

P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate

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Imatinib (Glivec[®], STI571) is an intracellular acting drug that demonstrates high activity against BCR-ABL-positive chronic myelogenous leukemia (CML) or acute lymphoblastic leukemia (ALL). However, many patients, especially with advanced disease, develop drug resistance. Here, we show by a novel high-performance liquid chromatography-based method that intracellular levels of imatinib decrease in P-glycoprotein (Pgp)-positive leukemic cells. In a model of K562 cells with gradually increasing Pgp expression, a Pgp-dependent decline of intracellular imatinib levels was observed. Decreased imatinib levels were associated with a retained phosphorylation pattern of the Bcr-Abl target Crkl and loss of effect of imatinib on cellular proliferation and apoptosis. The modulation of Pgp by cyclosporin A (CSA) readily restored imatinib cytotoxicity in these cells. Finally, we provide first data showing a biological effect of Pgp modulation in the imatinib treatment of a patient with BCR-ABL-positive ALL. MDR1 overexpression must therefore be considered as an important clinical mechanism in the diversity of resistance development to imatinib treatment.

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Introduction

In 95% of chronic myelogenous leukemia and about 25% of acute lymphoblastic leukemia patients – sequences of the tyrosine kinase ABL on chromosome 9 are under the transcriptional control of the BCR gene on chromosome 22. This translocation results in a fusion transcript with transforming activity (recent review).¹ Consequently, the inhibition of the tyrosine kinase function of Bcr-Abl with the specific imatinib compound leads to apoptosis in t(9;22)-positive cells.² Clinical trials indicate that imatinib is a highly active substance in CML with few side effects and is therefore used in all stages of the disease. However, the efficacy of the drug declines with its use in more advanced stages of the disease and most patients treated with imatinib in accelerated or blast phase develop resistance.^{3,4}

Molecular mechanisms of resistance towards imatinib are beginning to be unravelled. Gene amplification and overexpression of the BCR-ABL gene have been observed.⁵ Moreover, the occurrence of mutations of the kinase domain of ABL has been described recently as a crucial mechanism for the development of imatinib resistance.^{6,7} These mutations usually interfere with the ability of Bcr-Abl to bind imatinib; however, they leave a fully competent Bcr-Abl kinase, which is the hallmark of continuous growth stimulation even in the presence

of the inhibitor. It has been reported that such mutations may already reside in the original leukemic clone thus promoting the idea of a possible positive selection process of mutated clones by imatinib treatment. In addition, it has been shown that the inhibition of imatinib binding, associated with some mutations in the kinase domain of Bcr-Abl, is not complete.^{7–9} Rising intracellular levels of the drug should therefore result in a better clearance of BCR-ABL-positive cells. This finding is supported by the clinical results of a recently published trial in resistant CML patients. As reported by Kantarjian *et al*,¹⁰ increasing the dose of imatinib may reverse clinical resistance. Thus, intracellular levels of imatinib may be crucial even for the treatment of resistant BCR-ABL-positive patients.

It has been suspected that intramembranous drug transporters may reduce the critical concentration needed for the antileukemic effect.¹¹ The best-studied ABC transporter in human cells is the MDR1 gene product Pgp that effectively extrudes natural compounds out of the cell.¹² Pgp expression has been observed more frequently in patients with advanced stage CML.¹³ Moreover, the chemical structure of imatinib suggests lipophilic properties. These data indicate Pgp as a likely candidate contributing to imatinib resistance. We therefore established a chromatographical method to determine imatinib as a substrate for Pgp-mediated efflux. We furthermore explored a model system of different K562 cells with a gradual increase of Pgp and investigated the pharmacological and cellular effects of Pgp modulation on the treatment efficacy of imatinib.

Materials and methods

Patient blast samples

The patient was treated in accordance with the Helsinki declaration. Before the start of treatment, the patient gave written informed consent. Daily blood samples allowed changes in the peripheral blood count to be documented. Blast samples at days 0 and 1 of treatment were obtained using a standardized Ficoll isolation procedure.

Cell lines, kinase inhibitor and drugs

The parental HL-60, CCRF-CEM and K562 cell lines were obtained from DSMZ (DSMZ, Braunschweig, Germany). The drug-resistant CCRF-VCRI100 cell line was provided by Dr Stefan Neu (Department of Pediatric Hematology, University of Tübingen, Germany) and was cultured in the presence of 0.1 μM vincristine. Cell lines were grown in RPMI 1640 medium supplemented with penicillin, streptomycin and 10% FCS under standard conditions. Imatinib was donated by Novartis (Nuremberg, Germany) and was prepared as a stock solution of 10 mM in methanol. The solution was diluted in RPMI before use. Hence, only minimal amounts of methanol, not affecting the

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quality of the experiments (data not shown), were included in the investigations. VP-16 was purchased from Bristol-Myers Squibb (Munich, Germany), vincristine from Lilly (Bad Homburg, Germany), CsA from Sigma (Taufkirchen, Germany) and Ara-C from Mack (Karlsruhe, Germany).

Isolation of Pgp-expressing cell lines

Generation of resistant HL-60 and K562 cell lines was achieved after prolonged exposure of the cells to increasing concentrations of VP16 starting with concentration as low as 0.1 μM as described previously. Drug selection took place over a period of 3–5 months. Viable resistant cells were maintained under the continuous selection pressure of the respective drug. Cells surviving in the presence of the indicated drug concentration were cultured in fresh medium containing 10% FCS without the addition of any cytotoxic drug 12 h before performing the experiments described here.

Determination of MDR1 mRNA expression

RNA extraction, cDNA synthesis and real-time PCR were performed essentially as described previously.¹⁴

Rhodamin efflux

The detection of Pgp-related efflux was carried out according to a previously described method with minor modifications.¹⁵ Uptake was measured after a 30-min incubation with 0.5 $\mu\text{g/ml}$ rhodamin 123 (Sigma) at 37°C. Efflux was determined after 90 min at 37°C or at 4°C in the absence or presence of the MDR-reversing drug (CsA, 5 μM). Flow cytometry analysis (Cytoron; Ortho Diagnostic Systems, Raritan, NJ, USA) was performed after two further washes.

Cellular imatinib uptake

Cellular uptake experiments were performed in at least two independent experiments (in triplicate for each) in a time range of 6 weeks. For a single measurement, 20×10^6 cells were incubated in 5 ml RPMI containing the indicated concentrations of imatinib. After an incubation time of 5 h at 37°C and 5% CO_2 , cells were centrifuged at 1000 g and the resulting pellet resolved in 50 ml isotonic NaCl and subsequently again pelleted by centrifugation. After discharging the supernatant, the sample pellet was treated for imatinib extraction (as described below).

In addition to the intracellular imatinib analysis, the concentration of the incubation media was analyzed both at the starting and end point of the experiments. Furthermore, all washing solutions were analyzed for imatinib residues. No metabolism of imatinib was found after incubation, the system could therefore be defined as a closed system and imatinib quantities in all compartments should have totaled 100%. In fact, we found amounts of imatinib in all compartments ranging from 75 up to 125%, probably reflecting the measurement variations as well as the surface activity of imatinib in aqueous solutions. Furthermore, we analyzed the rediffusion from intracellular imatinib to the washing solution and found that about 10% of the intracellular imatinib was distributed back within 5 min to the surrounding media (data not shown). Based on this observation, the centrifugation time for the washing step was limited to exactly 5 min in every experiment.

High-performance liquid chromatography (HPLC) and sample preparation

The newly developed method for imatinib concentration determination was carried out as follows. The chromatographic system comprised two Knauer 64 analytical HPLC pumps (Knauer Corp. Berlin, Germany), a Shimadzu UV spectrometric detector SPD-SA (Shimadzu, Duisburg, Germany), a Waters WISP 712 autoinjector and an electric motor-driven autoswitch equipped with a rheodyne valve 7740-001 for online-enrichment switching (Besta-HPLC-Technik, Wilhelmsfeld, Germany). Recording, evaluation and quantification of chromatograms was carried out by a PC-supported GINA program from Raytest (Straubenhardt, Germany).

A ZirChrom analytical HPLC column (3 μm , PDB-ZrO₂, 3% carbon, $50 \times 4 \times 6 \text{ mm}^2$) was used with a precolumn of the same solid phase specificity. The system was designed as an online-enrichment system with another PDB-ZrO₂ precolumn as the enrichment column. Flow was set at 0.4 ml/min at room temperature in the analytical part and on 2 ml/min at room temperature in the enrichment part. The analytical eluent consisted of 600 ml 0.01 M $\text{KH}_2\text{PO}_4/0.09 \text{ M K}_2\text{HPO}_4 + 400 \text{ ml}$ methanol/litre (v/v), while the enrichment eluent was prepared with 450 ml 0.1 M $\text{KH}_2\text{PO}_4 + 350 \text{ ml H}_2\text{O} + 200 \text{ ml CH}_3\text{OH}$ (v/v). For quantification, the external standard method was by regression analysis of six spiked plasma samples with 10 ng/ml, 100 ng/ml, 500 ng/ml, 1000 ng/ml, 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ imatinib. The analysis was performed by UV detection adjusted to 260 nm.

This system has a detection limit of 10 ng/ml for imatinib. Within-day variation was 7% for imatinib. Day-to-day variation examined on 10 consecutive days with plasma exposed to 100 ng/ml of imatinib was 4%. At the detection limit, the coefficient of variation was 11% for imatinib as proven by 10 measurements with spiked plasma samples.

For sample preparation 30 μl concentrated perchloric acid was added to 300 μl plasma or RPMI and rapidly shaken with an autoshaker for 10 min. Subsequently, 200 μl of the enrichment eluent was added to the sample and the mixture was shaken for another 10 min. Proteins were then precipitated by centrifugation for 7 min at 4000 g. A measure of 200 μl of the resulting supernatant was injected into the HPLC system for analysis.

Antibodies

The following antisera were used with the respective concentration: Anti-p-Crkl (Cell Signalling Technology Inc., Beverly, MA, USA) 1:1000; anti-Crkl (Cell Signalling Technology Inc., Beverly, MA, USA) 1:1000; anti-Pgp (C-19), anti-Bcl-2 (100); Bcl-xl (H-62); anti-bax (B-9) (all Santa Cruz Biotechnology, Santa Cruz, CA, USA) all 1:200; anti- β -actin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) 1:1000; secondary goat, rabbit and mouse-HRP-coated antibodies were from DAKO (DAKO A/S, Denmark) and were all used in a concentration of 1:2000.

Western blotting

Cellular proteins were extracted according to a modified variant of the method previously described by Dignam *et al.*¹⁶ Proteins were quantified using the BCA protein assay (Pierce, Rockford, IL, USA). Protein (40 μg) were resolved by SDS-PAGE, transferred to Hybond ECL nitrocellulose membrane (Amersham

Pharmacia Biotech, Freiburg, Germany) and immunoblotted with the respective antibodies (see above). The antigen was detected using the HRP-coated secondary antibody (see above) and ECL reagent (Amersham Pharmacia Biotech). After primary antigen detection, the membrane was stripped and reprobed with either Crkl or β -actin to determine relative amounts of proteins in each lane. Signals were scanned with a Biometra device and analyzed with ScanPack3 software (Biometra, Göttingen, Germany). Relative amounts of p-Crkl were calculated using a ratio of the obtained OD values for p-Crkl/Crkl antigen.

Measurement of cell viability and apoptosis

Cellular proliferation was determined using the methyl-thiazol tetrazolium (MTT) assay essentially as described by the manufacturer (Roche, Mannheim, Germany). In brief, cells were plated at a density of 0.5×10^6 /ml in RPMI containing 10% FCS overnight. Exposure against the indicated drug was performed for 48 h. Aliquots of 100 μ l were subsequently incubated with the MTT dye and were dissolved 4 h later with MTT buffer for another 12 h. The determination of OD values was carried out using an Anthos photometer (Krefeld, Germany) at the recommended wavelength of 570 nm.

Trypan blue exclusion was carried out according to routine protocols. Cells were counted in Neubauer hemocytometers.

To determine apoptotic changes, cells were stained with FITC-conjugated Annexin V (Alexis, Grünberg, Germany), as suggested by the vendor. Stained cells were analyzed on a FACScalibur (Becton Dickinson, San Jose, CA, USA) and the results were analyzed using Cell Quest (Becton Dickinson, San Jose, CA, USA) software.

Results

Characterization of the MDR phenotype in CCRF, HL-60 and K562 cells

We initially investigated a panel of three sensitive leukemic cell lines (CCRF, HL-60 and K562) and their resistant counterparts to compare for sensitivity profiles against daunorubicin, etoposide and Ara-C (Table 1). All resistant cell lines developed cross-resistance against daunorubicin and etoposide, which is less pronounced in the HL-60 5 μ M VP16 cell line. So, different cell

types may acquire different functional aspects of the MDR phenotype after exposure to cytotoxic drugs (Table 1).

Resistant CCRF and K562 cell lines showed strong upregulation of both mRNA and protein levels of the *MDR1* gene product (Table 1 and Figure 1a). In contrast, resistant HL-60 cells did not show elevated *MDR1* or Pgp levels. As indicated in Figure 1a, other mechanisms including Bax downregulation may be responsible for the development of the MDR phenotype in HL-60 cells. The reduced ratio of Bax/Bcl-2 expression may therefore increase antiapoptotic properties of this cell line.

We observed a VP16 concentration-dependent effect on the expression of *MDR1* and Pgp in that K562-5 μ M VP16 cells demonstrated stronger upregulation than K562-1 μ M VP16 cells (Table 1 and Figure 1a). Accordingly, in these cells *MDR1* and Pgp upregulation was associated with increased rate of drug efflux as detected by the rhodamin efflux method (Figure 1b). In contrast, no upregulation of two other well-studied drug transporters MRP1 and LRP occurred in the K562 cell lines (PCR data not shown). Thus, the most prominent changes during the process of drug development in K562 cells exposed to VP16 is a gradual increase in *MDR1*/Pgp, which is accompanied by an increase in functional resistance against the investigated cytotoxic drugs.

The tyrosine kinase Crkl is a critical downstream target of Bcr-Abl kinase activity and the phosphorylation status of Crkl may therefore be indicative of the activity of the fusion protein.⁹ As expected, both CCRF and HL-60 cell lines do not show phosphorylated Crkl. However, K562 cells demonstrated strong immunoreactivity for p-Crkl (Figure 1c). As shown in Figure 1c, phosphorylation patterns of the downstream effector of Bcr-Abl-Crkl do not change simply as a result of acquisition of resistance during VP16 exposure. We therefore reasoned that K562 cells remain dependent on Bcr-Abl function and the modulation of parallel occurring resistance phenomena (such as a Pgp function) is the main reason to reverse imatinib resistance.

Intracellular imatinib levels vary according to *MDR1*-mediated drug efflux

We explored a novel HPLC method to determine intracellular imatinib levels. We investigated sensitive CCRF-CEM cells – with their Pgp-expressing resistant strains CCRF-VCR100 as well as sensitive HL-60 cells – with their Pgp-negative resistant HL-60-5 μ M VP16 cells. Comparison of sensitive and resistant

Table 1 Multidrug resistance in the investigated cell lines

Cell line	MTT-IC ₅₀			Real-time PCR
	Daunorubicin (μ M)	Etoposide (μ M)	Ara-C (μ M)	<i>MDR-1</i> cDNA copies (s.d.)
HL-60	0.075	0.75	0.8	0.96 (0.38)
HL-60-5 μ M VP16	0.4	7.5	0.75	0.02 (0.01)
CCRF-CEM	0.25	0.5	0.75	5 (7)
CCRF-VCR	150	120	1	25 500 (7400)
K562	7.5	20	> 1000	370 (470)
K562-1 μ M	50	> 200	> 1000	122 834 (27 639)
K562-5 μ M	> 100	> 200	> 1000	203 712 (4990)

Description of the MDR phenotype by IC₅₀ values. Cells were exposed against a broad range of the indicated drugs (daunorubicin, 0.1–100 μ M; VP16, 0.1–200 μ M; Ara-C, 1–1000 μ M) for 48 h. The MTT assay was performed as it is described in Materials and methods. IC₅₀ values were calculated from graphs comparing treatment concentrations and % of decrement in OD measurement. Untreated control was set 100%. The values resemble the mean of at least two independent experiments, where OD values were determined in triplicate for each experiment. *MDR-1* mRNA copy number was determined by a previously described real-time PCR method. Values resemble the mean of three independent determinations with s.d.

SPOTLIGHT

CCRF-CEM cell lines after 5 h treatment with imatinib demonstrated that the cytoplasmatic fraction of sensitive cell lines was enriched for imatinib with median levels of 1107 ± 164 ng/ 10^7 cells. In contrast, resistant CCRF-VCR 100 cells showed only marginal intracellular imatinib levels with 154.8 ± 30.8 ng/ 10^7 cells (Figure 2). Whereas combined treatment with known Pgp modulators (CsA and verapamil) had no positive effect on intracellular imatinib levels in the sensitive cell line, the resistant cell line demonstrated a clear increment in intracellular imatinib that was more efficiently achieved with CsA as compared to verapamil (Figure 2). In contrast, the resistant HL-60–5 μ M VP16 cell line exhibited no evidence of imatinib efflux as compared to sensitive HL-60 cells. No influence of Pgp modulators was observed when sensitive or resistant HL-60 cells were analyzed for their intracellular imatinib levels (data not shown). As expected, imatinib had no impact on viability of either CCRF or

HL-60 cell lines up to a concentration of 10 μ M tested for 48 h (data not shown).

Functional relevance of MDR1 expression in BCR-ABL-positive cell lines

We extended our studies on BCR-ABL-positive K562 cells and their Pgp-expressing counterparts K562–1 μ M VP16 and K562–5 μ M VP16. As demonstrated previously, sensitive K562 cells are dependent on Bcr-Abl function and undergo apoptosis when exposed to imatinib in a concentration of about 0.25 μ M.¹⁷ K562 cells show very similar results in our

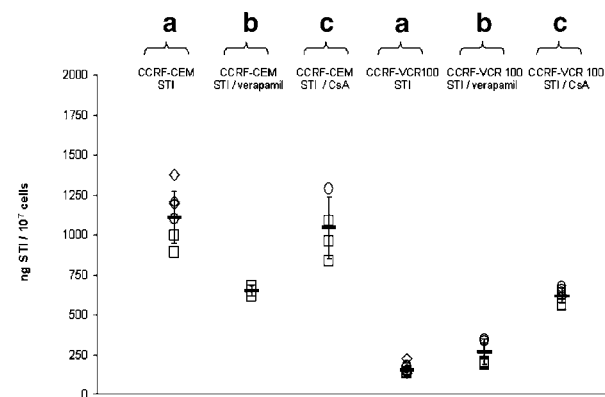
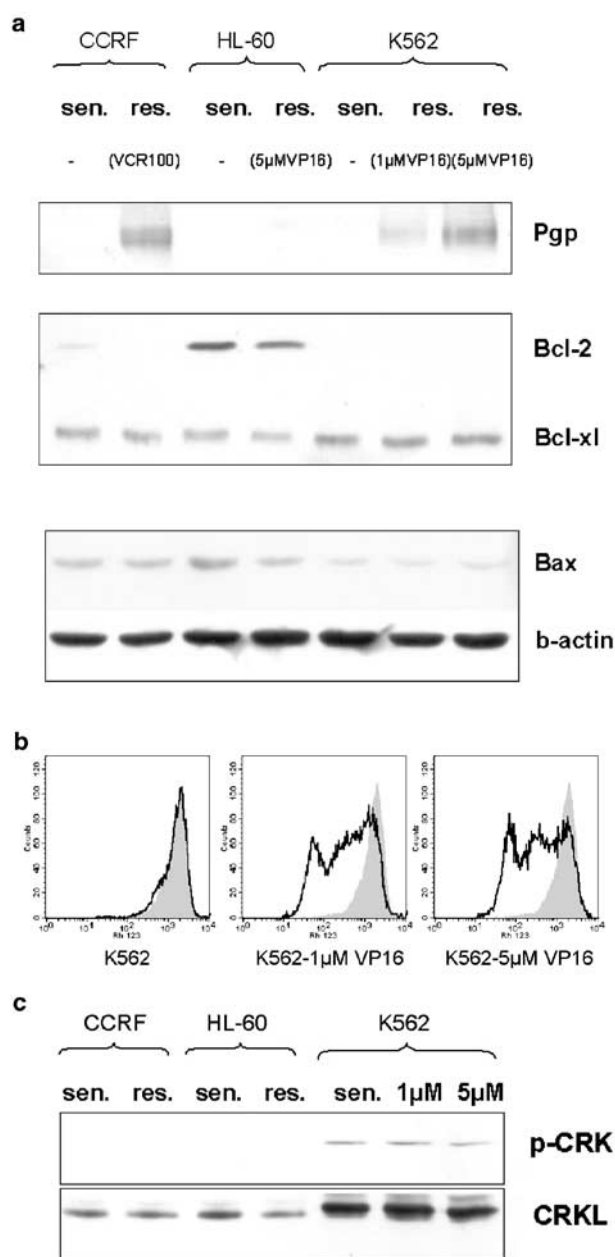


Figure 2 Intracellular imatinib levels in Pgp-positive resistant CCRF cells vs Pgp-negative resistant HL-60 cells. The determination of intracellular imatinib levels using a HPLC-based method in the parental CCRF-CEM cell line vs resistant CCRF-VCR100 cells – measurement was carried out as described in the Materials and methods. Cells were exposed to 5 μ g/ml (8.5 μ M) imatinib for 5 h and were immediately referred to the HPLC procedure. The means of three independent experiments that were determined in triplet each is indicated by a horizontal bar; s.d. is indicated. Different treatment conditions are denoted above and reflect the treatment of cells with either 5 μ g/ml (8.5 μ M) imatinib alone (a) or in combination with 5 μ g/ml verapamil (b) or in combination with 5 μ M CsA (c). Symbols reflect different time points of the independent experiments (\circ ; Δ ; \square).

Figure 1 Description of the MDR phenotype in the investigated cell lines. (a) The expression pattern of potential candidates for an MDR phenotype-related protein in the investigated cell lines CCRF, HL-60, K562 and their respective drug-resistant counterparts (level of resistance is indicated – italic letters). Cells were maintained under routine conditions (sensitive cell lines) or in the presence of the indicated drug (CCRF-VCR – 0.1 μ M vincristin, HL-60 and K562–1 μ M, and/or 5 μ M of VP16, respectively). Western blot conditions were as described in the Materials and methods. Membranes were loaded with equal amounts of protein and were exposed to the film in parallel. Therefore, expression patterns reflect relative amounts of expression (between sensitive and resistant cell lines, and between the different cell lines used). Actin controls are shown in the bottom to ensure equal loading of protein in each lane. One representative determination was chosen out of three with equivalent results. (b) Rhodamin efflux from the sensitive K562 cell line and their respective multidrug-resistant counterparts. Cell lines were analyzed as it is described in the Materials and methods comparing intracellular rhodamin staining in samples treated with rhodamin alone (black) or after the coadministration of 5 μ M CsA + rhodamin (gray). (c) The expression pattern of one critical downstream effector of Bcr-Abl in CCRF-CEM, HL-60 and K562 cells, and their resistant counterparts. P-Crkl denotes the phosphorylated variant of the Crkl protein. Whole Crkl antigen was detected to assure equal loading in the lane.

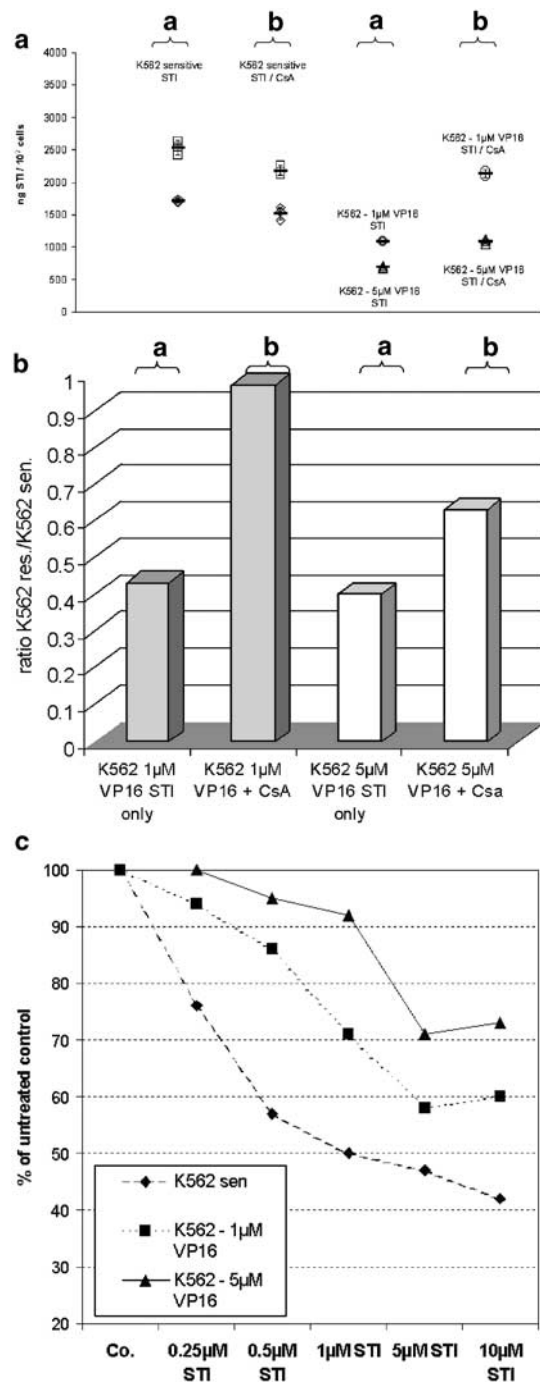
analysis (Figure 3c). In contrast, K562-1 μM VP16 and K562-5 μM VP16 cells show marked resistance to treatment with higher concentrations of imatinib. K562-1 μM VP16 do not respond to a concentration of 0.5 μM imatinib, whereas K562-5 μM VP16 tolerate imatinib concentrations even up to 1 μM without loss of viability (Figure 3c and trypan blue exclusion – data not shown).

As shown in Figure 3a, resistant K562 cells contain only about 30% of intracellular imatinib after incubation with 8.5 μM of imatinib as compared to the sensitive cell line. Absolute values indicate a stronger decline of intracellular imatinib levels in the K562-5 μM VP16 cells (Figure 3a). However, day-to-day variability may be responsible for this effect. Relative imatinib levels obtained by calculation of a ratio between the intracellular levels in resistant vs sensitive cell lines that were analyzed on the corresponding day demonstrated similar amounts of imatinib in K562-1 μM VP16 when compared to K562-5 μM VP16 cells (Figure 3b). However, when analyzing imatinib levels in K562-1 μM VP16 and K562-5 μM VP16 cells after the coadministration of CsA there is a clear increase in intracellular imatinib values in K562-1 μM VP16, which is similar to a sensitive cell line treated with imatinib (Figure 3a, b). In contrast, K562-5 μM VP16 cells show only a moderate increment of intracellular imatinib after the coadministration of CsA, indicating that Pgp is only partially blocked. We are thus able to demonstrate a dose-dependent relationship between the expression and function of Pgp in K562 cells and intracellular levels of imatinib, which supports the assumed properties of imatinib as a Pgp substrate.

Next, the functional effects of Pgp modulation in K562 cells with different resistance levels were tested. As shown in Figure 4a, the treatment of sensitive K562 cells lead to rapid dephosphorylation of p-Crkl. Scanning and densitometry analysis of immunoblots revealed a decrease in the ratio of phosphorylated/dephosphorylated Crkl from 72 to 3% (data from three independent experiments). In contrast, both resistant cell lines retained elevated levels of p-Crkl after imatinib treatment (phosphorylated/unphosphorylated in K562-1 μM VP16 vs K562-5 μM VP16–12 vs 23%). The modulation of Pgp function led to a reduction of p-Crkl (phosphorylated/unphosphorylated in K562-1 μM VP16 and K562-5 μM VP16–3 vs

10%, respectively). The same results were obtained when K562-1 μM VP16 cells were cotreated with the structurally unrelated compound verapamil (data not shown). Moreover, the modulation of Pgp function had a strong impact on the rate of apoptotic cells. As compared to sensitive K562 cells, K562-1 μM VP16 and K562-5 μM VP16 cells show marked resistance in apoptosis induction after imatinib treatment. However, CsA coadministration restores the response to imatinib and induces apoptosis in a similar proportion of resistant cells as compared to sensitive K562 cells. Again, the imatinib effect depends on the expression level of Pgp. High Pgp-expressing K562-5 μM cells show

Figure 3 Intracellular imatinib levels in K562 and drug-resistant K562 cells expressing different amounts of Pgp. (a) The determination of intracellular imatinib levels in parental K562 cells and in drug-resistant K562-1 μM VP16 and K562-5 μM VP16 cell lines. Cells were either treated with 5 $\mu\text{g/ml}$ (8.5 μM) imatinib alone (a) or in combination with 5 μM CsA (b) for 5 h. The means of two independent experiments that were determined in triplet each is indicated by a horizontal bar; s.d. is indicated. Determinations in resistant K562-1 μM VP16 cells (\square) were analyzed in parallel with sensitive K562 cells (\circ) at the corresponding day; resistant K562-5 μM cells (\triangle) were analyzed in another independent determination with the corresponding sensitive K562 cell line (\diamond). The determination of the ratio between intracellular imatinib levels detected in K562-1 μM VP16 cells, K562-5 μM VP16 cells vs K562-sensitive cells at the corresponding day. Cells were either treated with imatinib 5 $\mu\text{g/ml}$ (8.5 μM) (a) or treated with the combination of imatinib 5 $\mu\text{g/ml}$ (8.5 μM) and CsA 5 μM (b). MTT analysis of K562-sensitive, K562-1 μM VP16 and K562-5 μM VP16 cells that were exposed to different concentrations of imatinib. Cells were exposed for 48 h and MTT was performed as it is described in the Materials and methods. Results resemble the means of three independent experiments that were determined in triplet each. OD values of untreated control cells were set 100%. All other values refer to the untreated control.



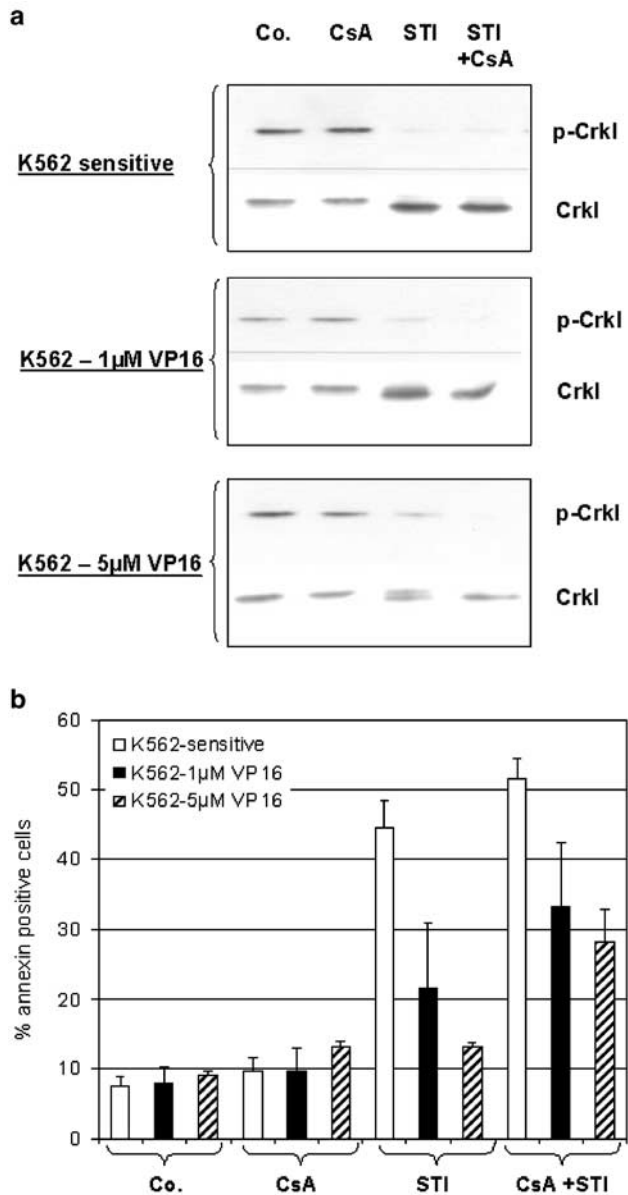


Figure 4 Modulation of Pgp function is restoring apoptotic response after STI571 treatment in resistant K562 cells. The effect of Pgp modulation treatment on phosphorylation pattern of Crkl. Sensitive and resistant K562 cell lines were treated either with CsA (5 μ M) alone for 3 h, imatinib (5 μ M) alone for 2.5 h or were pre-exposed to CsA (5 μ M) 30 min and then coincubated with imatinib (5 μ M) for additional 2.5 h. Thereafter, equal amounts of protein (40 μ g) were analyzed by immunoblotting against p-Crkl (where indicated). The blots were stripped and reprobated against Crkl (where indicated). The effect of CsA cotreatment on apoptotic cell death as determined by Annexin V staining. Sensitive and resistant K562 cell lines were treated either with CsA (5 μ M) alone for 48 h, imatinib (1 μ M) alone for 48 h or were pre-exposed to CsA (5 μ M) 30 min and then coincubated with imatinib (1 μ M) for additional 48 h. Values represent the means of three independent determinations \pm s.d.

decreased apoptotic cell rates after imatinib or imatinib and CsA treatment as compared to lower Pgp-expressing K562-1 μ M VP16 cells (Figure 4b).

Pgp modulation therapy in a patient with refractory BCR-ABL-positive ALL

A clinical protocol was developed that focused on Pgp modulation in relapsed BCR-ABL-positive patients. The protocol included a strategy to inhibit the Pgp-related drug efflux using CsA administration as previously reported in patients with relapsed AML.¹⁸ CsA administration was mainly directed to inhibit a potential imatinib efflux from leukemic blasts. Moreover, this strategy was combined with the application of high-dose cytosine arabinoside since the combination of imatinib and cytosine arabinoside could be shown to have synergistic antileukemic activity. Here, we report treatment results of a patient with BCR-ABL-positive ALL. A 24-year-old male patient with a BCR-ABL-positive c-ALL was referred to our department relapsing 2 months after allogeneic transplantation from an HLA-identical sibling. The patient presented with a high degree of marrow infiltration and a high peripheral blast count. The acquisition of a highly aberrant karyotype including another Philadelphia chromosome was seen (Table 2). Moreover, the patient's blasts were shown to effectively efflux rhodamin that was inhibited by CsA (Figure 5a). In parallel, a copy rate of MDR1 that was well above the median of eight healthy bone marrow donors was documented (Table 2). The patient had been treated with 800 mg imatinib about 2 weeks prior to admission to our hospital without any response. After informed consent, treatment was started with a bolus of CsA that was followed by continuous infusion of CsA for 72 h (Figure 5b). This combined treatment resulted in a decrease of blasts in the peripheral blood. Similarly, we observed a reduction in the phosphorylation status of the Bcr-Abl downstream effector P-Crkl (Figure 5c). Finally, the addition of cytosine arabinoside resulted in a clearance of blasts in the peripheral blood. There was no evidence of acute toxic side effects in the patient with respect to the renal or hepatic function. The only clinically apparent acute side effect was the development of mild edema.

Discussion

Resistance development is a multifactorial phenomenon in cells exposed to the kinase inhibitor imatinib.¹⁹ The amplification of the fusion gene can lead to increased Bcr-Abl activity thus overcoming the inhibiting function of imatinib. On the other hand, BCR-ABL-positive cells may become independent on Bcr-Abl function by the activation of other critical kinases as recently demonstrated for the SRC-LYN kinase pathway and Bruton's tyrosine kinase and show therefore reduced Bcr-Abl function.²⁰⁻²² Probably, the most important mechanism in imatinib resistance development is the occurrence of mutations in the ABL kinase domain that interfere with drug binding in the presence of retained ATP binding and catalytic activity. Recently, it was shown that mutations such as those at positions T315I cause an absolute refractoriness to imatinib, whereas other mutations (like E255V, H396P or Y253H) cause only intermediate drug resistance.⁷⁻⁹

The Pgp efflux pump enables efficient transport of a broad spectrum of lipophilic substances from the lipid bilayer to the outer leaflet of the cell membrane.²³ The chemical structure and lipophilic properties of imatinib suggest that the drug may be a substrate for Pgp. This suspicion was supported by recently published investigations by Mahon *et al*²⁴, who showed reduced cytotoxic activity of imatinib in BCR-ABL-positive and Pgp-expressing cells. Moreover, our data indicate that the intracel-

lular concentration of imatinib may be a critical component in the efficacy of the drug.

The results presented here clearly show that efficacy of imatinib treatment is associated with the accumulation of appropriate intracellular imatinib levels. Despite the fact that we cannot rule out completely other mechanisms of drug transport; the overexpression of Pgp and the obvious dependency of imatinib efficacy in the model of gradual increments in different resistant K562 cells suggest a causal role for Pgp in imatinib drug resistance. The results presented here indicate that targeting the Pgp efflux could modulate resistance to imatinib. This notion would be of clinical importance if BCR-ABL-positive cells express Pgp. As shown in a recent trial, Pgp expression is detected in about 60% of patients with advanced CML which therefore could possibly benefit from a combined therapy targeted against Bcr-Abl and Pgp.²⁵

From a clinical point of view, the development of resistance to imatinib may be further complicated by the diversity of mechanisms of drug resistance. Thus, increasing the intracellular levels of imatinib has been suggested in order to overcome the insensitivity of the Bcr-Abl kinase that is, for example, caused by mutation. This goal could be partially achieved by increasing extracellular concentrations of imatinib.¹⁰ However, in the presence of a significant MDR1 expression raising the extracellular imatinib concentration will not compensate the efficacy of the drug efflux pump. The upregulation of MDR1 has been associated with genetic instability that may even have given rise to secondary mutations in BCR-ABL-positive cells.²⁶ Indeed, the occurrence of markers of genetic instability has recently been shown to reduce the efficacy of imatinib therapy.²⁷ Therefore, it is possible that resistance to imatinib is associated with a complex mechanism that may be activated by

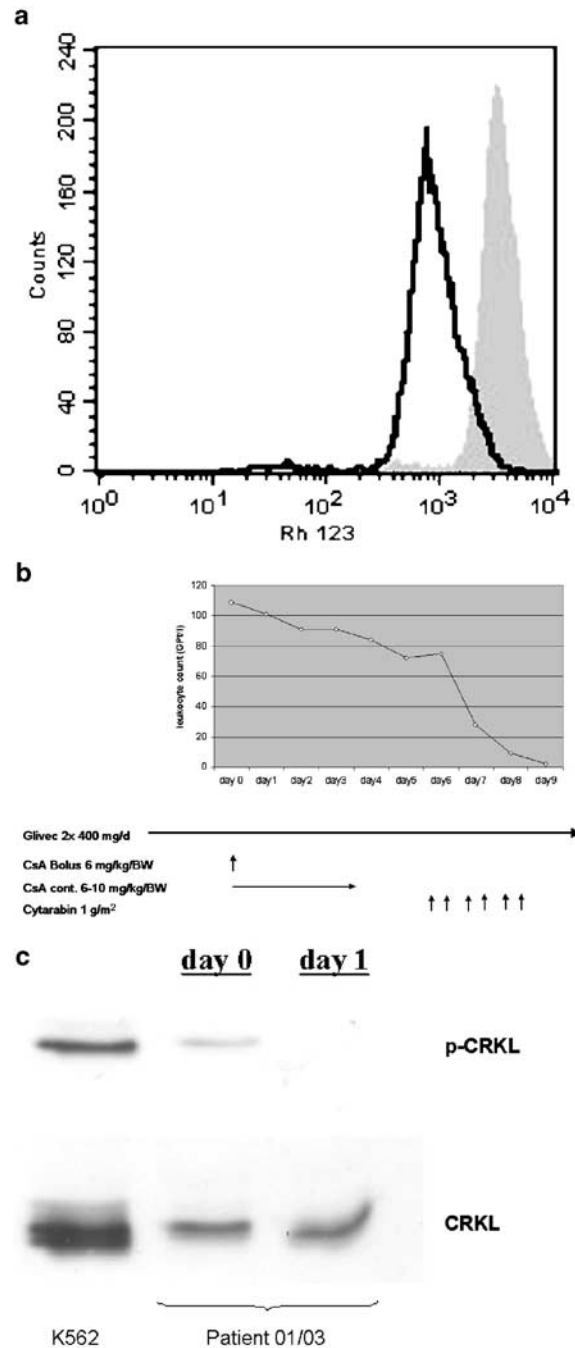


Figure 5 Effect of a CsA modulated imatinib therapy in a patient with relapsed BCR-ABL-positive ALL. (a) Rhodamin efflux in patient 01/03 blast samples. Cells were analyzed after a routine Ficoll isolation procedure as it is described in Materials and Methods comparing intracellular rhodamin staining in samples treated with rhodamin alone (black area) or after the coadministration of 5 μ M CsA + rhodamin (grey curve). (b) The treatment schedule of the patient 01/03 as compared to the blast amount in peripheral blood. (c) The phosphorylation pattern of Crkl in a patient treated with a combined treatment approach. Blast cells were analyzed while treating the patient with 800 mg imatinib/day (day 0) and 24 h after starting high-dose CsA administration + imatinib (day 1). Protein probes of patient 01/03 blasts were obtained as it is described in Materials and methods. Thereafter, equal protein amounts (40 μ g) were analyzed by immunoblotting against p-Crkl (where indicated). The blots were stripped and reprobated against Crkl (where indicated). K562 cells with previously shown strong phosphorylation of Crkl served as control.

Table 2 Patient characteristics

	Cytogenetics	Bone marrow	Peripheral blood	Immunophenotype	MDR expression (Taqman)
Patient 01/03	50,XY, t(9;22)(q34;q11.2), + ?17,+22, +der(22)t(9;22) (q34;q11)+i(7)(q10)	95% blasts, 5% lympho	Blast 94, Mye 0, Sta 0, Seg 0, Mon 0, Lym 6	CD 19: 84%, CD10: 83%	792 copies (median of eight healthy bone marrow donors: 364 copies)

genetic instability which even gives rise to mutations in the ABL kinase and MDR1/Pgp overexpression. So, a combination of MDR1/Pgp modulation and imatinib treatment would be beneficial especially for patients who acquired secondary chromosomal changes or show mutations in the ABL kinase. This notion is underlined here with a patient presenting both blasts with a high degree of genetic instability and MDR1 expression. In this case, clinical modulation of Pgp led to a biological response. Our data are in agreement with data obtained by Mahon *et al*²⁴, who recently reported *in vitro* efficacy of an MDR1 modulating strategy in blast samples of CML patients treated with imatinib. However, they could not demonstrate a significant Pgp expression raising the question of the specificity of the MDR1 modulating strategy in patient samples. Since we were able to show upregulated MDR1 transcripts by real-time PCR in this patient, we suspect a higher sensitivity for detecting potentially important quantities of MDR1 by PCR. Thus, even minor expression of MDR1, which is not detected by antibody-based methods, may be of clinical importance for critical concentrations of imatinib in BCR-ABL-positive cells. Clearly, the discovery of a positive effect of MDR1 modulation therapy in BCR-ABL-positive patients needs further substantiation to define its role in the treatment of patients who become resistant to treatment with commonly used dosing regimens of imatinib.

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References

- 1 Tsao AS, Kantarjian H, Talpaz M. STI-571 in chronic myelogenous leukaemia. *Br J Haematol* 2002; **119**: 15–24.
- 2 Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. Structural mechanism for STI-571 inhibition of Abelson tyrosine kinase. *Science* 2000; **289**: 1938–1942.
- 3 Druker BJ, Talpaz M, Resta DJ, Peng B, Buchdunger E, Ford JM *et al*. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001; **344**: 1031–1037.
- 4 Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM *et al*. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *N Engl J Med* 2001; **344**: 1038–1042.
- 5 le Coutre P, Tassi E, Varella-Garcia M, Barni R, Mologni L, Cabrita G *et al*. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. *Blood* 2000; **95**: 1758–1766.
- 6 Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN *et al*. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001; **293**: 876–880.
- 7 von Bubnoff N, Schneller F, Peschel C, Duyster J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. *Lancet* 2002; **359**: 487–491.
- 8 Azam M, Latek RR, Daley GQ. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. *Cell* 2003; **112**: 831–843.
- 9 Roumiantsev S, Shah NP, Gorre ME, Nicoll J, Brasher BB, Sawyers CL *et al*. Clinical resistance to the kinase inhibitor STI-571 in chronic myeloid leukemia by mutation of Tyr-253 in the Abl kinase domain P-loop. *Proc Natl Acad Sci USA* 2002; **99**: 10700–10705.
- 10 Kantarjian HM, Talpaz M, O'Brien S, Giles F, Garcia-Manero G, Faderl S *et al*. Dose escalation of imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukemia. *Blood* 2003; **101**: 473–475.
- 11 Mahon FX, Deininger MW, Schultheis B, Chabrol J, Reiffers J, Goldman JM *et al*. Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood* 2000; **96**: 1070–1079.
- 12 Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002; **2**: 48–58.
- 13 Giles FJ, Kantarjian HM, Cortes J, Thomas DA, Talpaz M, Manshour T *et al*. Multidrug resistance protein expression in chronic myeloid leukemia: associations and significance. *Cancer* 1999; **86**: 805–813.
- 14 Illmer T, Schuler US, Thiede C, Schwarz UI, Kim RB, Gotthard S *et al*. MDR1 gene polymorphisms affect therapy outcome in acute myeloid leukemia patients. *Cancer Res* 2002; **62**: 4955–4962.
- 15 Lamy T, Drenou B, Grulois I, Fardel O, Jacquelinet C, Goasguen J *et al*. Multi-drug resistance (MDR) activity in acute leukemia determined by rhodamine 123 efflux assay. *Leukemia* 1995; **9**: 1549–1555.
- 16 Bierhaus A, Illmer T, Kasper M, Luther T, Quehenberger P, Tritschler H *et al*. Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. *Circulation* 1997; **96**: 2262–2271.
- 17 Yu C, Krystal G, Varticovski L, McKinstry R, Rahmani M, Dent P *et al*. Pharmacologic mitogen-activated protein/extracellular signal-regulated kinase kinase/mitogen-activated protein kinase inhibitors interact synergistically with STI571 to induce apoptosis in Bcr/Abl-expressing human leukemia cells. *Cancer Res* 2002; **62**: 188–199.
- 18 List AF, Kopecky KJ, Willman CL, Head DR, Persons DL, Slovak ML *et al*. Benefit of cyclosporine modulation of drug resistance in patients with poor-risk acute myeloid leukemia: a Southwest Oncology Group study. *Blood* 2001; **98**: 3212–3220.
- 19 Gambacorti-Passerini CB, Gunby RH, Piazza R, Galiotta A, Rostagno R, Scapozza L. Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias. *Lancet Oncol* 2003; **4**: 75–85.
- 20 Hofmann WK, de Vos S, Elashoff D, Gschaidmeier H, Hoelzer D, Koefler HP *et al*. Relation between resistance of Philadelphia-chromosome-positive acute lymphoblastic leukaemia to the tyrosine kinase inhibitor STI571 and gene-expression profiles: a gene-expression study. *Lancet* 2002; **359**: 481–486.
- 21 Warmuth M, Simon N, Mitina O, Mathes R, Fabbro D, Manley PW *et al*. Dual-specific Src and Abl kinase inhibitors, PP1 and CGP76030, inhibit growth and survival of cells expressing imatinib mesylate-resistant Bcr-Abl kinases. *Blood* 2003; **101**: 664–672.
- 22 Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, Arlinghaus R *et al*. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. *Blood* 2003; **101**: 690–698.
- 23 List AF. Role of multidrug resistance and its pharmacological modulation in acute myeloid leukemia. *Leukemia* 1996; **10**: 937–942.
- 24 Mahon FX, Belloc F, Lagarde V, Chollet C, Moreau-Gaudry F, Reiffers J *et al*. MDR1 gene overexpression confers resistance to imatinib mesylate in leukemia cell line models. *Blood* 2003; **101**: 2368–2373.
- 25 List AF, Kopecky KJ, Willman CL, Head DR, Slovak ML, Douer D *et al*. Cyclosporine inhibition of P-glycoprotein in chronic myeloid leukemia blast phase. *Blood* 2002; **100**: 1910–1912.
- 26 Mickley LA, Spengler BA, Knutsen TA, Biedler JL, Fojo T. Gene rearrangement: a novel mechanism for MDR-1 gene activation. *J Clin Invest* 1997; **99**: 1947–1957.
- 27 Brummendorf TH, Ersoz I, Hartmann U, Bartolovic K, Balabanov S, Wahl A *et al*. Telomere length in peripheral blood granulocytes reflects response to treatment length with imatinib in patients with chronic myeloid leukemia. *Blood* 2003; **101**: 375–376.