Combination Treatment of Imatinib-sensitive and -resistant *BCR-ABL*-positive CML Cells with Imatinib and Farnesyltransferase Inhibitors

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Abstract. *Background: Resistance to imatinib monotherapy frequently emerges in advanced stages of chronic myelogenous leukemia (CML), supporting the rationale for combination drug therapy. In the present study, the activities of the farnesyltransferase inhibitors (FTIs) L744,832 and LB42918, as single agents and in combination with imatinib, were investigated in different imatinib-sensitive and -resistant BCR-ABL-positive CML cells. Materials and Methods: Growth inhibition of the cell lines and primary patient cells was assessed by MTT assays and colony-forming cell assays, respectively. Drug interactions were analyzed according to the median-effect method of Chou and Talalay. The determination of apoptotic cell death was performed by annexin V/propidium iodide staining. Results: Combinations of both FTIs with imatinib displayed synergism or sensitization (potentiation) in all the cell lines tested. In primary chronic phase CML cells, additive and synergistic effects were discernible for the combination of imatinib plus L744,832 and imatinib plus LB42918, respectively. Annexin V/propidium iodide staining showed enhancement of imatinib-induced apoptosis with either drug combination, both in imatinib-sensitive and -resistant cells. Conclusion: The results indicated the potential of L744,832 and LB42918 as combination agents for CML patients on imatinib treatment.*

Chronic myelogenous leukemia (CML) is a myelo-proliferative disorder which accounts for 15% of all

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leukemias in adults (1). The Philadelphia chromosome, the hallmark of the disease and present in nearly all CML cases, is the result of a t(9;22)(q34;q11) reciprocal translocation, which generates a *BCR-ABL* hybrid gene encoding the BCR-ABL oncoprotein (2-4). The constitutively enhanced tyrosine kinase (TK) activity of *BCR-ABL* is essential for malignant transformation and plays a crucial role in the pathogenesis of CML (4-6). Imatinib mesylate (formerly STI571; imatinib; Gleevec®; Glivec®), an orally administred specific tyrosine kinase inhibitor, is known to selectively suppress the proliferation of *BCR-ABL*-positive cells leading to sustained hematological, cytogenetic and molecular responses in all stages of the disease (7-9). Despite the high efficiency of imatinib as a single agent, development of imatinib resistance can frequently be observed, especially in advanced stages of disease (10).

The application of imatinib in combination with other antineoplastic agents may be a strategy to increase the antileukemic effect and overcome resistance towards imatinib monotherapy (11-13). Targeting downstream pathways and their molecules which are activated through BCR-ABL TK activity, such as the oncogenic Ras proteins, may be an approach. Ras signal transduction and Ras activation are involved in malignant transformation and were reported to be implicated in the pathogenesis of myeloid leukemias (1, 14, 15). The oncogenic activity of Ras requires several post-translational modifications, including protein prenylation catalyzed by farnesyl-transferase (16). Consequently, interrupting Ras processing by specific farnesyltransferase inhibitors (FTIs) may prove useful for the treatment of myeloid leukemias. Various FTIs with differing antiproliferative properties have been designed, of which several have entered clinical trials (16-18).

Based on these considerations, the activities of the FTIs L744,832 and LB42918 were investigated alone and in combination with imatinib in imatinib-sensitive and -resistant *BCR-ABL*-positive CML cell lines and primary CML cells.

Materials and Methods

Cell lines. BCR-ABL-positive EM3, K562 and LAMA84 cell lines, derived from patients in the blast crisis of CML, were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and are all sensitive to sub-micromolar concentrations of imatinib. The imatinib-resistant cell lines LAMA84-R and K562-R, were generated in the Laboratory of Dr. Melo (London, UK) and were characterized previously (19). The cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) under standard conditions (37˚C, fully humidified atmosphere of 95% air and 5% CO_2). The imatinib-resistant LAMA84-R and K562-R cells were cultured in medium containing 1 μM imatinib.

Patient samples. Primary CML cells were isolated by Ficoll density gradient centrifugation (Biocoll separation solution, density 1.077 g/ml; Biochrom, Berlin, Germany) from the peripheral blood of three patients with newly diagnosed CML (imatinib-sensitive, chronic phase) and three patients refractory to imatinib therapy [imatinib-resistant, chronic phase (one patient) and blast crisis (two patients)]. The study was approved by the local ethical committee and informed consent was obtained from all patients. After separation, mononuclear cells were frozen in Iscove's modified Dulbecco's medium with 50% FCS and 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and stored at –80˚C until investigation.

Drugs. Imatinib was kindly provided by Novartis Pharma (Basel, Switzerland), L744,832 was a gift from Merck & Co. Inc. (Linden, NJ, USA) and LB42918 was a gift from LG Chem (Seoul, South Korea). Imatinib and L744,832 were initially dissolved in sterile Dulbecco's phosphate-buffered saline (Dulbecco's PBS; Invitrogen) and LB42918 in DMSO. Stock solutions at a concentration of 10 mM of each drug were prepared and stored at –20˚C until use. For experiments, serial working dilutions were prepared.

Cell proliferation assay (MTT). Cell proliferation was assessed by the MTT assay as described previously (19, 20). Briefly, after 24 h of pre-incubation in tissue culture flasks at a density of 3×10^5 cells/ml (at logarithmic cell growth), 90-μl aliquots of the cell suspension were dispensed into 96-well flat-bottomed microtiter plates (Becton Dickinson, Heidelberg, Germany) containing 10 μl/well of serial drug dilutions. The plates were incubated for 48 h under standard conditions. After addition of 15 μl of a 5 mg/ml MTT solution (MTT; Sigma, Munich, Germany) to each well and incubation for another 4 h, 100 μl/well of a 10% sodium dodecyl sulphate solution in 0.01 M hydrochloric acid were added and the plates were incubated overnight under the same conditions (stain development). Subsequently, the absorbance of each well at 540 nm (reference wavelength 690 nm) was recorded with a Multiskan Bichromatic plate reader (Labsystems, Helsinki, Finland). Blank control values (no drug, no cells) were subtracted from the sample values and the means of four replicate wells for each drug dilution and the control (cells grown in absence of the drug) were used to calculate the extent of relative cell proliferation (as percentage of the control) and growth inhibition (defined as fraction affected (Fa); for details, see Topaly *et al.* (20)).

Table I. *Sensitivity of BCR-ABL-positive imatinib-sensitive and imatinib-resistant CML cell lines towards imatinib and the farnesyltransferase inhibitors L744,832 and LB42918.*

Cell line	IC_{50} (μM), mean±s.d.		
	Imatinib	L744,832	LB42918
EM3	0.10±0.01	25.37±1.86	3.52±0.77
K562	0.63±0.03	n.a.	n.a.
K562-R	16.36±2.64	n.a.	>100
LAMA84	0.29±0.03	n.a.	28.76±9.57
LAMA84-R	3.79±0.24	n.a.	30.29±5.19

(n.a., no activity: no dose response at doses up to 40 μM)

Each cell line was treated with six increasing concentrations (doubling with each increment) of imatinib, L744,832 and LB42918 alone or their combination (imatinib plus L744,832 or imatinib plus LB42918).

Analyses of drug effects and interactions. Analyses of the dose-effect relationships and the evaluation of drug interaction upon combination of imatinib and FTI were performed according to the median-effect method of Chou and Talalay (21) using the CalcuSyn Software (Biosoft, Cambridge, UK). Sufficient dose-response correlations of both drugs when given alone are a prerequisite for the applicability of this method. When feasible, for single-agent treatments, the mean IC_{50} values for each drug and cell line were calculated.

For combinations where both drugs (imatinib and FTI) displayed distinct cytotoxic effects, the mean combination index (CI) values based on equitoxic drug ratios were assessed with CI<1, CI=1 or CI>1 denoting synergism, additivity or antagonism of the substances, respectively. As CI values depend on the levels of growth inhibition (fraction affected, Fa) they are observed at, evaluable combination data are presented in CI *vs.* Fa plots.

In cell lines where the FTI showed no antiproliferative activity, an analysis in terms of sensitization (potentiation) or inhibition of imatinib activity according to Topaly *et al.* (13, 20) was conducted instead; here, for better comparability between the different cell lines, the results are shown as relative IC_{50} values (ratio of the IC_{50} value of the FTI in combination with imatinib to the IC_{50} value of single agent imatinib). A relative $IC_{50}>1$ indicates inhibition, whereas a relative $IC_{50}<1$ indicates potentiation of the active agent (*i.e.* imatinib) (for details, see Topaly *et al.* (13, 20)).

Colony-forming cell (CFC) assay. Primary patient cells were plated in duplicate in MethoCult GF H4534 methylcellulose (Stem Cell Technologies, Vancouver, BC, Canada) and incubated in the absence of drugs (untreated control samples), with three different concentrations of the single agents imatinib, L744,832 and LB42918, respectively, or their combinations (imatinib plus FTI). The number of plated cells was defined individually by titration (approximately 100 colony-forming units granulocyte/macrophage (CFU-GM) after 14 days of incubation in untreated control samples). CFU-GM were visualized and counted on day 14 after plating. The mean CFU-GM values for each treatment were calculated and depicted as the percentage of the untreated control and Fa *vs.* CI plots.

Rhodamine 123 efflux assay. The rhodamine 123 efflux assay was performed according to a previously described method (19).

Apoptosis assay. The determination of apoptotic cell death was performed by annexin V/propidium iodide (PI) staining employing the Annexin V-FITC Kit (Immunotech, Marseille, France), which allows for the distinction of viable cells (unstained) from apoptotic (annexin V^+ / PI^-) and necrotic (annexin V^+ / PI^+) cells. Twenty-four hours after drug treatment, the cells were processed according to the manufacturer's protocol and the data were collected and analyzed with a FACScalibur flow cytometer (Becton Dickinson) using the CellQuest Software (Becton Dickinson).

Statistics. The results are shown as the mean value ± standard deviation (s.d.) derived from three to five independent experiments. Statistically significant differences between values acquired under different experimental conditions were calculated using the Student's *t*-test. Significance levels were selected at $p < 0.05$ and $p < 0.01$.

Results

Single agent treatment. The *BCR-ABL*-positive cell lines EM3, K562 and LAMA84 were all sensitive towards imatinib and displayed a concentration-dependent inhibition of proliferation after 48-h imatinib monotherapy. The IC_{50} values are given in Table I. As expected, in the imatinib-resistant K562-R and LAMA84-R lines, higher imatinib concentrations were required for growth inhibition. The FTIs L744,832 and LB42918 (concentration range investigated 0 μM – 40 μM) showed variable effects on the proliferation of the cell lines tested. In imatinib-sensitive cells only the growth of EM3 was inhibited by L744,832, while in the imatinib-resistant K562-R and LAMA84-R cells no inhibitory effects could be observed. LB42918 showed antiproliferative activity in imatinib-sensitive EM3 and LAMA84 cells and in both imatinib-resistant lines (Table I).

Combination treatment. The combination treatment of EM3 cells with imatinib plus FTI (L744,832 and LB42918) resulted in enhanced growth inhibition as compared to imatinib monotherapy. Analysis of the combined effects revealed that both combinations were synergistic with CI values of 0.47±0.18 and 0.67±0.15 for imatinib plus L744,832 and imatinib plus LB42918, respectively, at the 75% growth inhibition level (Fa=0.75) (Figure 1a, b). Since L744,832 monotherapy showed no measurable antiproliferative effects in K562 and LAMA84 cells (Table I), the median-effect method was not applicable for investigating combination effects with imatinib. Here, a significant reduction of imatinib IC_{50} values (relative $IC_{50} < 1$) when combined with noncytotoxic doses of L744,832 uncovered a potentiation of imatinib (Figure 1c, e). Similar results could be observed for the combination treatment of K562 with imatinib and LB42918 (Figure 1d). In contrast, this combination was synergistic in imatinib-sensitive LAMA84 cells (CI=0.50±0.06 at Fa=0.75) (Figure 1f).

In imatinib-resistant K562-R and LAMA84-R cells, again the L744,832 monotherapy showed no measurable antiproliferative effect at concentrations up to 40 μM so that the median-effect method was not applicable; simultaneous application of imatinib and L744,832, however, potentiated imatinib activity (Figure 1g, i). The combination of imatinib and LB42918, on the other hand, was synergistic as denoted by CI<1 both in K562-R (CI=0.12±0.07 at Fa=0.75) and LAMA84-R cells (CI=0.65±0.09 at Fa=0.75) (Figure 1h, j).

Response of primary CML cells. Monotherapy of primary CML cells (imatinib-sensitive, chronic phase) with different concentrations of imatinib, L744,832 or LB42918 resulted in a dose-dependent inhibition of CFU-GM colony formation, with LB42918 being more effective than L744,832. The combination of imatinib plus FTIs at different concentrations produced a decrease in colony formation as compared to treatment with the respective drugs alone (Figure 2a). Further analysis revealed additive to slightly synergistic effects, with CI values ~1 for the combination imatinib plus L744,832 (Figure 2b). For imatinib plus LB42918 at Fa=0.5 and 0.75, CI values of 0.85±0.09 and 0.66±0.04 were observed, respectively, indicating synergism (Figure 2c).

In primary CML cells derived from three patients refractory or resistant to imatinib therapy [imatinib-resistant, chronic phase (one patient) and blast crisis (two patients)] no colony growth both in untreated and treated groups could be observed ($2x10^4$ - $4x10^5$ MNC / assay) (data not shown).

Effects on Pgp function. In a previous report, the imatinib-resistant LAMA84-R line was shown to display high Pgp activity as compared to the parental sensitive line (19). To study the effect on Pgp function, functional ^{123}Rh efflux assays were performed in imatinib-sensitive and -resistant LAMA84 cells employing L744,832 and LB42918. For both agents, no inhibitory effects on Pgp function were observed (data not shown).

Induction of apoptosis. Monotherapy of the imatinib-sensitive cell lines EM3, K562 and LAMA84 with imatinib, L744,832 or LB42918 increased the early apoptotic cell fractions (annexin V^+ / PI^- cells) as compared to the untreated controls. For imatinib, this effect was more pronounced with apoptotic fractions varying between 5% (for EM3) and 10% (for K562) as compared to L744,832- and LB42918-induced apoptosis (3%-5%). Compared to monotherapy, the combination treatment with imatinib plus L744,832 or imatinib plus LB42918 significantly increased apoptosis in the respective lines (15% - 19% and 14% - 21%, respectively) (Figure 3a).

In the imatinib-resistant K562-R and LAMA84-R lines as well, single agent imatinib, L744,832 and LB42918 slightly increased the proportion of apoptotic cells as compared to the untreated controls (Figure 3b). Here again, the
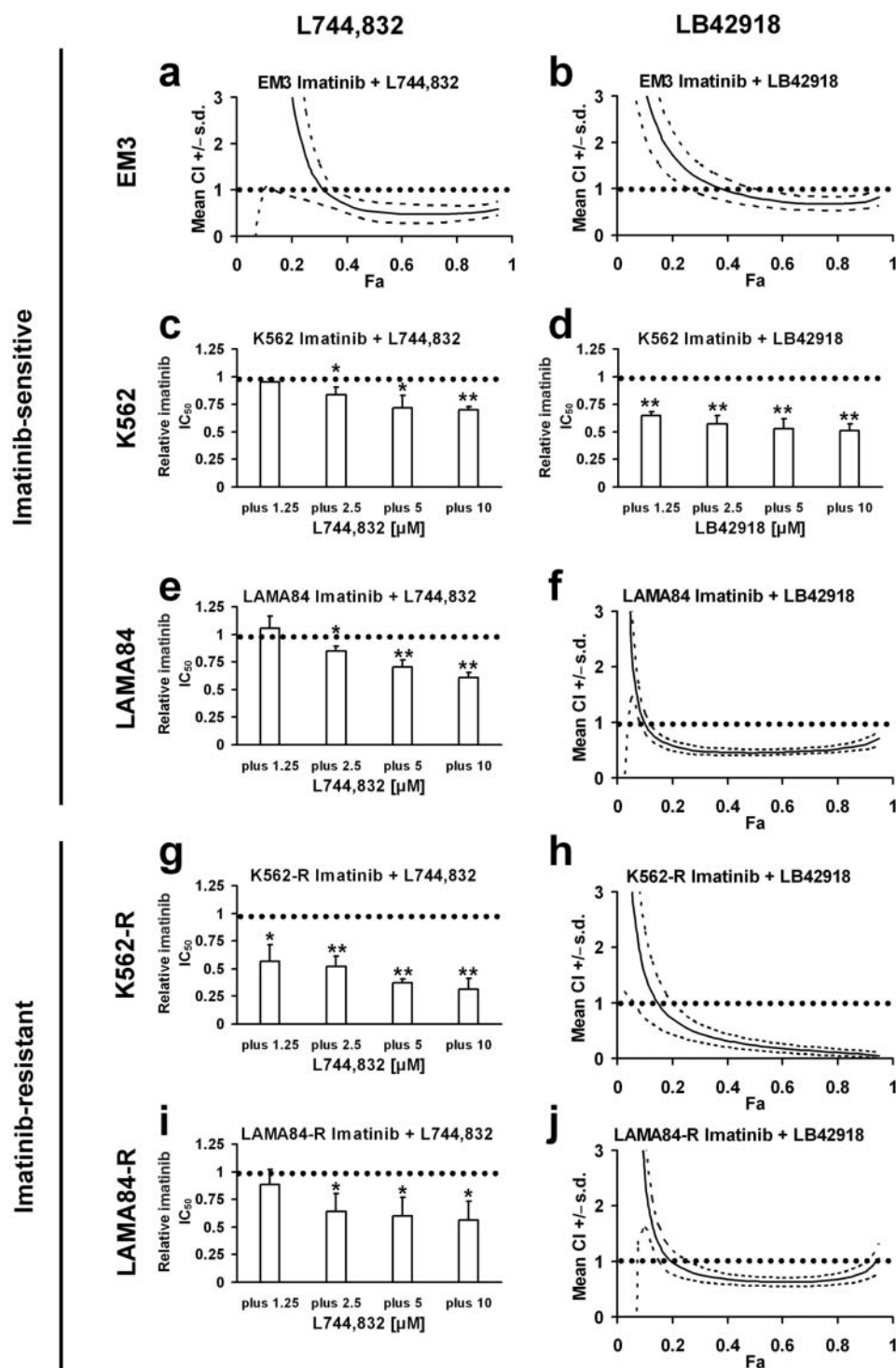
Figure 1. *Analysis of combination effects. (a, b, f, h, j) CI values by analysis according to the median-effect method of Chou and Talalay were determined when both single drugs displayed activity (cf. Table I). (c-e, g, i) In cases where single drug FTI treatment was inactive, the decrease of imatinib IC_{50} (in combination with a noncytotoxic dose of FTI) was assessed in terms of relative imatinib IC_{50} values (cf. Materials and Methods). (a, b) Treatment of imatinib-sensitive EM3 cells with combinations of imatinib plus L744,832 and imatinib plus LB42918 showed synergistic effects (CI<1) above affected fractions approximately ≥0.4 and 0.6, respectively. (c-e) In K562 and LAMA84 cells, noncytotoxic doses of L744,832 potentiated imatinib activity; the same was observed in K562 cells by treatment with imatinib plus LB42918 (d). Synergism between imatinib and LB42918 was observed in LAMA84 cells (f) with CI<1 above Fa> approx. 0.15. In the imatinib-resistant lines K562-R and LAMA84-R, noncytotoxic doses of L744,832 potentiated imatinib activity (g, i). In both lines, the combination of imatinib plus LB42918 showed synergism with CI<1 above Fa> approx. 0.2 (h, j). (*p<0.05, **p<0.01)*
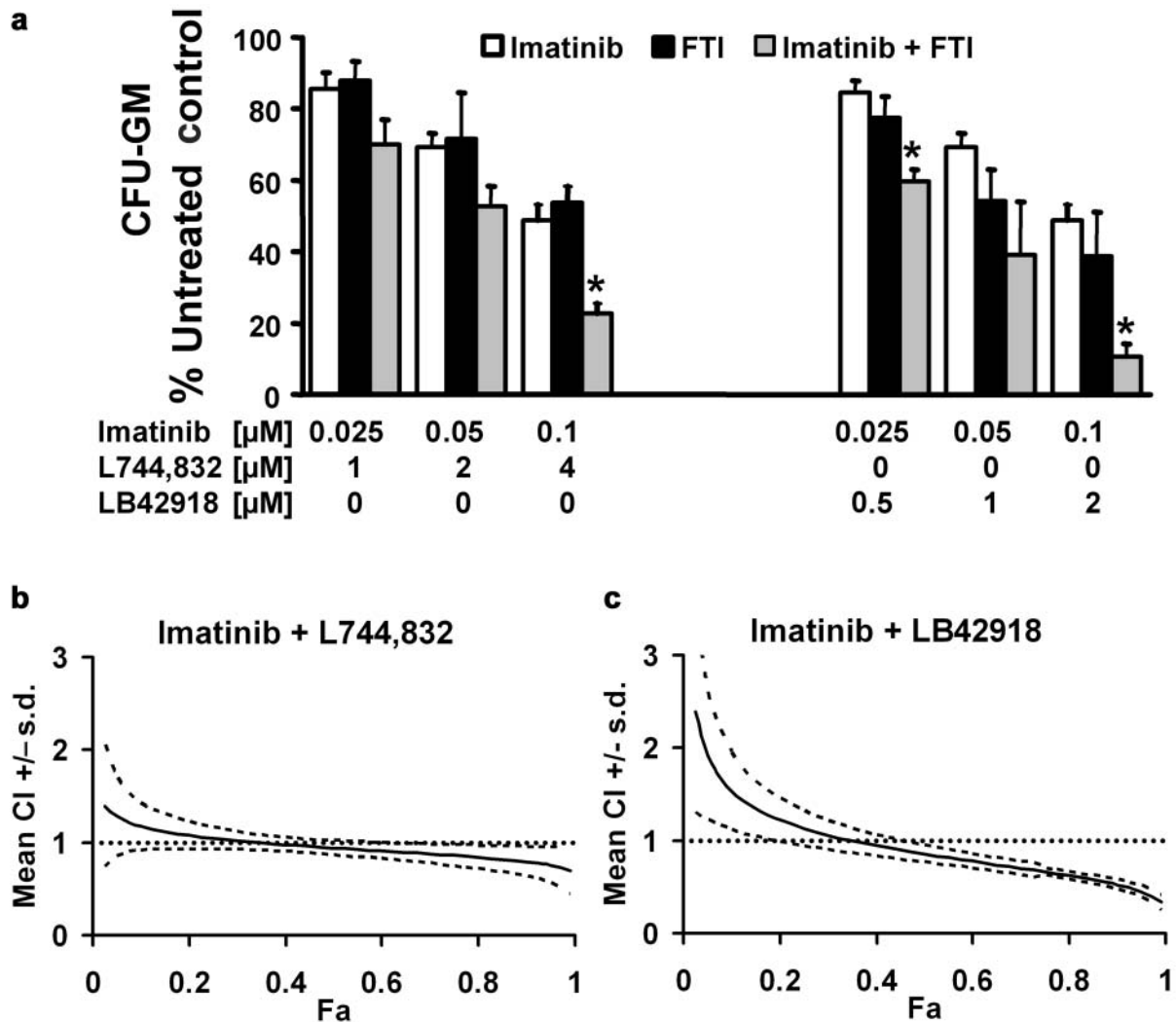
Figure 2. *Effects of imatinib plus FTIs on clonogenicity of primary human CML cells. (a) Colony formation of primary chronic-phase CML cells was more effectively inhibited by the combination of imatinib plus FTI as compared to treatment with each agent alone. CI plots calculated on the basis of colony-forming cell assays indicated (b) additive (CI ~ 1) and (c) synergistic (CI<1) effects of imatinib plus L744,832 and imatinib plus LB42918, respectively. Data for three patients, mean±standard deviation. (*p<0.05)*

combination treatment led to a substantial increase in the early apoptotic cell fraction in LAMA84-R (12% for imatinib plus L744,832 and 14% for imatinib plus LB42918). Comparable effects for both combinations were observed in K562-R (9% and 10%, respectively) signifying enhanced apoptosis in this imatinib-resistant line as well (Figure 3b).

Discussion

The tyrosine kinase inhibitor imatinib has revolutionized the drug therapy of CML. Recent reports from a phase III study demonstrated imatinib to be greatly superior to interferon plus low-dose cytarabine as first-line treatment in newly diagnosed chronic-phase CML with respect to hematological and cytogenetic responses, progression-free survival and quality of life outcomes (22, 23). However, in advanced stages of CML, the emergence of resistance under imatinib monotherapy has commonly been observed, causing frequent relapses and complicating disease treatment (10). Studies with *BCR-ABL*-positive cell lines and primary cells derived from patients who had become resistant to imatinib monotherapy identified multiple mechanisms of resistance, including genomic amplification of the *BCR-ABL* fusion gene, overexpression of BCR-ABL transcripts, point mutations of the *BCR-ABL* tyrosine kinase domain and non-specific multidrug resistance caused by P-glycoprotein (Pgp) overexpression (24-28). These findings point out that combination chemotherapy with imatinib plus drugs with
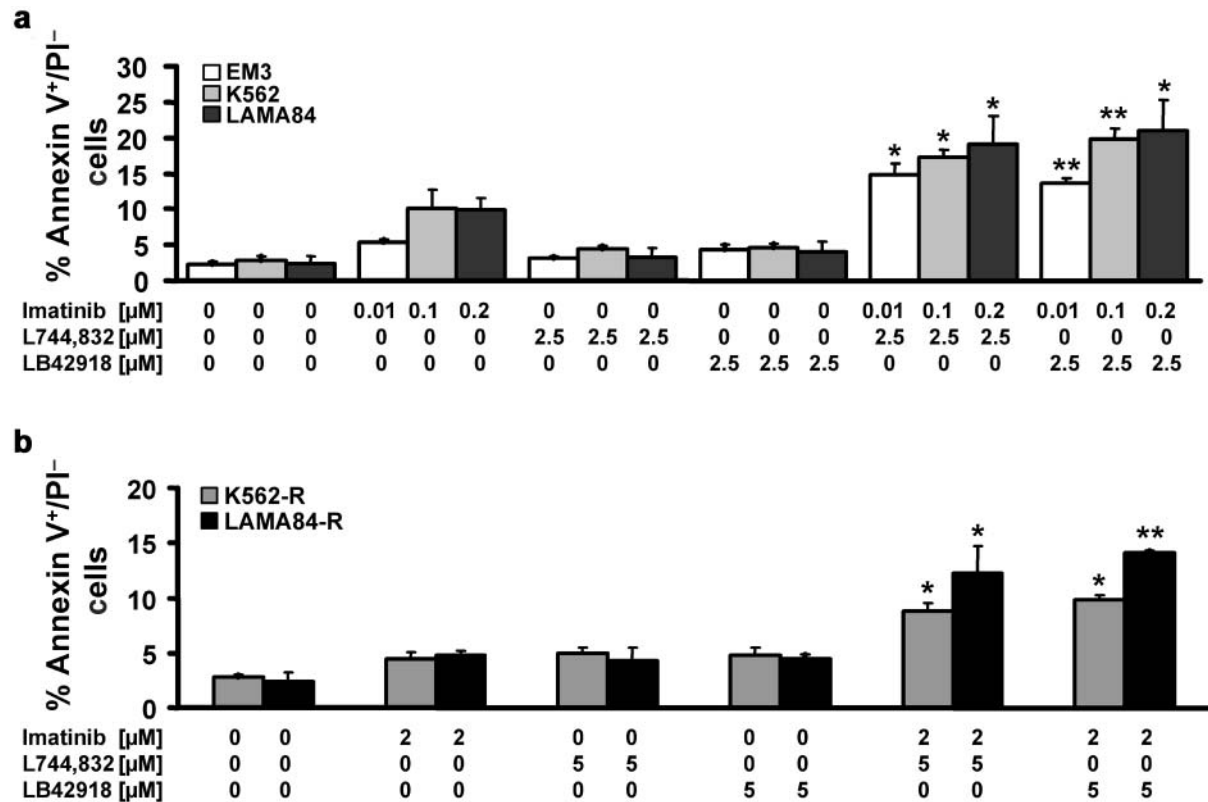
Figure 3. *Induction of apoptosis in imatinib-sensitive and -resistant CML cells after treatment with different concentrations of imatinib, L744,832 or LB42918 alone or in combination. (a) Combination treatment (imatinib plus L744,832 or imatinib plus LB42918) of imatinib-sensitive CML cell lines significantly increased apoptotic cell fractions (annexin V^+ / PI^- cells) as compared to imatinib or FTI monotherapy of the respective lines. (b) In imatinib-resistant K562-R and LAMA84-R cells for both combinations comparable effects as observed in imatinib-sensitive cells were discernible with significant enhancement of apoptosis. (*p<0.05, **p<0.01)*

different mechanisms of action may enhance the effect of imatinib and consequently increase the antileukemic efficacy.

Constitutive activation of Ras proteins plays an important role in the malignant growth of solid tumors and can frequently be observed in hematological malignancies (16, 29). Blocking the Ras signal transduction pathway by interfering with posttranslational Ras processing *via* farnesyltransferase inhibition was reported to have antiproliferative effects on *BCR-ABL*-positive cells and in *BCR-ABL*-induced murine leukemia models (30, 31). Besides Ras, several other proteins with importance in cell proliferation, survival and apoptosis, including the Rho family of proteins and the centromere active proteins CENP-E and CENP-F, are farnesyltransferase substrates and appear to contribute to the antiproliferative activity of FTIs (18, 32). Several groups have demonstrated the synergistic cytotoxic activity of a combination treatment with imatinib and FTI in imatinib-sensitive and -resistant cell lines suggesting that FTIs have the potential to overcome imatinib-resistance by targeting activated cellular signalling pathways downstream of BCR-ABL tyrosine kinase (33-35).

In the present study, the combination effects of two farnesyltransferase inhibitors (L744,832 and LB42918) with imatinib on imatinib-sensitive and -resistant cells were investigated. The imatinib-resistant cell lines were previously characterized in detail and both lines (LAMA84-R and to a lesser extent K562-R) represent two of the known mechanisms of resistance, *i.e.,* overexpression of BCR-ABL and increased Pgp activity (19). Monotherapy with L744,832 and LB42918 resulted in variable antiproliferative effects in imatinib-sensitive *BCR-ABL*-positive cell lines with only the EM3 cells being sensitive to both compounds (Table I). In the imatinib-resistant lines, only LB42918 reduced cell growth and was nearly equipotent in the LAMA84 pair revealing no cross-resistance in the imatinib-resistant line LAMA84-R (Table I). Combination therapy with imatinib showed increased antiproliferative effects resulting either from potentiation of imatinib activity or synergism. Simultaneous application of imatinib and LB42918 displayed synergistic activity (CI values <1) in EM3, in imatinib-sensitive LAMA84 and both imatinib-resistant LAMA84-R and K562-R lines; in imatinib-sensitive K562

cells potentiation of imatinib activity could be observed. In contrast, the combination of imatinib plus L744,832 demonstrated synergism in EM3 but potentiation in the other cell lines (Figure 1). The reason for the varying efficacies of the FTIs on the different cell lines, both in monotherapy and in combination with imatinib, is unclear but may possibly be attributed to the ability of FTIs to interfere with the function of a large number of proteins which are farnesyltransferase substrates and which, apart from Ras, are probably more or less relevant for cell growth in the particular lines.

The combination treatment of human primary cells derived from patients with chronic-phase CML resulted in stronger suppression of colony formation as compared to monotherapy and revealed additive and synergistic effects for the combination of imatinib plus L744,832 and imatinib plus LB42918, respectively, implying improved efficacy of the combinations in this stage of the disease as well (Figure 2). Comparable results were obtained by others with the combination of imatinib plus SCH66336 (33, 34). In a recent report by Morgan *et al.* (36) on combination effects of prenylation inhibitors in multiple myeloma cells, the IC_{50} of L744,832 towards normal $CD34^+$ cells was 8.2 μM, suggesting that the concentration applied in our colony-forming assays were not likely to cause unspecific hematotoxicity.

When combined with imatinib, for both L744,832 and LB42918, a significant increase in the number of early apoptotic cells as compared to single agent imatinib could be observed both in imatinib-sensitive and -resistant cells (Figure 3). These findings are comparable with previously published results for R115777 (35) and SCH66336 (34), signifying the potential of FTIs to enhance imatinib-induced apoptosis.

In a study by Wang *et al.* (37), SCH66336 was shown to be a potent inhibitor of Pgp, predicting improved efficacy of cancer therapeutics that are subject to Pgp-mediated drug efflux. Furthermore, imatinib was reported to be a substrate for Pgp (38). In functional ^{123}Rh efflux assays in imatinib-sensitive and -resistant LAMA84 cells, both FTIs tested in the present study showed no inhibitory effects on Pgp function, providing no evidence for a possible interaction with imatinib activity *via* this mechanism.

L744,832 was shown to have antiproliferative effects on various solid tumor cell lines (39). Moreover, the combination treatment with L744,832 and different drugs resulted in enhanced cytotoxic effects in several *in vitro* tumor models as well (40, 41). Recent results on the antileukemic effects of L744,832 indicated synergistic activity in combination with the checkpoint abrogator UCN-01 and other prenylation inhibitors in multiple myeloma cells (36, 42). For LB42918, no *in vitro* data are available to date. The present study is the first to address the antileukemic activity and efficacy of LB42918 alone and in combination with imatinib.

Several FTIs (*e.g.,* SCH66336, R115777, BMS-214662) have entered clinical trials and demonstrate tolerability and largely acceptable toxicity profiles upon single-agent treatment (17). However, due to the limited efficacy of FTI monotherapy in clinical studies on CML, these drugs did not meet the expectations. Cortes *et al.* (43) reported modest but demonstrable activity of R115777 monotherapy in one-third of patients with CML; in a pilot study of single-agent SCH66336 in patients with CML resistant or refractory to imatinib, comparable results were observed thus highlighting the necessity for ongoing studies combining FTIs and imatinib (44).

The present study demonstrated the *in vitro* activity of two FTIs (L744,832 and LB42918) as single agents and in combination with imatinib. Combination with imatinib led to enhanced antiproliferative effects both in imatinib-sensitive and -resistant cells. The efficacy of the combination in imatinib-resistant cells indicated the potential of L744,832 and LB42918 to overcome resistance towards imatinib and suggests that both FTIs investigated might find their place in combination with imatinib for CML treatment.

Acknowledgements

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