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Drug responses of imatinib mesylate-resistant cells: synergism of imatinib with other chemotherapeutic drugs

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Imatinib mesylate (STI571, Glivec, Gleevec) is a powerful inhibitor of the tyrosine kinase activity of Bcr-Abl, the oncoprotein responsible for chronic myeloid leukemia (CML). The drug shows great efficacy in chronic phase, but is less effective in maintaining hematologic remissions in blast crisis patients. Our group has previously described several cell lines made resistant to imatinib. We now examine the question of cross-resistance to other chemotherapeutic drugs used in CML. Four paired imatinib-sensitive/resistant CML cell lines were assessed by caspase-3 and MTS assays for their proliferative response to cytosine arabinoside (Ara-C), daunorubicin (DNR), homoharringtonine (HHT) and hydroxyurea (HU), either alone or in combination with imatinib. Primary blasts from advanced-stage CML patients refractory to imatinib therapy were studied by semi-solid media clonogenic assays. We found that these drugs are generally capable of major inhibition of proliferation of the CML cell lines, although differential responses to DNR and HHT were noted between some sensitive and resistant cell line pairs, implying that resistance to imatinib may confer a growth advantage under such conditions. The four drugs were also effective in preventing the formation of progenitor cell colonies from CML patients both before treatment with imatinib, and after relapse on the drug. Isobolographic analysis implied that these drugs will generally combine well with imatinib, and in some cases will be synergistic. We conclude that Ara-C, DNR or HHT, either alone or in combination with imatinib, are likely to be the best therapeutic alternatives in the management of patients who become resistant to imatinib monotherapy.
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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the presence of the BCR-ABL hybrid gene in the Philadelphia (Ph) chromosome, generated by the t(9;22)(q34;q11) reciprocal translocation. This oncogene is also present in around 25% of adults and 5% of children with acute lymphoblastic leukemia (ALL). The resulting Bcr-Abl fusion protein possesses deregulated tyrosine kinase activity and *in vivo* causes leukemia in a murine model system[1] and *in vitro* transforms cells to a growth factor-independent phenotype.[2] The human disease usually starts with a relatively benign chronic phase which is followed by blastic transformation, either with or without an intermediate accelerated phase.

Historically, treatment of CML has progressed through the use of drugs such as busulfan, hydroxyurea (HU), interferon-α (IFN-α), and cytosine arabinoside (Ara-C). These drugs exert an anti-leukemic effect via mechanisms which are largely undetermined. Recently, understanding of the underlying cellular defect in *BCR-ABL*-positive leukemias triggered the development of compounds rationally designed to specifically inhibit the Abl tyrosine kinase. The most effective of these, imatinib mesylate (STI571, Glivec, Gleevec), has been shown to potently inhibit this activity, as well as that of the Kit and PDGF receptors.[3] Inhibition of the Bcr-Abl kinase activity disrupts signaling via tyrosine phosphorylation cascades, preventing the suppression of apoptosis, a key component of the CML phenotype.[4] Imatinib is now being tested in clinical trials,[5,6] and shows remarkable potential as a component of novel front-line treatment regimens for CML.

Previous work in our[7] and other laboratories[8,9] has shown that imatinib is a powerful inhibitor of the growth of granulocyte–macrophage (CFU-GM) and erythroid (BFU-e) chronic phase CML progenitors. The compound also suppresses proliferation of most Ph-positive cell lines, leading them to an apoptotic death.[7] However, a sizeable proportion of CFU-GM colonies from CML patients survive in the presence of imatinib, and two of 10 Ph-positive cell lines were found to be naturally resistant to this drug.[7] This suggested that, as for other chemotherapeutic drugs, resistance to imatinib might be a problem in the clinic.

In order to study this phenomenon, we had generated cell lines resistant to imatinib at concentrations similar to pharmacological levels.[10] The mechanisms of resistance in these lines varied, and included amplification of the *BCR-ABL* gene, overexpression of the multidrug resistance P-glycoprotein (Pgp), or persistence of tyrosine phosphorylation of specific proteins implying compensatory signaling via Bcr-Abl-independent pathways. Similar findings were reported by other groups in independently generated imatinib-resistant cell lines.[11,12] Recent reports of similar mechanisms operating in imatinib-resistant patients[13,14] support the initial hypothesis that *in vivo* resistance to imatinib may be 'acquired' after extended exposure to the drug, via selection of a pre-existing resistant subclone.

In the current study we investigate the response of imatinib-resistant cell lines and primary CML progenitors to chemotherapeutic drugs used in different treatment modalities in CML. Two basic questions were addressed: (1) Do these cells exhibit cross-resistance to other drugs? and (2) Does addition of imatinib to the given drug enhance or reduce the efficacy of the treatment? We show here that resistance to imatinib is not associated with general resistance to other chemotherapeutic drugs, and that the combination of imatinib with these drugs is often synergistic. This implies a greater-than-additive effect which may allow improved clinical responses for any given dose schedule, or otherwise allow dose-reduction regimens to be implemented.

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Materials and methods

Cell lines and primary cells

The Bcr-Abl-positive cell lines LAMA84, AR230, K562 and KCL22[15] are sensitive to sub-micromolar concentrations of imatinib and are thus designated LAMA84-s, AR230-s and K562-s. Clones able to survive and proliferate in 1 μM imatinib were derived as previously described[10] and designated LAMA84-r, AR230-r and K562-r. The KCL22 cell line showed inherent resistance to imatinib and was thus cloned in methylcellulose in order to derive imatinib-sensitive (KCL22-s) and -resistant (KCL22-r) clones.[10] All cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 8.8 μg/ml streptomycin, 88 IU/ml penicillin, and 3.5 mM L-glutamine (here referred to as RF10 media), at 37ºC in a humid atmosphere with 5% CO_2. The imatinib-resistant clones were cultured in the presence of 1 μM imatinib.

Peripheral blood specimens were obtained after written informed consent from patients with CML who were entered in the Novartis imatinib clinical trials 0102, 0109, 0114 and 0115. Samples for this study were collected before initiation of imatinib and at the time of hematologic relapse. None of the patients exhibited mutations in the tyrosine kinase domain of Bcr-Abl or amplification of the *BCR-ABL* gene, and the mechanism of their imatinib-resistance is unknown. Total leukocytes were separated by red-cell lysis and cryopreserved in liquid nitrogen until processed for specific tests.

Drugs and compounds

Imatinib mesylate (gift from Dr Elisabeth Buchdunger, Novartis, Basel, Switzerland) was dissolved in sterile distilled H_2O as a 100 mM stock solution and stored at 4ºC in the dark. Working dilutions were prepared in RF-10 medium and passed through a 0.2 μm filter. Verapamil (Securon IV Knoll AG, Ludwigshafen, Germany) was obtained at a concentration of 2.5 mg/ml, aliquoted and stored at 4ºC. Cyclosporin A (Sandimmun IV concentrate, Novartis) was obtained at 50 mg/ml, aliquoted and stored at room temperature. Ara-C, daunorubicin (DNR), and HU (all Sigma Chemicals, Poole, UK) were initially dissolved in sterile distilled H_2O and stored at –20ºC. IFN-α Roferon-A, Roche Products, Welwyn Garden City, UK) was obtained at 3 MU/ml. Homoharringtonine (HHT) was a kind gift from JP Robin (Oncopharm, Le Mans, France). For all drugs, working dilutions were prepared in RF-10 medium as above.

Cell proliferation assay (MTS)

Cell proliferation was monitored by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay, with the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Southampton, UK). Cultures for the first experiments with each cell line and drug were set up with 1×10^5 cells/ml in tissue culture flasks. Proliferation was assessed on aliquots taken daily from days 1 to 4 in order to establish the most representative time-point. Replicate experiments were thereafter set up by plating the cells directly into 96-well plates and staining with MTS on day 3. A minimum of 95% viability as assessed by Trypan blue staining was required for the initiation of any experiment. For each cell line, 50 μl of a cell suspension in RF10 were seeded at 2×10^5 cells/ml into flat-bottomed wells (1×10^4 cells/well), to which was added 50 μl of a 2× drug dilution in RF10. Blank wells contained 100 μl RF10. All samples were plated in quadruplicate for each independent experiment, and at least three independent experiments were performed for each assay. The plate was incubated at 37ºC, 5% CO_2 in a humid environment. On day 3, 20 μl of MTS reagent was added to each well, and the plate was re-incubated for a further 3–4 h for stain development. At the end of this period, the plates were gently agitated and the absorbance of each well at 490 nm was recorded on an automatic microplate reader (MRX; Dynatech, Billingshurst, UK). Averaged blank values (no cells, no drug) were subtracted from sample values, and these corrected A_{490} values were calculated as percentages of the control cultures grown in the absence of drug. The SPSS for Windows v10.0.5 statistics package (SPSS Inc, Chicago, IL, USA) was utilized to analyze differences by the non-parametric two-tailed Mann–Whitney test. Statistical significance was considered as $P \leq 0.05$ in the two-tailed analysis.

CFU-GM assays

Mononuclear cells were separated on Lymphoprep (Nycomed, Oslo, Norway) from fresh or cryopreserved peripheral blood leukocytes, and plated at 5×10^4 cells/ml in Iscoves' methylcellulose medium (Methocult H4230; Stemcell Technologies, Vancouver, Canada) supplemented with 20 ng/ml each recombinant human interleukin-3, granulocyte colony-stimulating factor, granulocyte–macrophage colony-stimulating factor, interleukin-6, (Amgen, Thousand Oaks, CA, USA) and 100 ng/ml Flt3 ligand (R&D System, Abingdon, UK). Chemotherapeutic drugs were added to the methylcellulose at the required concentration. Colonies of at least 50 cells were visualized and counted on an inverted microscope on day 14 after plating. All clonogenic assays were done in duplicate or triplicate.

Drug combination analyses

For evaluation of the effect of combining a given drug with imatinib, the median effect isobolographic method of Chou and Talalay was utilized[16] via the CalcuSyn software (Biosoft, Cambridge, UK). The endpoint for these analyses was derived from MTS proliferation assays incorporating a third set of replicates, where cells were exposed to constant-ratio combinations of the test drug with imatinib. These ratios were selected, where possible, such that both drugs were in a range relevant to their achievable serum concentrations. Imatinib-sensitive and resistant cells were exposed to doubling dilutions of the individual drugs over a wide range. The degree of inhibition of cell proliferation in this assay relative to unexposed controls was designated the 'effect', which ranged from 0.0 (no inhibition) to 1.0 (no cellular conversion of the MTS reagent, denoting complete cell death). The effects of the drug treatments were plotted and analyzed to produce computed estimations of the relative responses to either drug alone or in combination. In this way it is possible to detect synergistic, additive or antagonistic effects. Duplicate or triplicate independent experiments were set up for each cell line/drug combination. In each case the analysis generates plots of CI (Combination Index) *versus* effect. A CI value of 0.9–1.1 denotes an additive combination, whilst CI >1.1 denotes

antagonistic interaction, and CI <0.9 indicates synergism between the drugs. These values are most relevant at high effect levels, as this is the desired physiological level of effect. Subsequently the CI curves can be represented as the average CI values at effects 0.5, 0.75 and 0.9 (IC50, IC75 and IC90, respectively).

Caspase-3 activation assays

Cell viability on day 0 and day 3 of exposure to individual drugs was measured by trypan blue staining, in parallel with the MTS assays. For determination of apoptotic cell death, the level of caspase 3 activation was assessed by measurement of its capacity to cleave a Ac-Asp-Glu-Val-Asp-amino-4-methyl-coumarine (Ac-DEVD) substrate, as modified from a previously published method.[17] Aliquots of 7.5×10^4 cells were cultured in triplicate in RF10 in 96-well plates in the presence or absence of 1 μM imatinib. Triplicate wells with RF10 only were used as background control. After 3 days the plate was centrifuged at 1500 r.p.m. for 5 min, the culture supernatant was removed and the cells resuspended in 50 μl of a buffer containing 10 mM HEPES, 5 mM DTT, 2 mM EDTA, 0.02% saponine, 1 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptine and 72 mM fluorogenic Ac-DEVD substrate (UBI Euromedex, Souffelweyerssheim, France). Development of the reaction was read on a spectrofluorometer (Victor 2 multilabel Counter, Wallac and Perkin Elmer, Akron, OH, USA), using λ_{exc} = 380 nm and λ_{em} = 480 nm. After this reading, 200 μl of a 15 mg/ml propidium iodide (PI) solution were added to each well, and to fresh replicate wells containing 7.5×10^4 cells as a day-zero control, and fresh replicate wells containing known decreasing cell numbers. New readings were taken at λ_{exc} = 360 nm and λ_{em} = 600 nm. In this manner, correction of the experimental PI readings allow calculation of the number of live cells present, and thus correction of the experimental caspase data to reflect the DEVDase activity present per 10^5 cells, irrespective of the degree of proliferation.

Results

Response of cell lines to individual drugs

Once it was verified that the MTS assay displayed a linear response over the cell density range utilized in this study (data not shown), the four pairs of imatinib-sensitive and -resistant lines were assayed for their ability to proliferate in the presence of the drugs, relative to their proliferation in the absence of treatment. Cells were initially exposed to 1 μM imatinib (the concentration used to select the resistant sublines) to establish the baseline profile of their response (Figure 1). In this assay, the most resistant line was LAMA84-r, whose proliferation was virtually unaffected by culture in 1 μM imatinib. We next assessed the responses of these pairs of cell lines to other cytotoxic drugs. The results are summarized on Table 1.

Ara-C: Treatment with Ara-C was effective in inhibiting the proliferation of most of the sensitive and resistant cell lines. Both K562 lines showed marked resistance to the drug, but with no differential responses (Figure 2a). Of the other pairs of cell lines, a slight increase in cell proliferation was noted for LAMA84-r at the lowest dose tested, although this was not statistically significant (P = 0.275). The responses of

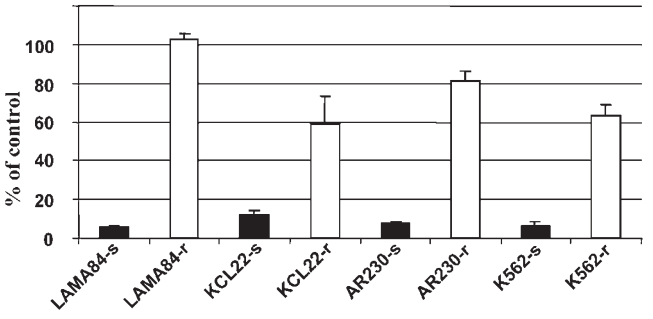

Figure 1 Proliferation of imatinib-sensitive and -resistant cell lines during a 3-day culture in 1 μM imatinib, as measured by the MTS assay. Each result is presented as the mean percentage of proliferation of unexposed control cultures, and represents three independent experiments. Error bars indicate one standard deviation from the mean.

LAMA84-s and -r cells were not significantly different either at the highest dose, implying that LAMA84-r is not cross-resistant to Ara-C (Figure 2a).

DNR: Responses to DNR were clearly observed, particularly at the highest doses tested. LAMA84-r was significantly resistant to the effects of 50 and 500 nM DNR as compared with the imatinib-sensitive line, although at 500 nM there is also clear inhibition of proliferation in the imatinib-resistant line. A differential response to DNR was also apparent between K562-s and K562-r, albeit with an overall marginally lower level of effect compared to LAMA84-r (Figure 2b and Table 1). However, the differential sensitivity of K562-s and -r was only statistically significant at 500 nM DNR.

HU: Low-dose (1 μM) HU did not produce a major anti-proliferative effect on any of the cell lines studied, but at 50 μM growth inhibition was apparent in LAMA84, KCL22 and AR230 sensitive and resistant lines (Table 1). In each case, this effect was enhanced by culture in 500 μM HU. Both K562 cell lines were entirely resistant to doses of up to 500 μM HU. At 50 μM HU, LAMA84-s showed greater proliferation than LAMA84-r, an effect which was less evident at 500 μM HU. Notably however, resistance to HU (relative to the sensitive line) was not observed in any of the imatinib-resistant lines (Table 1).

HHT: The responses of the imatinib-sensitive cells to HHT were marked at 18 nM for the KCL22 lines and LAMA84-s, and for all lines at 183nM. Significant cross-resistance to HHT was only observed for LAMA84-r, which showed greater proliferation in all doses of HHT when compared to LAMA84-s (Figure 2c), indicating cross-resistance to this drug. In contrast, KCL22-r showed no cross-resistance to HHT, proliferating significantly less than the sensitive counterpart in 183 nM HHT and above. AR230-r showed slightly greater proliferation than AR230-s in HHT only at the higher concentrations tested, although these concentrations were still effective in inhibiting the proliferation of AR230-r (Table 1).

IFN-α: No marked anti-proliferative effect of IFN-α was observed for any of the cell lines studied, regardless of their sensitivity to imatinib (Figure 2d).

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Table 1 Responses of cell lines to individual drugs

Concentration in culture	*AraC, μM (0.1–0.5 μM)*[36]			*DNR, nM (25–250 nM)*[37]		*HU, μM (500–1000 μM)*[38]			*HHT, nM (36 nM)*[39]			*IFN-α, IU/ml (25 IU/ml)*[40]
	0.02	*0.2*	*2*	*50*	*500*	*1*	*50*	*500*	*18*	*183*	*916*	*1000*
AR230-s	90	61	45	75	25	100	66	52	103	24	22	121
AR230-r	85	60	40	76	20	103	67	51	94	37	31	98
	(94%)	(98%)	(89%)	(101%)	(80%)	(103%)	(101%)	(98%)	(91%)	(154%)	(141%)	(81%)
K562-s	100	91	71	78	27	91	95	131	88	30	21	101
K562-r	106	93	73	90	43	82	98	126	121	56	30	100
	(106%)	(102%)	(103%)	(115%)	(159%)	(90%)	(103%)	(96%)	(138%)	(187%)	(143%)	(99%)
KCL22-s	86	39	26	42	9	100	77	32	45	20	18	85
KCL22-r	77	32	25	61	8	94	67	25	42	15	9	81
	(90%)	(82%)	(96%)	(145%)	(89%)	(94%)	(87%)	(78%)	(93%)	(75%)	(50%)	(95%)
LAMA84-s	68	39	26	49	7	100	71	33	18	5	5	94
LAMA84-r	81	26	12	77	26	98	40	28	87	28	15	88
	(119%)	(67%)	(46%)	(157%)	(371%)	(98%)	(56%)	(85%)	(483%)	(560%)	(300%)	(94%)

The average responses of each cell line to each drug concentration are presented as a percentage of the proliferation of an untreated control culture. Each result represents the mean of at least three independent experiments. Average plasma concentrations in standard therapeutic regimens according to referenced publications are shown in brackets underneath each drug. Numbers inside brackets under each value for the resistant cells indicate the mean percentage of proliferation relative to their sensitive counterpart; a result greater than 100% implies that the imatinib-resistant culture is cross-resistant to the drug at the concentration tested. In some instances additional doses of single drugs were also tested, and these results are mentioned in the text or figures where relevant.

Verapamil and cyclosporin A: The imatinib-resistant LAMA84-r cell line was markedly refractory to most of the compounds tested, as compared with its sensitive counterpart. In addition to Bcr-Abl overexpression, LAMA84-r also overexpresses the *MDR1* gene, and its imatinib-resistant phenotype can be partly reversed by verapamil, an inhibitor of the Pgp pump.[10] We therefore analyzed the effect of the Pgp inhibitors verapamil and cyclosporin A on the proliferative response of LAMA84-s and LAMA84-r to the Pgp substrates DNR and HHT. Both pump inhibitors produced a significant improvement in the anti-proliferative effect of HHT, but not of DNR (Figure 3), implying some involvement of Pgp-mediated drug efflux in the cross-resistance of LAMA84-r to HHT, but not DNR. Higher doses of the pump inhibitors were inherently toxic (data not shown). Thus, in LAMA84-r Bcr-Abl overexpression is implied to confer cross-resistance to both drugs.

Mechanism of drug-induced cell death

Overall, inhibition of proliferation as demonstrated by the MTS assay was accompanied by cell death as assessed by trypan blue exclusion (data not shown). In order to investigate the mechanism of this cell death, caspase-3 activation assays for apoptosis were performed after 72-h drug treatment. Data are presented for LAMA84-s and -r, and show clear activation of caspase-3 by imatinib in the sensitive, but not the resistant subline. Ara-C also induces greater caspase-3 activation in the sensitive subline relative to the resistant, whereas the level of induction by the other drugs appears to be low or negligible for both imatinib-sensitive and -resistant cells (Figure 4). Generally there was good correlation between the anti-proliferative and pro-apoptotic effects of each drug, where these were directly comparable. Exceptions to this were responses to HU and HHT in LAMA84-s and -r. In the case of HU, induction of caspase 3 activity was overall modest, but still significantly higher ($P = 0.05$) in LAMA84-s as compared with its resistant counterpart, whereas inhibition of proliferation was more accentuated in the latter cells (Table 1).

In the presence of 18 nM HHT, negligible caspase-3 activation was observed in both LAMA84 lines (Figure 4), despite significantly greater proliferation of the imatinib-resistant clone (Table 1, Figure 2c).

Response of primary imatinib-resistant cells to individual drugs

The response of progenitor cells from CML patients in blast crisis relapsing on imatinib therapy was investigated by CFU-GM assays in the presence or absence of imatinib, Ara-C, DNR, HU or HHT (Figure 5). Interestingly, there appeared to be negligible imatinib-mediated inhibition of colony formation in material obtained before treatment with the drug, with the *in vitro* response to imatinib not significantly different from that exhibited by cells collected at relapse. In contrast, a high degree of sensitivity to the other chemotherapeutic drugs was observed in progenitors from these patients both before and after relapse, strongly implying that in CML blast crisis cells refractory to imatinib there is no significant cross-resistance to the drugs studied.

Drug combinations

Two of the imatinib-resistant lines were selected for these analyses, in order to represent two distinct models for imatinib resistance: Bcr-Abl and Pgp overexpression (LAMA84-r), and an unidentified mechanism of resistance (K562-r). The combination index (CI) *vs* level of effect (F_A, fraction affected) plots are presented in Figure 6, together with the average CI values for 50, 75 and 90% effects (Table 2). The drug–response curves allowed generation of IC_{50} values for the individual drug treatments (Table 2). Overall, combinations of the individual chemotherapeutic drugs with imatinib were near-additive or synergistic at high effect levels, and the degree of synergy was greater in the imatinib-resistant than in the sensitive lines (Table 2). An exception to this was represented by the combination of HU + imatinib in the K562 cell lines. Here,

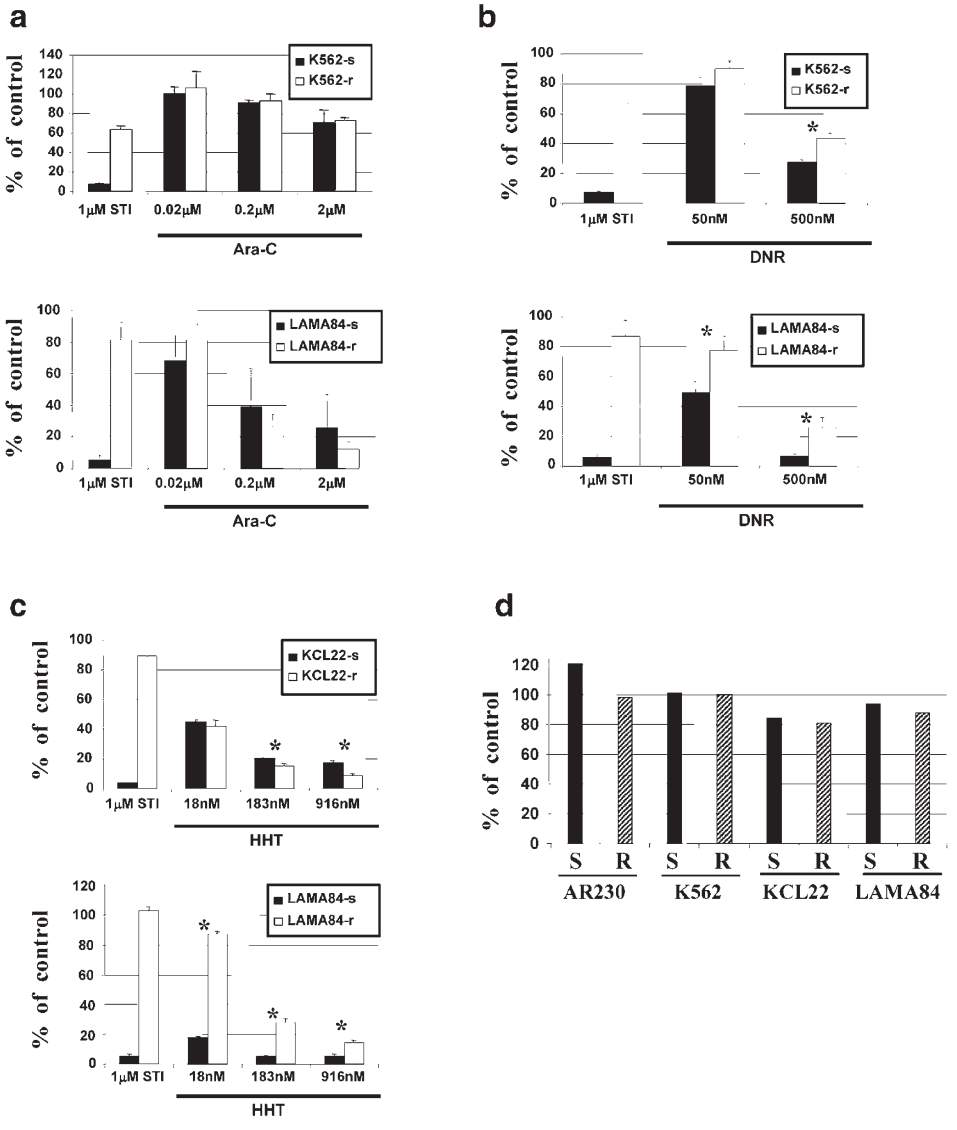

Figure 2 Proliferation of imatinib-sensitive and -resistant cell lines during a 3-day culture in the presence of either 1 μM imatinib (STI), or indicated doses of (a) Ara-C, (b) DNR, (c) HHT and (d) IFN-α. Each result is presented as the mean percentage of proliferation of unexposed control cultures, and represents three independent experiments. Error bars indicate one standard deviation from the mean. For Ara-C, DNR and HHT asterisks above the error bars indicate statistically significant cross-resistance to the chemotherapeutic drug ($P \leq 0.05$).

addition of imatinib to HU was antagonistic in both cases, but markedly more so in the resistant counterpart. For LAMA84, mild antagonistic effects of such combination in the sensitive line appeared to have been converted into a moderate synergism in the resistant counterpart.

Discussion

The development of imatinib has raised hopes of an effective low toxicity front-line therapy for CML, tempered only by the identification of acquired *in vitro* and *in vivo* resistance to the drug. The current work provides a unique insight into the relative effectiveness of other therapeutic drugs in killing imatinib-resistant leukemic cells, both alone and in concert with imatinib. The findings of this study are important since they suggest alternative therapies that can be investigated in a clinical setting for individuals who acquire resistance to imatinib, as well as those who respond to the drug. If resistance to imatinib is associated with cross-resistance to other chemotherapeutic drugs, irrespective of genetic background, there are obvious and important implications for patients who relapse under treatment with the tyrosine kinase inhibitor.

The first important finding of this work is that resistance to imatinib is not generally associated with resistance to near-therapeutic doses of Ara-C, DNR and HHT in our cell line models, suggesting that these drugs may be clinically useful in the management of imatinib-resistant patients. Whilst similar data have been published for the drug responses of imatinib-sensitive cell lines,[18–21] to our knowledge this work represents the first investigation of the effect of acquired resistance to imatinib on these drug responses. LAMA84-r showed some cross-resistance to these drugs relative to the sensitive line, but the levels of anti-proliferative effect obtained are still generally substantial. The detection of dose-related cross-resistance in only one of our model imatinib-resistant lines is encouraging, but implies that a similar phenomenon may occur in some patients resistant to imatinib. However, it

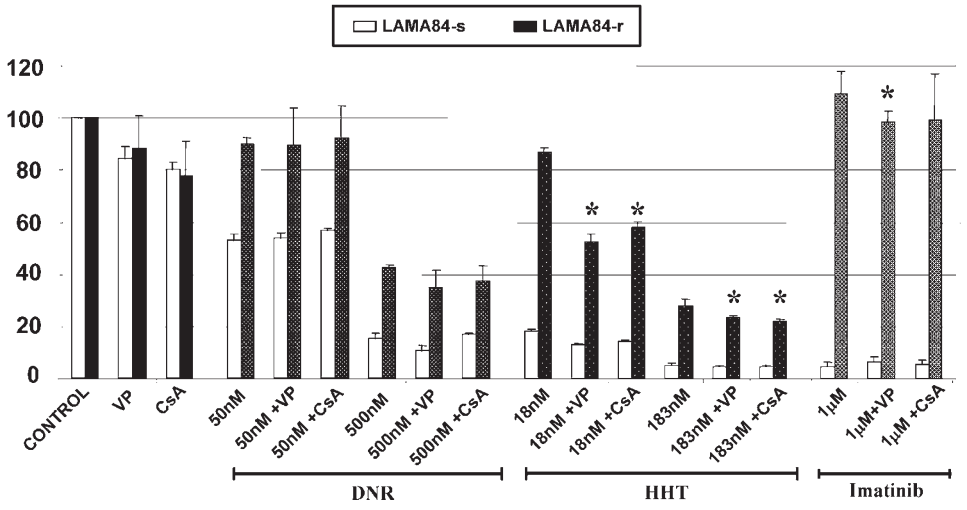

Figure 3 Effects of verapamil (5 μg/ml) and cyclosporin A (2 μM) on the proliferation of LAMA84-s and LAMA84-r during 3 days culture in DNR, HHT or imatinib. Each result is presented as the mean percentage of proliferation of unexposed control cultures, and represents four independent experiments. Error bars indicate one standard deviation from the mean. Statistically significant ($P \leq 0.05$) cross-resistance in the imatinib-resistant line is detected at all doses of DNR and HHT treatment, irrespective of verapamil or cyclosporin A. Asterisks indicate a statistically significant enhancement of effect of co-treatment with verapamil or cyclosporin A as compared with the respective cytotoxic alone at a given concentration ($P \leq 0.05$).

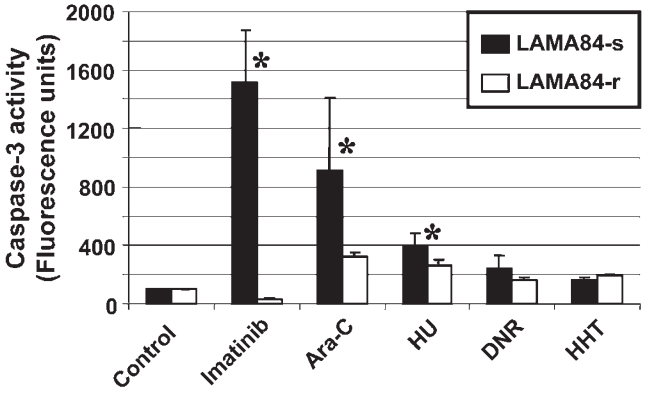

Figure 4 Caspase-3 activation in LAMA84-s and LAMA84-r after 3 days culture in the presence of 1 μM imatinib, 200 nM Ara-C, 50 μM HU, 100 nM DNR or 36 nM HHT. Each result is presented as arbitrary fluorescence units per 10^5 cells, normalized for cell proliferation. Each column represents the mean of three independent experiments, with error bars indicating one standard deviation from the mean. Asterisks indicate statistically significant differences between the sensitive and the resistance counterparts ($P \leq 0.05$).

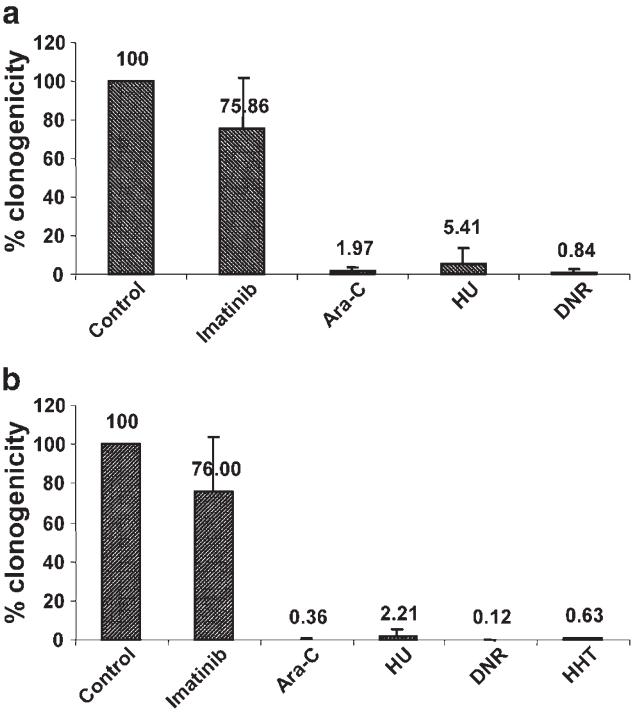

Figure 5 CFU-GM assay in the presence of 1 μM imatinib, 200 nM Ara-C, 50 μM HU, 100 nM DNR or 36 nM HHT. Each result is presented as the mean percentage of clonogenicity of unexposed control cultures from either seven (imatinib, Ara-C, HU, DNR) or six (HHT) different patients (a) prior to imatinib exposure or (b) at the time of relapse on imatinib therapy. Error bars indicate the range of responses observed. HHT responses were not evaluated in the pre-imatinib samples due to limiting number of cells.

appears from our data that under these conditions dose escalation may prove effective in inhibiting the resistant clone. The good responses of both imatinib-sensitive and -resistant lines to HHT are particularly encouraging, since this drug has not yet been extensively used in CML.[22–24]

Two contrasting and notable exceptions to the general trend are represented by HU and IFN-α. Responses to HU were generally modest and complex; the behavior of LAMA84 sensitive and resistant cells in 50 μM HU shows that resistance to imatinib appears to confer sensitivity (and not cross-resistance) to HU. The reasons for this phenomenon are unknown, but may reflect an increased requirement for ribonucleotide reductase activity in the imatinib-resistant line.[25] By contrast, IFN-α was totally ineffective in inhibiting cell proliferation on all cell lines tested. Since all these lines are derived from blast crises, this is not surprising, as only minimal responses have been previously described in CML patients in advanced stage disease[26] and in blast crisis cell lines.[18,20,27]

It is interesting to note that, since co-treatment with HHT and verapamil or cyclosporin A failed to thoroughly recapitulate the sensitive phenotype, it is likely that Bcr-Abl over-

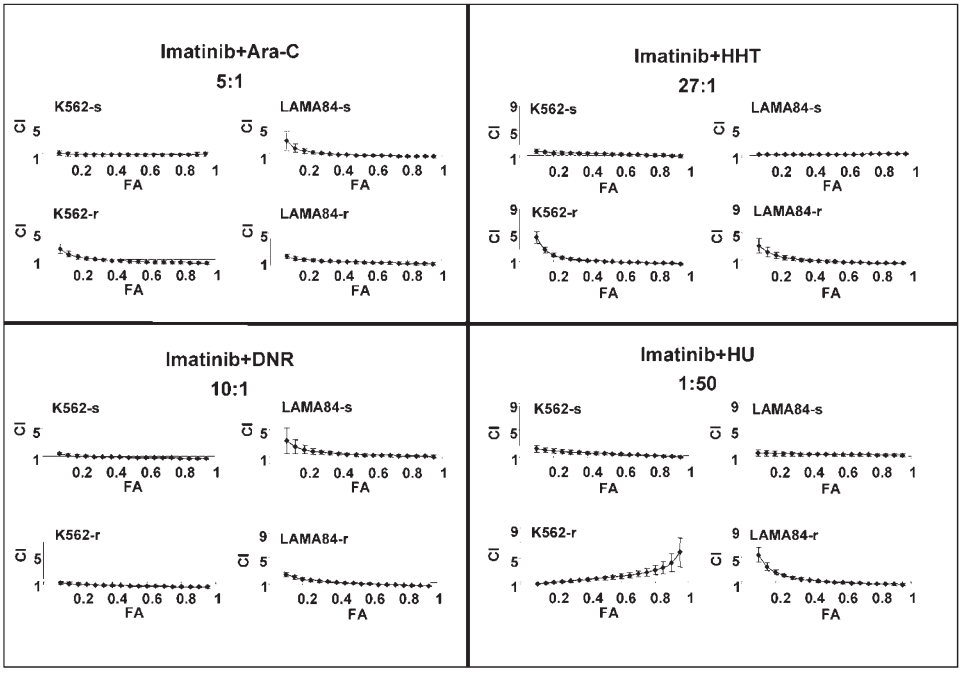

Figure 6 Combination index (CI) *vs* F_A (fraction affected) simulation plots derived from isobolographic drug combination analyses of K562 and LAMA84. The ratio of imatinib to each secondary drug is shown above each set of plots. Where CI $\approx$ 1 over the range of F_A values, additivity is suggested. CI values of significantly<1 or >1 suggest synergism or antagonism respectively (the x-axis intersects at y = 1 in these plots). Error bars indicate one standard deviation generated by CalcuSyn from the derived simulation data.

Table 2 Average CI (combination index) at F_A values of 0.5, 0.75 and 0.9[a] and approximate IC_{50} values from interpolations of the dose–response curves derived for each cell line/drug combination[b]

Drug (ratio)	*K562-s*		*K562-r*		*LAMA84-s*		*LAMA84-r*	
	CI	IC_{50}	*CI*	*IC_{50}*	*CI*	*IC_{50}*	*CI*	*IC_{50}*
Ara-C (Imatinib:Ara-C = 5:1)	1.0	5.5 μM	0.5	>12.8 μM	0.8	15 nM	0.4	20 nM
DNR (Imatinib:DNR = 10:1)	0.8	150 nM	0.4	200 nM	1.2	12 nM	0.6	200 nM
HHT (Imatinib:HHT = 27:1)	1.1	55 nM	0.6	110 nM	1.2	12 nM	0.6	50 nM
HU (Imatinib:HU = 1:50)	1.1	>1.6 mM	2.8	0.6 mM	1.2	0.2 mM	0.8	0.05 mM

[a]F_A denotes fraction affected (ie an F_A of 0.9 is equivalent to a 90% reduction in cell proliferation). Threshold values are as follows: CI > 1.1 denotes antagonism, CI 0.9–1.1 denotes additivity, and CI <0.9 denotes synergy.
[b]Data are derived from at least two independent, representative experiments.

expression in itself is the main contributor to the cross-resistance of LAMA84-r to HHT and DNR by an as yet unknown mechanism. The minor effect of verapamil on the imatinib resistance of LAMA84-r cells is statistically significant only due to the previously described loss of viability in this line on withdrawal of imatinib.[28] Whether *MDR1* overexpression is a common mode of acquisition of resistance to imatinib in patients treated *in vivo* is still an open question. Thus far, reports of *de novo* imatinib-resistance *in vivo* have implicated only Bcr-Abl overexpression or mutations in its kinase domain resulting in persistent phosphotyrosine signaling.[13,14,29–34]

Extension of our investigation into primary cells revealed that, in general, loss of *in vivo* response to imatinib is not associated with failure of other chemotherapeutic drugs to inhibit progenitor cell colony formation. The lack of *in vitro* response to imatinib in the primary material harvested prior to exposure of the patient to imatinib might be interpreted as innate resistance to the drug. In fact, the seven blast crisis patients analyzed in this study showed initially a good hematologic response to imatinib therapy, but never achieved cytogenetic remission. This implies that, in blast crisis, either CFU-GM assays of samples exposed to relatively low (1 μM) concentrations of imatinib may not reflect the *in vivo* hematologic response of the non-primed (pre-imatinib-treated) cells, or alternatively, they may have a predictive value for patients who respond initially, but will inevitably relapse. The responses of the primary CML cells to chemotherapeutic drugs is encouraging, and supports our hypothesis that, in general, imatinib-resistant cells are not cross-resistant to other chemotherapy.

The observation that other chemotherapeutic agents are

likely to be effective in the management of resistance to imatinib is strengthened by our work on the combination of these drugs with imatinib. Other groups have recently investigated the same issue in standard CML blast crisis cell lines sensitive to imatinib.[18–21] Our findings are generally in concert with theirs, in that most of these chemotherapeutic drugs are additive or synergistic in combination with imatinib. This means that these drugs are unlikely to antagonize the effects of imatinib, and vice versa. The conclusions from such isobolographic analyses may be expected to vary depending on the analytical methodology utilized. Our assays were designed to investigate the interaction of these drugs in cell line models at the approximate ratios defined by plasma concentrations resulting from standard dose regimens. Other studies have utilized equitoxic ratios of these drugs in median effect analysis,[18,19] but clinically this may be rarely achievable in patients resistant to the standard tolerable doses of imatinib. It is therefore reassuring that these different experimental designs have, in the cell line/drug combinations studied, given such similar results.

Our study is the first to address the issue on whether combination chemotherapy is a viable and/or better alternative once imatinib resistance has emerged. In fact we observed that in general, combinations of the investigated drugs with imatinib are more synergistic in the imatinib-resistant than in the -sensitive lines. The reasons for this are intriguing, as clearly the resistant lines tolerate imatinib very well, and yet, the presence of the kinase inhibitor appears capable of sensitizing the cells to treatment with the second drug. It is likely that the effects of low doses of the secondary drugs on the sensitive cells are masked by the high sensitivity of these lines to imatinib, a phenomenon which may be reversed in the resistant cells. We have previously shown that in both K562 and LAMA84 imatinib-resistant cells Bcr-Abl is partially dephosphorylated by imatinib treatment.[10] Whilst this limited reduction in the Bcr-Abl tyrosine kinase activity is insufficient in itself to induce apoptosis, it likely sensitizes the imatinib-resistant cells to the antiproliferative/cytotoxic effect of the combination drug. Similar observations were made for the combination of imatinib and SCH66336, a farnesyl transferase inhibitor, which led to a synergistic increase in apoptosis on cells resistant to imatinib.[35] Whatever the mechanisms, the implications of such synergy for potential dose-reduction regimens are obvious, as this interaction should be clinically exploitable in the management of CML.

In conclusion, we have demonstrated the efficacy of conventional anti-neoplastic drugs in CML cell line models and in primary cells from imatinib-relapsed patients. We have extended these analyses and found clear indications of synergism when some of these drugs are combined with imatinib therapy. It is our experience that Bcr-Abl overexpression, a resistance determinant described in cell line models[10–12] and in patients treated *in vivo*,[13,14] can be reversed by the cessation of imatinib exposure.[28] A similar observation was reported by Gorre *et al*[13] in one patient from their series whose *BCR-ABL* gene amplification was no longer detectable after 4 weeks after discontinuation of imatinib therapy. This phenomenon appears to reflect the removal of selective pressure followed by the loss of amplified copies of the *BCR-ABL* fusion. Thus, if the same occurs *in vivo*, it may be more advantageous to temporarily cease treatment with imatinib and pursue therapy with another of the drugs shown to be active against the resistant clone. Alternatively, combination therapy may still represent a more effective initial clinical approach to preventing imatinib resistance, as it should affect both the sensitive and resistant leukemic clones. Forthcoming clinical trials of combinations such as imatinib plus Ara-C or imatinib plus HHT will give the first indication of the relative success of combination *vs* single drug therapies involving this most exciting new drug.

SPOTLIGHT

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