Imatinib and leptomycin B are effective in overcoming imatinib-resistance due to Bcr-Abl amplification and clonal evolution but not due to Bcr-Abl kinase domain mutation

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ABSTRACT

Treatment with imatinib is very effective in Bcr-Abl positive leukemia. However, development of resistance to this drug is a common phenomenon in late stage disease. The Bcr-Abl protein localizes to the cytoplasm in transformed cells but can enter the nucleus upon treatment with imatinib. Using leptomycin B, a nuclear export blocker, it has been shown that reactivated nuclear Bcr-Abl kinase activity can induce cell death, thus presenting an interesting potential treatment option for imatinib resistant disease. Here we show that the combination of imatinib and leptomycin B effectively induces cell death in imatinib-resistant Ba/F3 cells which display Bcr-Abl amplification or signs of clonal evolution. However, no such synergism is observed in imatinib-resistant Ba/F3 cells carrying the T315I mutation of Bcr-Abl or those which have lost Bcr-Abl expression. Thus, a partial inhibition of Bcr-Abl by imatinib is required for this approach in agreement with the proposed mode of action.

Key words: chronic myeloid leukemia, imatinib resistance, leptomycin B.

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Introduction

Philadelphia chromosome results from the reciprocal chromosomal translocation t(9;22)(q34;q11) and generates a *Bcr-Abl* fusion gene.[1] The product of *Bcr-Abl* is a cellular oncoprotein responsible for chronic myelogenous leukemia (CML) as well as Philadelphia-positive acute lymphoblastic leukemia (Ph+ ALL).[2] Bcr-Abl is a constitutively active tyrosine kinase that induces a myriad of downstream signaling events resulting in clonal expansion of leukemic progenitors.[3,4] The introduction of imatinib, a small molecule inhibitor of Bcr-Abl kinase activity has revolutionized the treatment of CML.[5] However, development of drug resistance is a common event in advanced phases of the disease.[6] Point mutations in the kinase domain, Bcr-Abl amplification and cytogenetic clonal evolution were shown to be the main mechanisms underlying the development of imatinib resistance.[7] While resistance due to point mutations can be overcome by novel kinase inhibitors, treatment of advanced refractory disease arising from clonal evolution remains a challenge in many patients.[8-13] Several strategies have been employed to overcome imatinib resistance, including the use of combination treatment and alternate inhibitors. One such strategy exploits the nuclear targeting of the Bcr-Abl protein. It is well-known that Bcr-Abl remains exclusively localized in the cytoplasm despite the presence of three nuclear localization signals.[14] Recent reports have shown that Bcr-Abl is imported into the nucleus following imatinib treatment and its export to the cytoplasm can be prevented by the addition of the nuclear export inhibitor leptomycin B (LMB). Reactivation of nuclear Bcr-Abl activity by removing imatinib or metabolic decay of imatinib subsequently induces cell death.[15]

As it has been shown that a fraction of total Bcr-Abl kinase in the nucleus is sufficient to induce apoptosis, we asked if this could be exploited to treat imatinib resistant disease due to Bcr-Abl amplification, point mutation or clonal evolution.

Design and Methods

Cell culture and inhibitors

Ba/F3 cells were grown in RPMI 1640 (Gibco, Karlsruhe,

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Germany) containing 10% fetal calf serum (Gibco) and P/S (200 U/mL penicillin, 200 μg/mL streptomycin; Gibco). Parental Ba/F3 cells were cultured in the presence of 2 ng/mL interleukin-3 (R&D, Wiesbaden, Germany). Imatinib mesylate stocks were prepared in water (Aqua Delta Select) and stored at -20°C. Leptomycin B was purchased from LC Labs. Both drugs were diluted to specified concentrations in RPMI 1640. Imatinib-resistant Ba/F3 cell lines were derived from a cell-based screen as previously described.[16]

Cell viability assay

Untransfected and Bcr-Abl positive BaF/3 cells were plated at a density of 1×10^6 cells per well in a 12-well plate. Cells were treated with the indicated concentration of imatinib mesylate or leptomycin B or both for 48 h. Cells were then washed free of drugs and replated. Cell viability was assessed every 24 h by adding propidium iodide and subjecting cells to FACS analysis

Western blot

Ba/F3 cells were cultured for 2.5 h without or in the presence of imatinib at the indicated concentrations. Cell lysis, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were carried out as previously described.[16] Abl antibodies were obtained from Pharmingen (8E9) (BD Biosciences, Heidelberg, Germany) and Calbiochem-Novabiochem (Ab3) (Schwalbach, Germany). Antibodies for phosphotyrosine were purchased from Upstate Biotechnology (4G10) (Biozol, Eching, Germany) and BD Biosciences (PY20). Anti-phospho-signal transducer and activator of transcription 5 (Stat5; Tyr694) was obtained from Cell Signaling (New England Biolabs, Frankfurt/Main, Germany), and anti-stat5 (G-2) was obtained from Santa Cruz (Santa Cruz Biotechnology, Heidelberg, Germany). Bands were visualized using the enhanced chemiluminiscence (ECL) system (Amersham, Braunschweig, Germany).

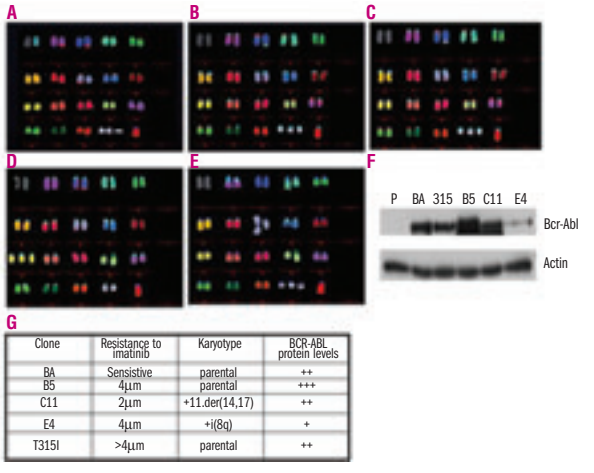
Ex vivo *colony formation assay*

1×10^6 Bcr-Abl positive Ba/F3 cells were mixed with 1×10^6 murine bone marrow cells or plated alone in 4 mL medium per well in a 12-well plate. Cells were left alone or treated with 2 μM imatinib and 10 nM leptomycin B for 48 hours. One thousand cells were then plated in 1mL methylcellulose with growth factors (Stem Cell Technologies) per well in a 12-well plate in duplicate and incubated at 37°C, 5% CO_2. After three weeks, plates were scored for the presence of colonies by morphological criteria using an inverted microscope in duplicates.

Results and Discussion

Characterization of imatinib resistant cell clones

Imatinib resistant Bcr-Abl postive Ba/F3 cell clones were chosen from a systematic *in vitro* screen as previously described.[17] Cytogenetic and molecular data for a large panel of resistant cell lines were generated and four experimental cell lines (B5, C11, E4 and T315I)



Clone	Resistance to imatinib	Karyotype	BCR-ABL protein levels
BA	Sensistive	parental	++
B5	4μm	parental	+++
C11	2μm	+11.der(14,17)	++
E4	4μm	+i(8q)	+
T315I	>4μm	parental	++

Figure 1. Cytogenetic characterization of Bcr-Abl expressing Ba/F3 cells. Karyotypes of parental Ba/F3 cells (A), imatinib sensitive Bcr-Abl expressing Ba/F3 cells (B) and imatinib resistant Ba/F3 sublines expressing unmutated Bcr-Abl B5 (C), C11 (D) and E4 (E) are shown. Expression levels of Bcr-Abl protein were analyzed in parental Ba/F3 cells (P), imatinib sensitive cells expressing unmutated Bcr-Abl (BA), imatinib-resistant cell lines expressing the Bcr-AblT315I mutation and in the sublines expressing unmutated Bcr-Abl B5, C11 and E4 (F). The same blot was stripped and probed for actin levels as a control for loading. Resistant cell lines are summarized in (G).

were selected based on their different resistance mechanisms. Analysis of these cell lines showed altered Bcr-Abl protein levels, abnormal cytogenetics or point mutations in the Bcr-Abl kinase domain consistent with the *in vivo* setting at the time of relapse in patients during imatinib treatment. Clone B5 exhibits no additional cytogenetic abnormality compared to parental or imatinib sensitive Bcr-Abl positive Ba/F3 cells (compare Figure 1C with Figures 1A and 1B). However, this clone shows an overexpression of the Bcr-Abl protein (Figure 1F). Clone C11 displays additional cytogenetic abnormalities [+ 11,der(14;17)] but normal Bcr-Abl protein expression (Figure 1F). Clone E4 harbors an additional chromosomal aberration [+ i(8q)]. In addition, this clone shows diminished Bcr-Abl protein expression, indicating that these cells may no longer depend on Bcr-Abl (Figure 1E, 1F). Clone T315I contains a point mutation in the Bcr-Abl kinase domain conferring resistance to imatinib. Features of the resistant clones are summarized in Figure 1G.

The combination of imatinib and LMB induces cell death only in certain cases of imatinib-resistant Ba/F3 cells

The combination of imatinib and LMB was tested on the four characterized imatinib-resistant cell lines. Treatment with imatinib alone had no effect on B5 (Bcr-Abl amplification) cells as expected (Figure 2A). LMB as a single agent showed substantial toxicity in this clone comparable to parental Ba/F3 cells (*data not shown*). However, about 20% of the cells did survive the treatment so that the clone could recover after washout of LMB. These data are in agreement with the level of toxicity of LMB on cell lines such as TonB

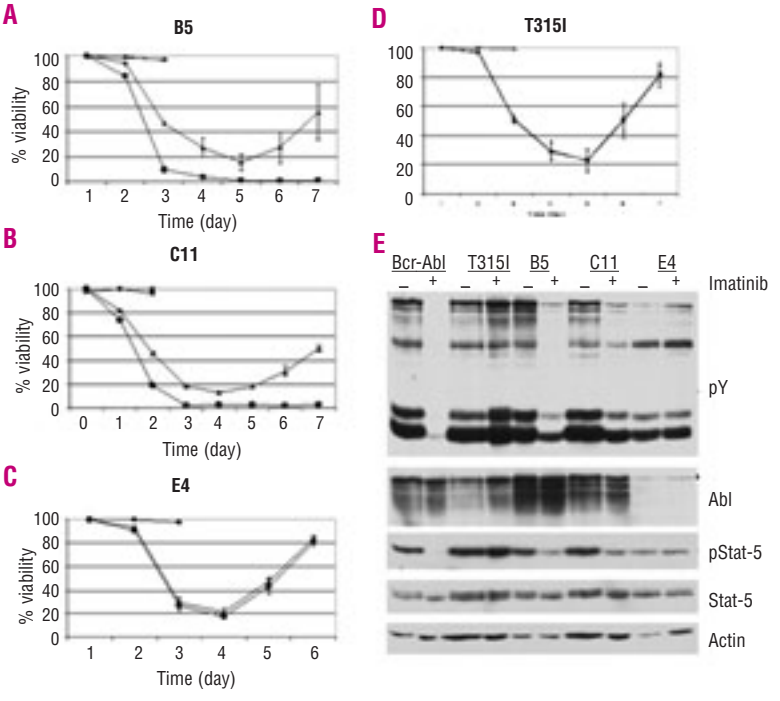


Figure 2. Effect of the combination of imatinib and leptomycin B on Ba/F3 cells expressing Bcr-Abl. Imatinib resistant Bcr-Abl positive Ba/F3 sublines B5 (A), E4 (C) and T315I mutant cells (D) were left untreated (◆) or treated with 4 μM imatinib (■) or 10 nM leptomycin B (▲) or both (●) for 48 hours and then assessed for cell viability. Cell line C11 (B) was resistant to 2 μM imatinib and hence was treated with either 2 μM imatinib or 10nM leptomycin B or both. Viability was assessed daily by propidium iodide staining. Untreated cells and cells treated with imatinib alone were measured up to 48 hours and then were discarded due to overgrowth. Ba/F3 cells expressing either wild-type (BA) or T315I mutated Bcr-Abl and imatinib-resistant sublines as indicated were treated with imatinib alone for two hours and analyzed. Bcr-Abl autophosphorylation was analyzed using phosphotyrosine antibody. The same blot was stripped and probed for Bcr-Abl, p-Stat5, Stat5 and actin (E).

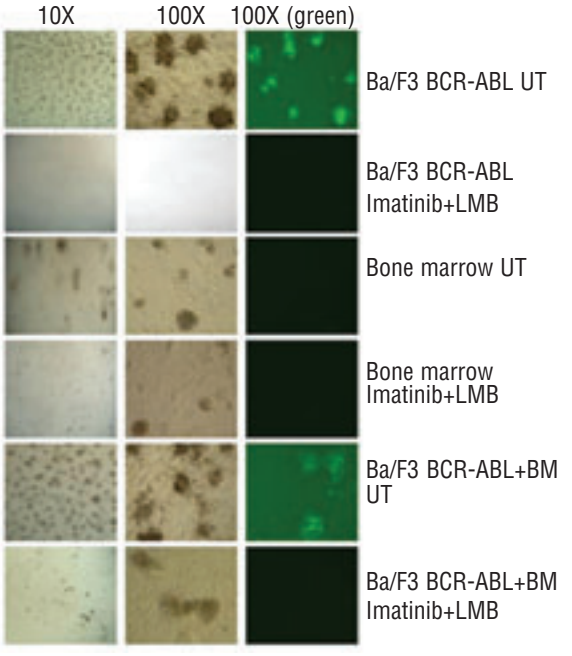
and Ba/F3 cells as previously reported (15, 18). We next tested the combination of imatinib and LMB for 48 hours. As previously reported, this combination is able to efficiently induce cell death with no recovery of cells over time in imatinib resistant cells with Bcr-Abl amplification (B5). Very similar data were obtained with the imatinib resistant cell clone C11 that displays additional cytogentic abnormalities (Figure 2B). On the contrary, clone E4 (additional cytogentic abnormalities and loss of Bcr-Abl expression) could not be eradicated by the combination of LMB and imatinib (Figure 2C). These cells did recover even after treatment with the combination of LMB and imatinib. Very similar results were obtained with the clone T315I (Figure 2D). Thus, the combination of LMB and imatinib was able to overcome resistance in imatinib resistant cell lines due to clonal evolution or Bcr-Abl protein overexpression. However, this approach was not successful in imatinib resistant cells with loss of Bcr-Abl expression or cells resistant due to a point mutation in the Bcr-Abl kinase domain.

Next, we aimed to clarify the mechanisms responsible for the differential response to combination treatment by imatinib and LMB observed in the resistant cell lines. It has been clearly shown, that the combined effect of LMB and imatinib is exerted through the inhibition of the Bcr-Abl kinase by imatinib which leads to its nuclear relocalization, entrapment in the nucleus by LMB and, finally, reactivation of the kinase in the nucleus which leads to apoptotic cell death.[15] Therefore, we analyzed the extent of inhibition of Bcr-Abl and its downstream target STAT5 by imatinib alone in the selected cell clones, since this is a prerequisite for the nuclear relocalization. Imatinib inhibited Bcr-Abl autophosphorylation and STAT5 phosphorylation in imatinib sensitive Bcr-Abl expressing cells and in the cell clones B5 and C11. No inhibition was observed in E4 cells and cells expressing the T315I mutant (Figure 2E). Thus, the combination of LMB and imatinib efficiently induced cell death in cell lines resistant to imatinib due to Bcr-Abl overexpression or clonal cytogenetic evolution alone. However, the clones which harbored a point mutation in the kinase domain, or which may have become Bcr-Abl independent, did not respond to the combination treatment. These results are in line with the proposed mode of action of the imatinib/LMB combination. Prerequisite for cell killing is Bcr-Abl inhibition, nuclear translocation and consecutive reactivation of Bcr-Abl in the nucleus. Thus, this approach requires the presence of Bcr-Abl protein and at least a partial inhibition of Bcr-Abl kinase activity by imatinib to allow LMB to exert its effect. This model clearly explains the negative results for the cell clones E4 and T315I.[15,18]

The combination of imatinib and LMB selectively kills Bcr-Abl positive cells with only marginal effects on normal bone marrow cells

Since LMB as a single agent showed toxic effects even in Ba/F3 cells (Figure 1 and *data not shown*), we examined its effect on normal bone marrow mononuclear cells at concentrations which, in combination with imatinib, efficiently kill Bcr-Abl positive Ba/F3 cells. Since Ba/F3 cells were derived from a Balb/c mouse background, we used bone marrow from this strain for our experiments. The ability of normal bone marrow cells to survive the combined treatment of imatinib and LMB was assessed in an *ex vivo* colony forming assay by mixing mouse bone marrow mononuclear cells with Bcr-Abl and EGFP positive Ba/F3 cells. Bcr-Abl expressing colonies were visualized by the expression of green fluorescent protein. As shown in Figure 3A, untreated Ba/F3-Bcr-Abl

A



B

Experimental well	Total n. of colonies	N.of green colonies
Ba/F3 BCR-ABL untreated	45±2	45±2
Ba/F3 BCR-ABL Imatinib+LMB	0	0
Bone marrow (BM) untreated	25±5	0
Bone marrow (BM) Imatinib+LMB	18±2	0
Ba/F3 BCR-ABL+BM untreated	40±5	24±4
Ba/F3 BCR-ABL+BM Imatinib+LMB	29±3	0±1

Figure 3. Effect of the combined treatment of imatinib and leptomycin B on primary mouse bone marrow cells. (A) Ba/F3 cells expressing wild-type Bcr-Abl or Balb/c bone marrow mononuclear cells or a 1:1 mixture of both were left untreated or treated with 2 μM imatinib and 10 nM leptomycin B for 48 hours. Cells were then washed free of drugs and plated in duplicates in methylcellulose. The number of colonies was counted after three weeks using 10X and 100X magnification. Bcr-Abl expressing colonies were visualized by green fluorescence by virtue of GFP coexpression. (B) Number of counted colonies are summarized.

cells alone formed abundant colonies in methylcellulose, while no growth was seen in the presence of imatinib and LMB. In contrast, primary bone marrow mononuclear cells treated with the drug combination showed only a moderate decrease in colony formation compared to untreated controls (Figure 3A, GFP, right column). In the experiment containing a mixture of primary bone marrow cells and Bcr-Abl positive cells, the combination of imatinib and LMB led to preferential eradication of Bcr-Abl (EGFP) positive cells while sparing most of the normal bone marrow cells. Results are quantified and summarized in Figure 3B. Recently, it was shown that the combination of imatinib and leptomycin B is effective in *ex vivo* treatment of bone marrow cells derived from CML patients. In that study, a combination of 10 μM imatinib and 10 nM leptomycin B was used in colony forming assays using a different schedule.[18] In an MTT-based experiment, Aloisi *et al.* have also shown that the combination is effective in inhibiting Bcr-Abl point mutant D276G but not Y253F and T315I. These data are in accordance with our observations in the T315I clone.

Despite the introduction of novel Abl kinase inhibitors, treatment of resistant Bcr-Abl positive leukemia remains a challenge, and hence various strategies are currently being tested to overcome this problem.[19] Here we show that the combination of imatinib with a nuclear export blocker will only be effective in selected patients with imatinib resistance. Combination treatment of imatinib and leptomycin B seems an attractive option in treating imatinib-resistant CML due to Bcr-Abl amplification or clonal evolution. Given that clonal cytogenetic evolution is a frequent finding when resistance occurs in CML patients treated with imatinib, the potential option of a combination treatment with imatinib and a nuclear export blocker may have a role in overcoming imatinib resistance in the future. However, less toxic nuclear export blockers need to be developed in order to make this a feasible clinical approach. Anguinomycin C might be such a compound since it shows low toxicity towards normal cells compared to leptomycin B.[20] Thus, further testing of combinations of imatinib and alternative nuclear export blockers seems warranted.

Authorship and Disclosures

RK: performed experiments and wrote the manuscript; NvB: designed research and contributed reagents; CM: performed experiments; CP: discussed data and designed the figures; KG: designed research; JD: designed research and wrote the manuscript.

The authors reported no potential conflicts of interest.

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