is a useful substrate also for these two multidrug resistance proteins.

The highest daunorubicin accumulation occurred in imatinib-sensitive K-562 cell line, which was characterized also by the lowest PGP and MRP1 expression. The accumulation was lower in imatinib-resistant cell lines (with higher PGP and MRP1 expression). In the study of Legrand et al. [25], simultaneous activity of PGP and MRP1 correlated with *in vitro* resistance to daunorubicin and with *in vivo* clinical resistance in adult acute myeloid leukemia. On the other hand, there are opposite data suggesting that overexpression of PGP in K-562 cells does not confer resistance to imatinib *in vitro,* and imatinib is a poor substrate for PGP [26].

In conclusion, we suggest that drug efflux caused by multidrug resistance proteins may play an important role in development of resistance to imatinib in CML cells. Continuous exposition to low concentrations of imatinib can induce resistance to this drug in CML cells, while higher continuous imatinib concentrations may overcome the resistance. These data suggest that imatinib is a useful substrate for testing multidrug resistance proteins, and an increased expression of PGP, MRP1 and LRP may play a role in development of resistance to imatinib in CML.

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References

[1] Huntly BJ, Guilhot F, Reid AG et al. Imatinib improves but may not fully reverse the poor prognosis of patients with CML with derivative chromosome 9 deletions. Blood 2003; 102: 2205–2212. doi:10.1182/blood-2002-09-2763

[2] Ferrari G, Pastorelli R, Buchini F et al. Comparative proteomic analysis of chronic myelogenous leukemia cells: inside the mechanism of imatinib resistance. J Proteome Res 2007; 6: 367–375. doi:10.1021/pr0603708

[3] Apperley JF. Part I: mechanisms of resistance to imatinib in chronic myeloid leukaemia. Lancet Oncol. 2007; 8: 1018–1029. doi:10.1016/S1470-2045(07)70342-X

[4] O'Dwyer ME, Druker BJ. STI571: an inhibitor of the BCR-ABL tyrosine kinase for the treatment of chronic myelogenous

leukaemia. Lancet Oncol 2000; 1: 207–211. doi:10.1016/S1470-2045(00)00149-2

[5] Mahon FX, Belloc F, Lagarde V et al. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood 2003; 101: 2368–2373. doi:10.1182/blood.V101.6.2368

[6] O'Brien SG, Guilhot F, Larson RA et al. IRIS Investigators. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med. 2003; 348: 994–1004. doi:10.1056/NEJMoa022457

[7] Baccarani M, Saglio G, Goldman J et al. European LeukemiaNet. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. Blood 2006;108: 1809–1820. doi:10.1182/blood-2006-02-005686

[8] Litzow MR. Imatinib resistance. Arch Pathol Lab Med 2006; 130: 669–679.

[9] Hochhaus A, Kreil S, Corbin AS et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 2002; 16: 2190–2196. doi:10.1038/sj.leu.2402741

[10] Gambacorti-Passerini C, Barni R, le Coutre P et al. Role of alpha1 acid glycoprotein in the in vivo resistance of human BCR-ABL(+) leukemic cells to the abl inhibitor STI571. J Natl Cancer Inst 2000; 92: 1641–1650. doi:10.1093/jnci/92.20.1641

[11] le Coutre P, Kreuzer KA, Na IK et al. Determination of alpha-1 acid glycoprotein in patients with Ph1 chronic myeloid leukemia during the first 13 weeks of therapy with STI571. Blood Cells Mol Dis 2002; 28: 75–85. doi:10.1006/bcmd.2002.0493

[12] Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood 2004; 104: 3739–3745. doi:10.1182/blood-2003-12-4276

[13] Illmer T, Schaich M, Platzbecker U et al. P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate. Leukemia 2004; 18: 401–408. doi:10.1038/sj.leu.2403257

[14] Styczynski J, Wysocki M, Debski R et al. In vitro sensitivity of leucemic cells to nucleoside derivates in childhood acute leukemia: good activity In leukemic relapses. Neoplasma, 2005; 52: 74–78.

[15] Styczynski J, Wysocki M, Debski R et al. The influence of intracellular idarubicin and daunorubicin levels on drug cytotoxicity in childhood acute leukemia. Acta Biochim Pol 2002; 49: 99–107.

[16] Lee SM, Bae JH, Kim MJ et al. Bcr-Abl-Independent imatinib-resistant K562 cells show aberrant protein acetylation and increased sensitivity to histone deacetylase Inhibitors. J Pharmacol Exp Ther 2007; 322: 1084–1092. doi:10.1124/jpet.107.124461

[17] Hegedus T, Orfi L, Seprodi A, et al. Interaction of tyrosine kinase inhibitorswith the human multidrug transporter proteins, MDR1 and ABCC1. Biochim Biophys Acta 2002; 1587: 318–325.

[18] Che XF, Nakajima Y, Sumizawa T et al. Reversal of P-glycoprotein mediated multidrug resistance by a newly synthesized 1,4-benzothiazipine derivative, JTV-519. Cancer Lett 2002; 187: 111–119. doi:10.1016/S0304-3835(02)00359-2

[19] Mahon FX, Belloc F, Lagarde V et al. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. Blood 2003; 101: 2368–2373. doi:10.1182/blood.V101.6.2368

[20] Burger H, Nooter K. Pharmacokinetic resistance to imatinib mesylate: role of the ABC drugpumps ABCG2 (BCRP) and ABCB1 (MDR1) in the oral bioavailability of imatinib. Cell Cycle 2004; 3: 1502–1505.

[21] Ferrao PT, Frost MJ, Siah SP, Ashman LK. Overexpression of P-glycoprotein in K562 cells does not confer resistance to the growth inhibitory effects of imatinib (STI571) in vitro. Blood 2003; 102: 4499–4503. doi:10.1182/blood-2003-01-0083

[22] Lange T, Gunther C, Kohler T et al. High levels of BAX, low levels of MRP-1, and high platelets are independent predictors of response to imatinib in myeloid blast crisis of CML. Blood 2003; 101: 2152–2155. doi:10.1182/blood-2002-05-1366

[23] Styczynski J, Wysocki M, Debski R et al. Predictive value of multidrug resistance proteins and cellular drug resistance in childhood relapsed acute lymphoblastic leukemia J Cancer Res Clin Oncol 2007; 133: 875–893. doi:10.1007/s00432-007-0274-1

[24] Qadir M, O'Loughlin KL, Fricke SM et al. Cyclosporin A is a broad-spectrum multidrug resistance modulator. Clin Cancer Res 2005; 11: 2320–2326. doi:10.1158/1078-0432.CCR-04-1725

[25] Legrand O, Simonin G, Beauchamp-Nicoud A et al. Simultaneous activity of MRP1 and Pgp is correlated with in vitro resistance to daunorubicin and with in vivo resistance in adult acute myeloid leukemia. Blood 1999; 94: 1046–1056.

[26] Ferrao PT, Frost MJ, Siah SP, Ashman LK. Overexpression of P-glycoprotein in K562 cells does not confer resistance to the growth inhibitory effects of imatinib (STI571) in vitro. Blood 2003; 102: 4499–4503. doi:10.1182/blood-2003-01-0083