Imatinib activity *in vitro* in tumor cells from patients with chronic myeloid leukemia in chronic phase and blast crisis

Ulla Olsson-Strömberg[a], Anna Åleskog[a], Anneli Björnberg[b], Martin Höglund[a], Bengt Simonsson[a], Mats Bengtsson[c], Gisela Barbany[d], Rolf Larsson[b] and Elin Lindhagen[b]

The aims of this study were to evaluate the feasibility of using the non-clonogenic fluorometric microculture cytotoxicity assay in drug sensitivity testing of tumor cells from patients with chronic myeloid leukemia. In nine samples (six chronic phase, three blast crisis), the drug sensitivities in tumor cells from blood versus from bone marrow and fresh tumor cells versus cryopreserved were compared. In 26 samples obtained in chronic phase (pretreatment), in six samples from patients in blast crisis and in the K 562 cell line, the activity of imatinib alone and in combination with cytarabine, vincristine, daunorubicin, interferon, arsenic trioxide and homoharringtonine was evaluated. All chronic myeloid leukemia chronic phase samples were sensitive to imatinib, with a mean IC_{50} at 10.3 μmol/l. The chronic myeloid leukemia samples from blast crisis ($n=6$) were significantly more sensitive to imatinib than the samples from chronic phase ($n=26$) ($P<0.05$), with an IC_{50} mean at 0.4 μmol/l. In blast crisis samples, significant positive interaction effects were observed between imatinib and all other tested drugs except for interferon. In chronic phase samples, interferon, daunorubicin and arsenic trioxide were the drugs with the highest frequency of positive interactions with imatinib ($P<0.05$). We conclude that the fluorometric microculture cytotoxicity assay may be a useful method for drug sensitivity testing in chronic myeloid leukemia patient samples from both chronic phase and blast crisis, and that testing primary tumor cells may have advantages over cell line studies. Imatinib shows a higher *in vitro* activity and more positive drug interactions in cells from blast crisis than chronic phase chronic myeloid leukemia patients. Combinations between imatinib and interferon, daunorubicin and arsenic trioxide may be interesting for future clinical trials in patients with chronic myeloid leukemia chronic phase. *Anti-Cancer Drugs* 17:631–639

Anti-Cancer Drugs 2006, **17**:631–639

Keywords: chronic myeloid leukemia, drug combinations, fluorometric microculture cytotoxicity assay, imatinib mesylate, *in vitro* drug sensitivity

Departments of [a]Hematology, [b]Clinical Pharmacology, [c]Clinical Immunology and [d]Clinical Genetics, University Hospital, Uppsala, Sweden.

Correspondence to U. Olsson-Strömberg, Department of Hematology, University Hospital, 751 85 Uppsala, Sweden.
Tel: +46 18 611 00 00; fax: +46 18 51 01 33;
e-mail: ulla.olsson.stromberg@akademiska.se, ulla.stromberg@talk21.com

Sponsorship: Imatinib was kindly supplied from Novartis Pharma.

Received 16 November 2005 **Revised form accepted** 11 March 2006

Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder. More than 95% of the patients show a reciprocal translocation between chromosome 9 and 22, the so-called Philadelphia (Ph) chromosome [1]. This translocation results in the head-to-tail fusion of the breakpoint cluster region (BCR) gene on chromosome 22 at band q11 with the ABL proto-oncogene located on chromosome 9 at band q34 [2]. The p210 BCR/ABL transcript is expressed in almost all Ph^+ CML cases. The BCR/ABL protein has an antiapoptotic activity and is believed to play a central role in the development of CML [3,4]. At present, allogeneic stem cell transplantation is the only curative approach for CML [5–9]. Treatment with interferon alone or in combination with hydroxyurea or cytarabine prolongs the time to blast crisis (BC) as well as survival, compared with treatment with hydroxyurea or busulfan alone [10–13]. Imatinib (imatinib mesylate, STI 571 Glivec; Novartis, Basel, Switzerland), a signal transduction inhibitor that acts specifically on the BCR/ABL tyrosine kinase [14,15], has emerged as the first-line treatment in CML with a complete cytogenetic remission rate of 76% after a median of an 18-month follow-up [16–18].

In the development of a new drug or drug combinations, *in vitro* studies of the effect on human cell lines and, in many cases, on primary tumor cells are performed. Several methods for testing cellular drug sensitivity *in vitro* have been described. They can be divided into two main groups: cell proliferation assays (clonogenic assay and short-term assays) and total cell kill (TCK) assays. TCK assays are believed to measure cell death in the whole population of tumor cells [e.g. methyl-thiazol-tetrazolium assay, differential staining cytotoxicity assay and fluorometric microculture cytotoxicity assay (FMCA)]. In most *in vitro* studies on primary CML chronic phase (CP) cells clonogenic assays are used, but this method is quite time consuming [19,20]. The

0959-4973

clonogenic assay relies on the proliferative capacity of a small fraction of cells, presumed to be stem cells. The drug effects are measured only in a small fraction of proliferating cells and effects on resting cells, which might have proliferative potential *in vivo*, are not measured. Clonogenic and differential staining cytotoxicity assays have shown good predictive capacity, but are intensive and time consuming [21–24]. The FMCA is a sensitive TCK assay capable of detecting small numbers of cells, and is easy to perform and has a high capacity [24]. The non-clonogenic cytotoxicity assay FMCA has been found to be suitable for drug sensitivity testing of leukemia and lymphoma cells [24–27] as well as of solid tumors, but has not previously been evaluated in primary CML tumor cells.

Most *in vitro* studies of imatinib have been performed in CML cell lines, which originate from patients in BC and not in the CP. Established cell lines, however, might have lost some of their original tumor characteristics. Thus, the use of *in vitro* assays based on patient tumor cells might be an attractive alternative. In the clinic, a small fraction of CML patients are primarily resistant to imatinib, and responding patients may ultimately develop resistance and relapse. Combining imatinib mesylate with other antileukemic agents may be one way to further increase the remission rate and to prevent the outgrowth of resistant clones. Indeed, *in vitro* studies, using BCR/ABL-positive cell lines, have shown synergistic effects of imatinib and cytarabine [19,28], vincristine [29], and interferon [19,29] as well as both additive and synergistic effects of imatinib combined with daunorubicin [19] and homoharringtonin [20]. Alternatively, agents that are still active in imatinib-resistant cells, such as BMS-354825 (Bristol Myers Squibb, Wallingford, Connecticut, USA) and AMN 107, may be used [30].

One aim of this study was to evaluate the feasibility of using the non-clonogenic cytotoxicity assay FMCA in drug sensitivity testing of tumor cells from patients with CML in CP and BC. The other aim was to study the effect of the BCR/ABL tyrosine kinase inhibitor imatinib alone and in combination with other antileukemic agents.

Materials and methods

Patient population

The local ethics committee at the Uppsala University Hospital approved the study.

Methodological study

In nine samples (six CP, three BC), the drug sensitivities in tumor cells from blood versus from bone marrow and fresh tumor cells versus cryopreserved were compared. The CML diagnosis was established by morphology, conventional karyotyping and reverse transcriptase-polymerase chain reaction [31]. All samples, both diagnosis and BC, were from patients with 100% Ph^+, as assessed by karyotyping. Samples were taken from both bone marrow and peripheral blood at the same occasion. After cell preparation, one portion of the cells was used directly, and the rest was frozen and analyzed later.

Imatinib single-drug study

For this study, tumor cells from 37 newly diagnosed, untreated CML patients in CP, eight from CML patients in BC and three from acute myeloid leukemia (AML) patients were used. The presence of t(9;22) was assessed by bone marrow cytogenetic analysis and blood real-time reverse transcriptase-polymerase chain reaction in all CML patients. Both the diagnosis and BC samples were 100% Ph^+, and the BCR/ABL mRNA levels were very high, as seen in newly diagnosed CML patients.

Cytogenetic analysis revealed the classical balanced reciprocal translocation t(9;22) as the sole abnormality in all but one CP sample. One patient had a variant translocation with the presence of additional material of unknown origin on derivative chromosome 9. The same variant translocation and no additional aberrations were observed at BC. The karyotypes from the samples in BC are presented in Table 1. The BC and the CP samples were from patients not treated with imatinib. All cells were cryopreserved. In addition to the primary tumor cells, the K 562 cell line (kind gift from Dr A. Gruber, Karolinska Hospital), a BCR/ABL-positive human cell line derived from a CML patient in BC, was used. Triplicate cultures of the cell line were used. The K 562 cell line was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

Imatinib combination study

Tumor cells from 37 CML patients in the CP at diagnosis and eight from CML patients in BC (the same samples as in the imatinib single-drug study) were used. In 11 of the samples, additional data were collected for arsenic trioxide.

Table 1 Karyotype at diagnosis and at blast crisis for six chronic myeloid leukemia patients

Patient no.	At diagnosis	Karyotype at blast crisis	Blast crisis
1	46,XY,t(9;22)(q34;q11)	46,XY,del(6)(q?21), – 7,t(9;22)(q34;q11),del(13)(q?22), + mar	myeloid
2	46,XX,t(9;22)(q34;q11)	45,XX, – 5,t(9;22)(q34;q11), – 18, + mar	myeloid
3	46,XX,add(9)(q34),der(22)t(9;22)(q34;q11)	46,XX,add(9)(q34),der(22)t(9;22)(q34;q11)	myeloid
4	46,XY,t(9;22)(q34;q11)	46,XY,t(9;22)(q34;q11)i(17)(q10)[12]/48;XY,idem + der(22)t(9;22)q34;q11)[8]	myeloid
5	46,XY,t(9;22)(q34;q11)	55–56,XY, + 6,t(9;22)(q34;q11), + 11, + 13, + 14, + 15, + 15, + 17, + 19, + 20, + 21, + der(22)t(9;22)(q34;q11), + 2 ~ 6 mar[cp5]	lymphoblastic
6	46,XX,t(9;22)(q34;q11)	not done	myeloid

Five patients had a myeloid blast crisis and one patient had a lymphoid blast crisis.

Cell preparation

Tumor cells from bone marrow or peripheral blood were isolated within 24 h after collection by 1.077 g/ml Ficoll-Isopaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Viability was determined by the Trypan blue exclusion test and the proportion of tumor cells was judged by the inspection of May–Grünwald–Giemsa-stained cytocentrifugate preparations on days 0 and 3. Culture medium RPMI 1640 (Hyclone, Northumberland, UK) supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/l glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin was used throughout. Cells were cryopreserved in 10% dimethylsulfoxide (Sigma, St Louis, Missouri, USA) and 90% fetal calf serum by initial freezing for 24 h in −70°C followed by storing in a −150°C freezer. Both fresh and cryopreserved cells were used in the methodological study, and only cryopreserved in the imatinib studies.

Drug preparation

The cytotoxic drugs were in general tested at a concentration associated with a large scatter of survival indices as observed in previous studies on hematological human cells [25]. The choice of concentration was made purely from *in vitro* data aiming at optimizing the conditions for differentiation between sensitive and resistant tumor samples [32].

Methodological study

For the methodological study, all drugs were chosen on the basis of data from previous studies in CML cells; cytarabine 2 and 10.2 µmol/l, melphalan 8.2 µmol/l, cladribine 0.7 µmol/l, fludarabine 8.7 µmol/l, mitoxantrone 1.1 µmol/l, cisplatin 6.6 µmol/l, etoposide 8.5 µmol/l, vincristine 6 µmol/l, 6-thioguanin 59 µmol/l, prednisolone 138 µmol/l, idarubicin 0.2 µmol/l, amsacrine 2.5 µmol/l, and doxorubicin 0.9 µmol/l.

Imatinib single-drug study

Imatinib was tested at a 10-fold serial dilution ranging from 100 to 0.01 µmol/l. The concentration of 1.0 µmol/l imatinib resulted in approximately 50% cell survival of Ph^+ cells and was chosen for the combination study.

Imatinib combination study

The cytotoxic agents in this study were selected on the basis of clinical usefulness in CML or from the literature data. To study whether the response of imatinib was enhanced when acting together with other antileukemic agents, the leukemia cells were exposed to imatinib in combination with the following six cytotoxic drugs: cytarabine, vincristine, daunorubicin, interferon, arsenic trioxide and homoharringtonine. The concentrations for the other drugs were based on data from previous *in vitro* studies on CML cells and chosen to induce intermediate cytotoxicity; cytarabine 10 µmol/l, vincristine 0.5 µmol/l, daunorubicin 0.2 µmol/l, arsenic trioxide 1 µmol/l, interferon 50 000 IE/ml and homoharringtonine 0.1 µmol/l.

Nine different concentrations of imatinib ranging from 0.12 to 32 µmol/l and nine different concentrations of arsenic trioxide ranging from 0.06 to 8 µmol/l were used for a more thorough interaction evaluation using the median effect method.

All drugs were obtained from commercial sources. Imatinib was kindly provided from Novartis and dissolved in dimethylsulfoxide to a concentration of 10 mmol/l. Ninety-six-well V-shaped microtiter plates were prepared with 20 µl/well of drug solution in triplicate at 10 times the desired final concentration with the aid of a programmable pipetting robot (Biomech 2000; Beckman Instrument, Fullerton, USA).

Fluorometric microculture cytotoxicity assay

The FMCA is a TCK assay, based on the measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes as described in detail previously [24,33]. Briefly, 180 µl of the tumor cell suspension (50 000–100 000 cells/well) was added into 96-well micro-titer plates preprepared with drug solution. The culture plates were incubated for 72 h, after which the plates were centrifuged (200 *g*, 5 min) and the medium was removed. After one wash with phosphate-buffered saline (PBS), 100 µl/well of *N*-2-hydroxyl piperazine-*N*′-2-ehane sulfonic acid, buffered saline containing FDA (10 µl/ml) was added column wise. The plates were incubated for 40 min before reading the fluorescence in a scanning fluorometer, Fluoroscan II (Lab Systems, Helsinki, Finland). The fluorometer was blanked against wells containing PBS including the dye, but without cells. Standard quality criteria for a successful assay included a fluorescence signal in control cultures of above 5 × mean blank values and a mean coefficient of variation (CV) in control cultures below 30%. The results obtained were presented as survival index (SI) defined as fluorescence in percent of control cultures with blank values subtracted. Low numerical values indicate a high cytotoxic effect.

Definitions and statistical analysis

IC_{50} was defined as the drug concentration resulting in 50% cell survival compared with the control, interpolated from the concentration–effect curve. Mean values for different groups were compared using the Mann–Whitney test for unpaired comparisons. To compare samples from peripheral blood versus bone marrow and cryopreserved samples versus fresh, Wilcoxon's test was used. Two-sided *P*-values with a significance limit of 0.05 were used throughout.

When the effect of drug combinations was analyzed, the cytotoxic effect of the combination was compared with

the effect of the most active constituent alone (best drug). If the ratio between the observed SI and the SI after treatment with the most effective single agent was 0.8–1.2, the effect of the combination was considered to be caused by the best drug only. Ratios below 0.8 were defined as a positive interaction and above 1.2 as antagonism. All statistical analyses were performed using Stat View 4.01 software program (Abacus Concept, Berkeley, California, USA).

The 'best drug model' for interaction between two drugs was compared with the median effect method proposed by Chou and Talalay [34]. In 11 of the samples, a more thorough evaluation was performed of the drug interaction between imatinib and arsenic trioxide. We did not have enough tumor cells from patients to do it with all drugs, so we choose arsenic trioxide. In this study, nine concentrations (2-fold dilutions) of imatinib (0.12–32 μmol/l) and arsenic trioxide (0.06–16 μmol/l) and a combination between the two drugs in a fixed ratio (2:1) was tested. The concentrations were chosen to span a large effect range and the combination ratio was chosen to represent equitoxic doses of the two drugs. Drug interaction was evaluated using the combination index (CI) equation with the software CalcuSyn (Biosoft, Ferguson, Missouri, USA) [35], assuming that the substances act mutually non-exclusive (conservative isobologram) [34]. A CI was simulated for 50% drug effect and a CI of 1 indicated additive interaction. A CI below 0.8 was arbitrarily considered as synergy and above 1.2 as antagonism. A comparison between the result of this model and the effect of the best constituent alone for imatinib 4 μmol/l + arsenic trioxide 2 μmol/l was performed using data from the same experiment.

Results

Methodological study

The overall technical success rate was 90%. The most common cause of assay failure both in CP and BC samples was too low signal in controls compared with blanks. No difference in drug sensitivity between tumor cells obtained from peripheral blood and bone marrow was found (not shown). On the other hand, there was a tendency for the cryopreserved cells to be more drug sensitive than fresh cells, reaching statistical significance only for vincristine and fludarabine (not shown). The median viability for the fresh cells was 98%, compared with 87% for the cryopreserved cells, and FMCA was performed only when the viability was above 70%. Considering this, only cryopreserved cells were used in the imatinib single and combination studies.

Imatinib single-drug study

The technical success rate of samples with sufficient number of cells was 32/45 (70%). The cause of assay failure both in CP and BC samples was too low signal in controls compared with blanks ($n = 13$). All CML samples were sensitive to imatinib. IC_{50} (mean) was 10.3 μmol/l for the CP samples, 0.4 μmol/l for the CML BC samples and 0.7 μmol/l for the K 562 cell line (Fig. 1). The CML samples from BC were significantly ($P < 0.05$) more sensitive to imatinib than the samples from diagnosis at the concentrations 1, 10 and 100 μmol/l (Mann–Whitney test). The AML samples tended to be less sensitive than the CML BC samples (NS).

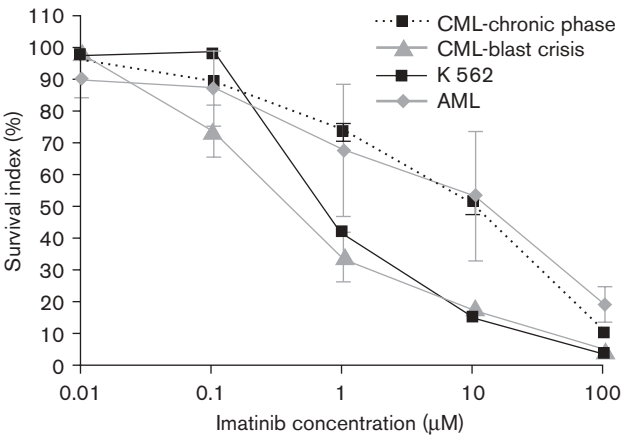
Imatinib combination study

Arsenic trioxide, interferon and daunorubicin were the drugs with the highest frequency of positive interactions with imatinib in CML CP samples, defined as an effect of the combination higher than the effect of the most active constituent alone. According to the 'best drug model', imatinib was found to have positive interactions with arsenic trioxide in 54%, with interferon in 48% and with daunorubicin in 42% of the CML CP samples. No samples showed antagonistic interactions. (Table 2 and Fig. 2a). Imatinib in combination with cytarabine, vincristine, arsenic trioxide and homoharringtonine was found to have positive interactions in 100% of the BC samples tested (Table 3 and Fig. 2b). Remarkably, interferon in combination with imatinib had an antagonistic effect in two of the BC samples (33%). Notably, tumor cells from patients obtained in BC were more sensitive to combinations (imatinib + other cytotoxic drug) than samples from CP ($P < 0.05$, Mann–Whitney test).

Imatinib in combination with arsenic trioxide

When investigating imatinib in combination with arsenic trioxide, the effect of the combination in sample 6 tended to be higher than that of the single agents alone at

Fig. 1



Mean concentration–effect curve for imatinib in samples from chronic myeloid leukemia (CML) patients in chronic phase ($n = 26$), in blast crisis ($n = 6$), in the K 562 cell line and acute myeloid leukemia (AML) samples ($n = 3$). The error bars show ± SEM.

Table 2 Interaction between imatinib and six other anticancer agents in tumor cells from patients with chronic myeloid leukemia in chronic phase

Drug combination Imatinib and	*n*	Positive interaction	As best drug	Antagonistic
Cytarabine	25	5 (20%)	20 (80%)	0
Arsenic trioxide	24	13 (54%)	11 (46%)	0
Vincristine	26	5 (19%)	21 (81%)	0
Daunorubicin	26	11 (42%)	15 (58%)	0
Interferon	25	12 (48%)	13 (52%)	0
Homoharringtonine	26	3 (12%)	23 (88%)	0

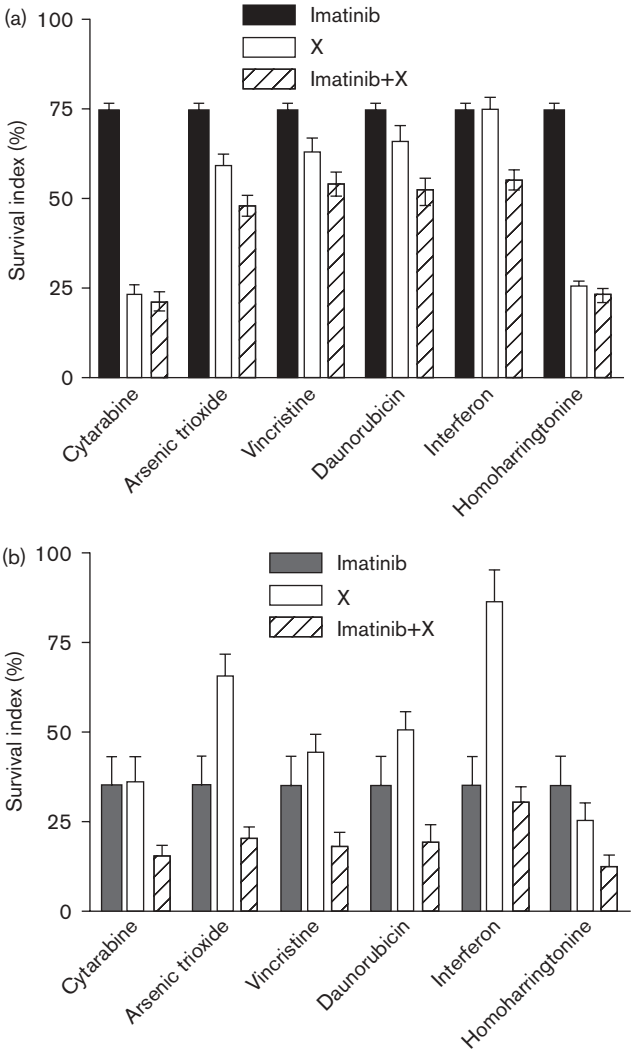
The ratio between the measured cell survival index of the drug combination and best single drug was calculated. A ratio below 0.8 was interpreted as a positive interaction, between 0.8 and 1.2 as best drug, and above 1.2 as antagonistic effect according to the 'best-drug model'.

most concentrations (Fig. 3a). The CI analysis showed an additive interaction at low effect levels, but synergy at higher levels (Fig. 3b). This was reflected in a CI of 0.88 at ED_{50} and a best drug ratio of 0.75 (Table 4). On the other hand, the sample from patient 10 showed a combination effect closer to the effect of arsenic trioxide alone (Fig. 3c), which was reflected as CI values above or around 1 at most effect levels (Fig. 3d) (CI 1.28 at ED_{50}, best drug ratio 0.95, Table 4). When the two methods of interaction evaluation for arsenic trioxide in CML CP samples were compared, both methods indicated a modest combination effect. Both methods resulted in an additive (six out of 11 for CI method) or best drug (nine out of 11 for best drug method) interaction for most samples (Table 4). Two samples showed a slightly positive interaction in the best drug model and two were considered synergistic using the median effect method with the limit of 0.8 used for synergy. Three samples showed an antagonistic interaction using the CI method.

Discussion

In vitro cytotoxicity assays are important tools in the preclinical development of new anticancer agents. In previously published *in vitro* experiments with CML patient samples, the clonogenic assay has been most commonly used, measuring the proliferative capacity of a small fraction of cells presumed to be stem cells. The FMCA, used in this study, is a TCK assay and is believed to measure cell death in the whole tumor cell population. The FMCA is easier to perform and less time consuming than a clonogenic assay, but requires a fairly pure tumor cell population, as it cannot distinguish between normal and malignant cells. In this experimental setting, therefore, we chose to assay only CML samples that were 100% Ph^+. Others have shown that 100% cytogenetic Ph-positivity corresponds to 80–92% BCR/ABL fusion measured with fluorescence *in situ* hybridization [36]. This can still be considered a high purity of malignant cells and a tumor cell fraction exceeding 70% is the quality criterion normally used for the FMCA. The need for a high proportion of Ph^+ cells in the cell preparation currently makes testing of samples from drug-treated

Fig. 2



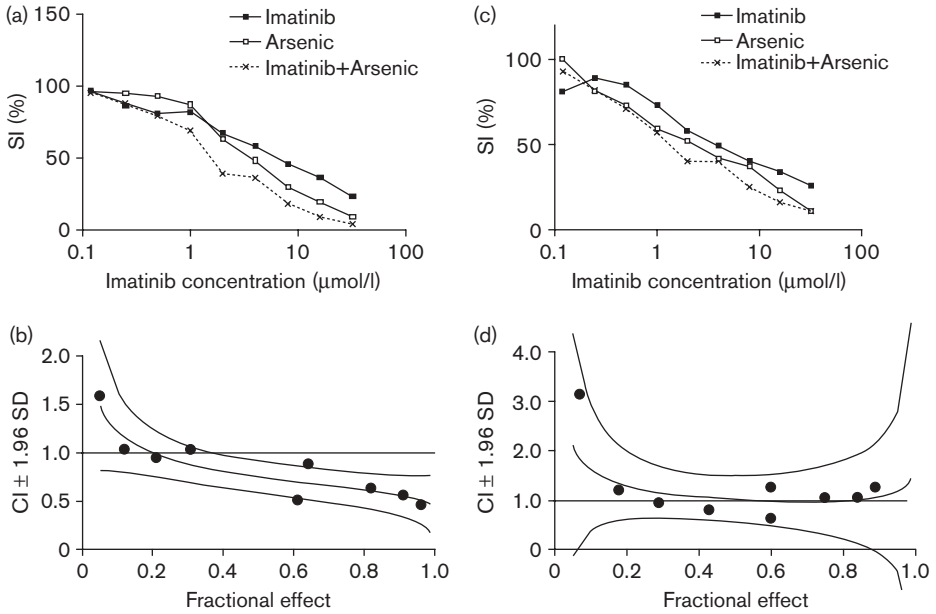
The mean survival index in samples from chronic myeloid leukemia (CML) patients in chronic phase ($n=26$) for imatinib and six drugs as single drugs, and in combination (a). Samples from CML patients in blast crisis ($n=6$) for imatinib and six drugs as single drugs, and in combination (b). The error bars show ±SEM.

Table 3 Interaction between imatinib and six anticancer agents in tumor cells from patients with chronic myeloid leukemia in blast crisis ($n=6$)

Drug combination Imatinib and	*n*	Positive interaction	As best drug	Antagonistic
Cytarabine	6	6 (100%)	0	0
Arsenic trioxide	6	6 (100%)	0	0
Vincristine	5	5 (100%)	0	0
Daunorubicin	5	4 (80%)	1	0
Interferon	6	1 (17%)	3 (50%)	2 (33%)
Homoharringtonine	5	5 (100%)	0	0

The ratio between the measured cell survival index of the drug combination and best single drug was calculated. A ratio below 0.8 was interpreted as a positive interaction, between 0.8 and 1.2 as best drug, and above 1.2 as antagonistic effect according to the 'best-drug model'.

Fig. 3



Cell survival after treatment with different concentrations of imatinib, arsenic trioxide and the combination of the two in chronic myeloid leukemia chronic phase samples from patient nos 6 (a) and 11 (c). Data analyzed with the combination index (CI) method assuming non-mutual exclusivity using CalcuSyn. CI as a function of effect level is shown for samples from patient nos 6 (b) and 11 (d). SI, survival index.

Table 4 Interaction between imatinib and arsenic trioxide in tumor cells from 11 patients with chronic myeloid leukemia in chronic phase

Patient no.	Combination index (ED_{50})	Best drug ratio
1	1.09	1.02
2	1.03	0.86
3	1.00	0.98
4	0.6	0.73
5	1.00	0.98
6	0.88	0.75
7	0.64	0.89
8	1.41	0.90
9	1.04	0.80
10	1.28	0.95
11	2.03	1.17

Two different methods of data evaluation are presented. The combination index is simulated at 50% drug effect using CalcuSyn software assuming mutual non-exclusivity. Best drug ratio is the ratio between the observed survival index after treatment with a fixed combination of imatinib and arsenic trioxide (4 μmol/l + 2 μmol/l), and the effect of the most effective single agent. Ratios below 0.8 are marked as positive interaction

patients difficult, because the separation of malignant cells from normal granulocytes is not possible. Many studies have been performed using the FMCA in other hematological malignancies as well as in solid tumors [24–27,33], but this is the first systematic investigation of the feasibility of assaying primary CML tumor cells.

Some limitations of the present approach should be noted. From a theoretical point of view, short-term *in vitro* assays (methyl-thiazol-tetrazolium, FMCA, ATP) measuring early cell damage in the entire CML cell population (the majority being Ph^+ quiscent G_0 cells) will primarily reflect short-term clinical responses although it has been argued that such assays may also accurately predict for a multiple-log stem cell kill and meaningful (long-term) clinical responses in several diagnoses [23]. Emprically, these short-term assays have in general been shown to compare favorably with clonogenic assays in predicting clinical responses. One problem with classical clonogenic assays is the possibility that true tumor stem cells determining long-term outcome may be largely non-dividing (G_0) cells, whereas cells forming colonies are exclusively dividing cells. This appears to be the case for CML containing a small fraction of very early BCR/ABL-positive quiescent progenitors sustaining the disease. It would be interesting, albeit technically difficult, to compare the bulk mass of mature CML cells with those of the Ph^+ quiescent stem cells with respect to imatinib sensitivity. Speculatively, the mature CML cells might reflect the sensitivity of the target quiescent stem cells better than the dividing stem cells detected by the clonogenic assay. In any case, the short-term assays like the FMCA will at least be able to mirror the short-term clinical effects of imatinib on the bulk mass of CML cells.

The observation that cryopreserved cells tended to be more drug sensitive than fresh cells suggests that it may not be optimal to use fresh and cryopreserved samples together in the same study. For our future prospective studies, fresh samples may be preferred because of better

viability. No difference in drug sensitivity between tumor cells obtained from peripheral blood and bone marrow was observed, indicating that peripheral blood can be used to make sampling easier for the patient.

Tumor cell lines are the most commonly used model system in cancer drug development, but the use of primary cultures of tumor cells from patients has gained increased interest [37]. A difference exists in using cell lines and primary cells, as the human tumor cell lines are generally high proliferating, which is not the case for primary tumor cells. The primary patient cells in general share more properties with the tumor cells to be treated in the clinical situation. Primary cultures have been shown to more adequately reflect the disease-specific activity of new compounds and may therefore be more relevant than cell lines when the effect of new drugs on different tumor types is studied [37].

The diagnosis-specific activity of cytotoxic drugs measured in tumor cells from patients has been shown to correlate well with the clinical disease-specific activity pattern, indicating usefulness in the selection of diagnoses for clinical phase II studies [37,38]. FMCA on primary tumor cells has also been used to differentiate the *in vitro* drug effects between different diagnostic subgroups in chronic lymphocytic leukemia, AML and acute lymphoblastic leukemia [39–41], and to relate *in vitro* drug resistance to prognosis and survival in childhood AML and ALL [27,42]. In CML primary cells, the relationship to clinical outcome has not been studied.

Most of the published *in vitro* studies of imatinib are performed in CML cell lines, which originate from patients in BC [28,29]. Some studies also use a small ($n = 1$–5) number of samples from CML patients [19,20]. The current study is thus the first investigation showing the activity of imatinib in a larger material of primary CML samples from both CP and BC. All CML cell lines available originate from patients in BC, and the current results indicate a similar activity of imatinib in the K 562 cell line and the BC samples. Using CML cell lines to draw conclusions concerning CML in CP may be suboptimal, risking an overestimation of drug efficacy and combination effects. Our data suggest that primary CML CP samples, as in this study, are preferred for use in *in vitro* studies.

Imatinib has a good activity in patients with CML in CP [17,18], whereas, in accelerated or phases of CML, the clinical effects of imatinib are moderate and transient, and remissions are short [43,44]. In this study, however, the BC samples were significantly more sensitive to imatinib than the samples taken in CP at diagnosis, including the two samples with additional Ph chromosomes, which is a known mechanism to render the cells relatively resistant to imatinib. The reasons for this are still partly unclear. One reason may be that BC cells are more immature cells, which in general have less mechanisms of protection against foreign stimuli. This phenomenon has also been observed in chronic lymphocytic leukemia, where the more immature unmutated subgroup is clinically more aggressive but also tend to be more drug-sensitive *in vitro* [39]. Another potential explanation may be a higher proliferation capacity of BC cells, but this explanation may in this case be less likely as most primary cells do not seem to proliferate under the current assay conditions. In the clinical setting, factors other than cellular drug sensitivity also come into play. For example, the tumor cell regrowth potential may be higher in BC cells, indicated by transient drug effects in this patient group. This can result in treatment failure, but cannot be measured by a TCK assay as the FMCA. Maybe the CML CP samples reflect the clinical situation better, and therefore it would be of importance to use patient samples. The K 562 cell line had a drug sensibility comparable to that of the BC samples.

As imatinib is an inhibitor of the BCR/ABL tyrosine kinase expressed by the Ph chromosome, diseases with Ph^+ cells would be expected to be more imatinib-sensitive than Ph^- diseases. This was the case in the diagnoses tested in the current study, and was observed also in the study by Hallbook *et al.* [41] in which normal lymphocytes responded to imatinib only at 100 µmol/l. The same study also showed a difference in response between Ph^+ and Ph^- ALL cells, when exposed to imatinib.

As primary and acquired resistance to imatinib therapy is a major problem in CML therapy today, combination of imatinib with other CML active agents is of large interest. Most previous combination studies have been performed in BCR/ABL-positive cell lines, and have shown the synergistic effects of imatinib and cytarabine, vincristine and interferon, and both additive and synergistic effects of imatinib combined with daunorubicin and homoharringtonin [19,20,29,45,46]. In the current study, higher combination effects were generally observed in BC cells than in cells from CP. The BC results are similar to previous results published using CML cell lines, as well as results on Ph^+ ALL cells [41]. Generally, smaller combination effects exist in cells from CP, indicating that cell line studies may overestimate the effects in CML CP.

In the current combination study, interferon, daunorubicin and arsenic trioxide were the drugs with the highest frequency of positive interactions with imatinib in the samples in CP. In general, the combination effects were larger in BC cells, but interestingly this was not the case for interferon. Interferon in combination with imatinib even showed an antagonistic effect in two of the BC samples. This may reflect the clinical situation, where interferon is used only in patients in CP. The exact

molecular mechanism behind the antileukemic activity of interferon remains to be elucidated, but as a large fraction of the effect is indirect and not a cellular cytotoxicity, it may not be possible to measure the full effect of this agent by using the FMCA. In clinical studies of combinations between imatinib and interferon and cytarabine [47] in CML CP, the dose of imatinib has been maintained at or near 400 mg/day, whereas the second drug dose had to be reduced.

The information obtained by testing single concentrations in combination studies is limited, because combination effects may differ between effect levels and drug ratios. It may, however, be a valuable tool for screening and getting indications of interesting combinations for further testing. It is also feasible to perform when there is a limited amount of primary tumor cells as in this study. The CI method using a larger range of concentrations gives more information. The current data indicate similar results for the combination between imatinib and arsenic at intermediate drug effect levels with both methods.

We conclude that the FMCA may be a useful method for drug sensitivity testing in CML patient samples from both CP and BC, and that testing primary tumor cells may have advantages over cell line studies. Imatinib shows a higher *in vitro* activity and more positive drug interactions in cells from BC than CP CML patients. Combinations between imatinib and interferon, daunorubicin and arsenic trioxide may be interesting combinations for future clinical studies in patients with CML in CP.

References

1 Kantarjian HM, Deisseroth A, Kurzrock R, Estrov Z, Talpaz M. Chronic myelogenous leukemia: a concise update. *Blood* 1993; **82**:691–703.

2 Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of *abl* and *bcr* genes in chronic myelogenous leukaemia. *Nature* 1985; **315**:550–554.

3 Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the $P210^{bcr/abl}$ gene of the Philadelphia chromosome. *Science* 1990; **247**:824–830.

4 Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of *bcr–abl* oncogene products. *Science* 1990; **247**:1079–1082.

5 Speck B, Bortin MM, Champlin R, Goldman JM, Herzig RH, McGlave P, *et al.* Allogeneic bone-marrow transplantation for chronic myelogenous leukaemia. *Lancet* 1984; **1**:665–668.

6 McGlave PB, De Fabritiis P, Deisseroth A, Goldman J, Barnett M, Reiffers J, *et al.* Autologous transplants for chronic myelogenous leukaemia: results from eight transplant groups. *Lancet* 1994; **343**:1486–1488.

7 Gale RP, Grosveld G, Canaani E, Goldman JM. Chronic myelogenous leukemia: biology and therapy. *Leukemia* 1993; **7**:653–658.

8 Goldman JM. Optimizing treatment for chronic myeloid leukemia. *N Engl J Med* 1997; **337**:270–271.

9 Simonsson B, Oberg G, Bjoreman M, Bjorkholm M, Carneskog J, Karlsson K, *et al.* Intensive treatment and stem cell transplantation in chronic myelogenous leukemia: long-term follow-up. *Acta Haematol* 2005; **113**:155–162.

10 The Italian cooperative study group on chronic myeloid leukemia. Interferon alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid leukemia. *N Engl J Med* 1994; **330**:820–825.

11 Allan NC, Richards SM, Shepherd PC. UK Medical Research Council randomised, multicentre trial of interferon-alpha n1 for chronic myeloid leukaemia: improved survival irrespective of cytogenetic response. The UK Medical Research Council's Working Parties for Therapeutic Trials in Adult Leukaemia. *Lancet* 1995; **345**:1392–1397.

12 Talpaz M, Kantarjian H, Kurzrock R, Trujillo JM, Gutterman JU. Interferon-alpha produces sustained cytogenetic responses in chronic myelogenous leukemia. Philadelphia chromosome-positive patients. *Ann Intern Med* 1991; **114**:532–538.

13 Hehlmann R, Heimpel H, Hasford J, Kolb HJ, Pralle H, Hossfeld DK, *et al.* Randomized comparison of interferon-alpha with busulfan and hydroxyurea in chronic myelogenous leukemia. The German CML Study Group. *Blood* 1994; **84**:4064–4077.

14 Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Regenass U, *et al.* Selective inhibition of the platelet-derived growth factor signal transduction pathway by a protein-tyrosine kinase inhibitor of the 2-phenylaminopyrimidine class. *Proc Natl Acad Sci U S A* 1995; **92**:2558–2562.

15 Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, *et al.* Inhibition of the Abl protein-tyrosine kinase *in vitro* and *in vivo* by a 2-phenylaminopyrimidine derivative. *Cancer Res* 1996; **56**:100–104.

16 Kantarjian H, Sawyers C, Hochhaus A, Guilhot F, Schiffer C, Gambacorti-Passerini C, *et al.* Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med* 2002; **346**:645–652.

17 Talpaz M, Silver RT, Druker BJ, Goldman JM, Gambacorti-Passerini C, Guilhot F, *et al.* Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. *Blood* 2002; **99**:1928–1937.

18 O'Brien SG, Guilhot F, Larson RA, Gathmann I, Baccarani M, Cervantes F, *et al.* Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. *N Engl J Med* 2003; **348**:994–1004.

19 Thiesing JT, Ohno-Jones S, Kolibaba KS, Druker BJ. Efficacy of STI571, an abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against bcr–abl-positive cells. *Blood* 2000; **96**:3195–3199.

20 Scappini B, Onida F, Kantarjian HM, Dong L, Verstovsek S, Keating MJ, *et al.* *In vitro* effects of STI 571-containing drug combinations on the growth of Philadelphia-positive chronic myelogenous leukemia cells. *Cancer* 2002; **94**:2653–2662.

21 Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977; **197**:461–463.

22 Weisenthal LM, Dill PL, Kurnick NB, Lippman ME. Comparison of dye exclusion assays with a clonogenic assay in the determination of drug-induced cytotoxicity. *Cancer Res* 1983; **43**:258–264.

23 Weisenthal LM, Lippman ME. Clonogenic and nonclonogenic *in vitro* chemosensitivity assays. *Cancer Treat Rep* 1985; **69**:615–632.

24 Larsson R, Kristensen J, Sandberg C, Nygren P. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). *Int J Cancer* 1992; **50**:177–185.

25 Nygren P, Kristensen J, Jonsson B, Sundstrom C, Lonnerholm G, Kreuger A, *et al.* Feasibility of the fluorometric microculture cytotoxicity assay (FMCA) for cytotoxic drug sensitivity testing of tumor cells from patients with acute lymphoblastic leukemia. *Leukemia* 1992; **6**:1121–1128.

26 Nygren P, Hagberg H, Glimelius B, Sundstrom C, Kristensen J, Christiansen I, *et al.* *In vitro* drug sensitivity testing of tumor cells from patients with non-Hodgkin's lymphoma using the fluorometric microculture cytotoxicity assay. *Ann Oncol* 1994; **5 (Suppl 1)**:127–131.

27 Frost BM, Nygren P, Gustafsson G, Forestier E, Jonsson OG, Kanerva J, *et al.* Increased *in vitro* cellular drug resistance is related to poor outcome in high-risk childhood acute lymphoblastic leukaemia. *Br J Haematol* 2003; **122**:376–385.

28 Topaly J, Zeller WJ, Fruehauf S. Synergistic activity of the new ABL-specific tyrosine kinase inhibitor STI571 and chemotherapeutic drugs on BCR-ABL-positive chronic myelogenous leukemia cells. *Leukemia* 2001; **15**:342–347.

29 Kano Y, Akutsu M, Tsunoda S, Mano H, Sato Y, Honma Y, *et al.* *In vitro* cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agents. *Blood* 2001; **97**:1999–2007.

30 O'Hare T, Walters DK, Stoffregen EP, Jia T, Manley PW, Mestan J, *et al.* *In vitro* activity of Bcr–Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. *Cancer Res* 2005; **65**:4500–4505.

31 Barbany G, Hagberg A, Olsson-Stromberg U, Simonsson B, Syvanen AC, Landegren U. Manifold-assisted reverse transcription-PCR with real-time detection for measurement of the BCR-ABL fusion transcript in chronic myeloid leukemia patients. *Clin Chem* 2000; **46**:913–920.

32 Larsson R, Nygren P. Prediction of individual patient response to chemotherapy by the fluorometric microculture cytotoxicity assay (FMCA) using drug specific cut-off limits and a Bayesian model. *Anticancer Res* 1993; **13**:1825–1829.

33 Larsson R, Nygren P, Ekberg M, Slater L. Chemotherapeutic drug sensitivity testing of human leukemia cells *in vitro* using a semiautomated fluorometric assay. *Leukemia* 1990; **4**:567–571.

34 Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**:27–55.

35 Steel GG, Peckham MJ. Exploitable mechanisms in combined radiotherapy–chemotherapy: the concept of additivity. *Int J Radiat Oncol Biol Phys* 1979; **5**:85–91.

36 Amare PS, Baisane C, Saikia T, Nair R, Gawade H, Advani S. Fluorescence *in situ* hybridization: a highly efficient technique of molecular diagnosis and predication for disease course in patients with myeloid leukemias. *Cancer Genet Cytogenet* 2001; **131**:125–134.

37 Fridborg H, Jonsson E, Nygren P, Larsson R. Relationship between diagnosis-specific activity of cytotoxic drugs in fresh human tumour cells *ex vivo* and in the clinic. *Eur J Cancer* 1999; **35**:424–432.

38 Jonsson E, Fridborg H, Csoka K, Dhar S, Sundstrom C, Nygren P, *et al.* Cytotoxic activity of topotecan in human tumour cell lines and primary cultures of human tumour cells from patients. *Br J Cancer* 1997; **76**: 211–219.

39 Aleskog A, Tobin G, Laurell A, Thunberg U, Lindhagen E, Roos G, *et al.* VH gene mutation status and cellular drug resistance in chronic lymphocytic leukaemia. *Eur J Haematol* 2004; **73**:407–411.

40 Aleskog A, Hoglund M, Pettersson J, Hermansson M, Larsson R, Lindhagen E. *In vitro* activity of the flt3-inhibitor SU5614 and standard cytotoxic agents in tumour cells from patients with wild type and mutated flt3 acute myeloid leukaemia. *Leuk Res* 2005; **29**:1079–1081.

41 Hallbook H, Barbany G, Aleskog A, Bjornberg A, Larsson R, Sundstrom C, *et al. In vitro* activity of imatinib in cells from patients with adult acute lymphoblastic leukemia. *Anticancer Drugs* 2005; **16**:631–634.

42 Zwaan CM, Kaspers GJ, Pieters R, Ramakers-Van Woerden NL, den Boer ML, Wunsche R, *et al.* Cellular drug resistance profiles in childhood acute myeloid leukemia: differences between FAB types and comparison with acute lymphoblastic leukemia. *Blood* 2000; **96**: 2879–2886.

43 Kantarjian HM, O'Brien S, Cortes JE, Smith TL, Rios MB, Shan J, *et al.* Treatment of philadelphia chromosome-positive, accelerated-phase chronic myelogenous leukemia with imatinib mesylate. *Clin Cancer Res* 2002; **8**:2167–2176.

44 Sawyers CL, Hochhaus A, Feldman E, Goldman JM, Miller CB, Ottmann OG, *et al.* Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood* 2002; **99**:3530–3539.

45 Tipping AJ, Mahon FX, Zafirides G, Lagarde V, Goldman JM, Melo JV. Drug responses of imatinib mesylate-resistant cells: synergism of imatinib with other chemotherapeutic drugs. *Leukemia* 2002; **16**:2349–2357.

46 Porosnicu M, Nimmanapalli R, Nguyen D, Worthington E, Perkins C, Bhalla KN. Co-treatment with As_2O_3 enhances selective cytotoxic effects of STI-571 against Brc–Abl-positive acute leukemia cells. *Leukemia* 2001; **15**:772–778.

47 Guilhot F, Gardembas M, Rousselot P, Tulliez M, Vigier M, Buzyn AF, *et al.* Imatinib in combination with cytarabine for the treatment of Philadelphia-positive chronic myelogenous leukemia chronic-phase patients: rationale and design of phase I/II trials. *Semin Hematol* 2003; **40**:92–97.