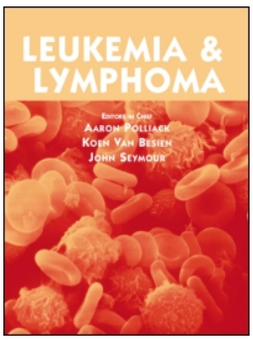

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Modulation of leukotriene signaling inhibiting cell growth in chronic myeloid leukemia

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ABSTRACT

Although tyrosine kinase inhibitors (TKIs) have dramatically improved clinical outcome in chronic myeloid leukemia (CML), cure rarely occurs. This may be due to *BCR-ABL*-independent, aberrant signaling pathways, one of which leads to leukotriene (LT) formation. Well-recognized as inflammatory mediators, LT can also affect oncogenic mechanisms of several tumors. We have previously discovered elevated LT-synthesis and up-regulated cysteinyl-LT-inducing enzyme in CML. Here we report on dose-dependent inhibition of CML cell growth exerted by specific blockers of LT-signaling. Thus, the cysteinyl-LT1-receptor-antagonist montelukast significantly reduced the growth of K562, KCL22, and KU812 cells, as well as primary $CD34^+$ blood cells from two CML patients. Adding montelukast to the TKI imatinib caused combined inhibition. No effect was seen on normal bone marrow cells. Similarly, growth inhibition was also observed with the 5-lipoxygenase (LO)-inhibitor BWA4C, the 5-LO-activating-protein-(FLAP)-inhibitor licofelone and the LTB_4(BLT1)-receptor-antagonist LY293111. Thus, blocking of aberrant LT-signaling may provide an additional, novel therapeutic possibility in CML.

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Chronic myeloid leukemia; leukotrienes; pharmacotherapeutics

Introduction

Chronic myeloid leukemia (CML) is a malignancy triggered by the fusion oncogene *BRC-ABL1*, expressed as a constitutively activated tyrosine kinase and believed to take its origin in the pluripotent bone marrow stem cell.[1] Although the introduction of imatinib, and other tyrosine kinase inhibitors (TKIs), has markedly improved the outcome for patients with CML,[2,3] some major clinical problems still remain. Despite continuous, long-term TKI administration signs of persistent leukemia are commonly detectable, primarily thought to be due to persistence of the leukemic stem cell (LSC), thus preventing cure. Furthermore, TKI resistance and intolerance, as well as disease progression to blastic phase, constitute remaining clinical challenges. There is thus a need to further improve the current therapy, to make it more curative, rather than palliative. Several signaling pathways, in addition to that directly associated with the well-established *BCR-ABL* tyrosine kinase fusion protein, have been found to be aberrantly expressed, and presumably involved in the initiation and maintenance of the leukemic clone, particularly in LSC.[4] Among these pathways are e.g. Wnt/β-catenin, PML, and sonic hedgehog, but also one that leads to the formation of the bioactive leukotrienes (LT).[4] These compounds are synthesized from membrane bound arachidonic acid (AA) *via* the 5-lipoxygenase (5-LO) pathway (Figure 1), for review.[5] AA is converted to the leukotriene epoxide LTA_4 by 5-LO, aided by 5-LO activating protein (FLAP) and coactosin-like protein (CLP).[6] Further metabolism of LTA_4 can proceed *via* LTA_4 hydrolase (LTA4H) to LTB_4, or *via* LTC_4 synthase (LTC4S) to the cysteinyl (cys) leukotrienes C_4, D_4, and E_4. Leukotriene B_4, which is an important pro-inflammatory mediator, binds to two types of surface receptors denoted BLT1 and BLT2.[7] Recently, LTB_4 has also been shown to be involved in atherosclerosis, myocardial infarction, and stroke.[8,9] The CysLTs are prominent products of myeloid cells such as activated macrophages, mast cells, eosinophils, and basophils.

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Supplemental data for this article can be accessed here.

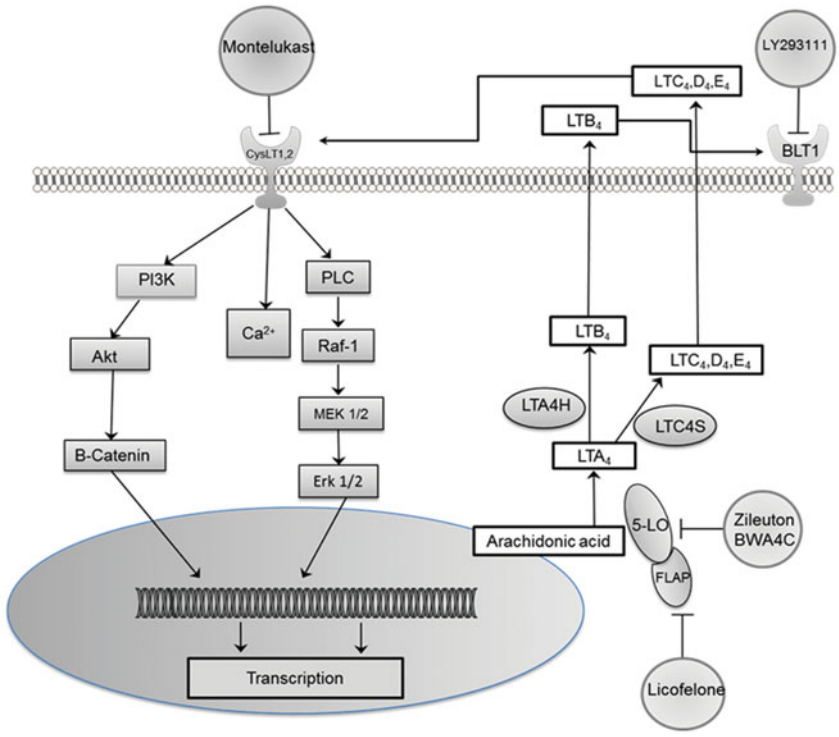

Figure 1. Schematic depiction of leukotriene biosynthesis from arachidonic acid, including receptors for leukotriene B_4 and cysteinyl leukotrienes with major signal transduction pathways upon inhibition of this receptor by montelukast.

The physiologic effects of the CysLTs are mediated through G-protein-coupled receptors, CysLT1, and CysLT2. Leukotriene D_4 has been reported to promote cell survival by increasing cellular Ca^{2+} and activating PKCα and cAMP response element-binding proteins and promoting proliferation by activation of PKCε and ERK1 and 2.[10] An additional pathway leading to the activation of Akt, results in an increase of nuclear β-catenin (Figure 1).[11] The CysLT1 receptor has a clear role in respiratory diseases, such as nasal allergies and asthma, but its activation has also been coupled to an up-regulation of proatherosclerotic genes.[12] Selective CysLT1 receptor antagonists of the leukast family have been in clinical use for both prophylaxis and treatment of asthma since several years.[13] The CysLT2 receptor mediates pro-inflammatory responses and has recently been implicated in a variety of cardiovascular functions.[12]

Our group has previously described a markedly increased capacity of CML myeloid cells to synthesize LT from endogenous substrate.[14] We also discovered that both LTB_4 and LTC_4, at picomolar concentrations, were capable of stimulating the growth of normal myeloid progenitor cells.[15] Furthermore, we observed that the expression and activity of the LTC4S enzyme was increased in myeloid cells, including neutrophils, from CML patients, as compared with that in corresponding cells from healthy donors.[16,17] More recently, Chen et al detected an up-regulation of the arachidonate 5-LO gene (*ALOX5*) in mouse CML stem cells, and noted that mice transplanted with *ALOX5*-deficient *BCR-ABL*-positive bone marrow cells were resistant to CML induction.[18] Furthermore, they found that treatment of CML mice with the 5-LO inhibitor zileuton impaired functions of their LSCs (but not of normal stem cells) as well as improved the overall survival of the mice, to the same extent as the TKI imatinib.[19] These data are of interest since leukotrienes, in addition to their well-known pathophysiological role in inflammation and asthma, have also been shown to be involved in various malignancies, such as colon cancer and prostate cancer [20,21] – see below.

In view of these data, we set out to further investigate the role of LT signaling in the growth of human CML cells, by interfering with specific LT enzyme activities and LT ligand binding. We used several pharmacologic compounds, some of which have already been clinically tested and approved in patients with non-hematologic disorders. Employing three different human CML cell lines and freshly drawn, CD34-separated mononuclear blood cells from two CML patients, we show that the CysLT receptor antagonist montelukast induced a clear and dose-dependent inhibition of cell growth. This was achieved at montelukast concentrations mimicking a clinical *in vivo* situation. Furthermore, other

inhibitors of key proteins involved in LT-signaling were also found to reduce CML cell growth in a dose-dependent fashion. When adding LT-modulating compounds to the TKI imatinib an amplified inhibitory effect was noted. Taken together, these data indicate a possible role for montelukast, and conceivably also other LT-signaling modulators, as novel therapeutic agents combined with TKIs in the quest to cure CML.

Materials and methods

Cell lines

The CML cell lines were obtained from Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures. K562 cells (DSMZ No. ACC 10), KCL22 (DSMZ No. ACC 519), KU812 (DSMZ No. ACC 378) were all derived from human CML in blast crisis. HCT-116 cells (ATCC No. CCL-247), derived from human colon carcinoma, were obtained from the American Type Culture Collection (Manassas, VA). The lung fibroblast cell line WI-38 was obtained from Coriell Cell Line Repository (CCR, Camden, NJ).

All tumor cell lines were cultured in RPMI 1640 with 10% FBS, 2 mmol/L L-glutamine (Invitrogen, CA) Pen Strep (Gibco, Life Technologies, CA). Lung fibroblasts WI-38 were maintained in MEM (Invitrogen) with 15% FBS and 2 mmol/L of L-glutamine.

The cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were conducted with cells at passages 5–15.

Primary cells

Peripheral blood was collected from two chronic phase, Philadelphia positive CML patients at diagnosis. Bone marrow aspirate from the iliac crest was obtained from an adult volunteer healthy donor. All procedures were performed in accordance with the principles of the Helsinki declaration, with written informed consent obtained from the respective donors and with approval from the Karolinska University Hospital Ethics Review Board. To obtain mononuclear cell populations, the blood samples were processed through Ficoll paque (GE Healthcare, IL) and the bone marrow sample through Lymphoprep separation (StemCell Technologies, Vancouver, Canada).

Mononuclear cells were then purified further using the CD34 MicroBead Kit according to the manufacturer's protocol and passed through MACS MS column (Miltenyi Biotec, Bergisch Gladbach, Germany) in order to separate $CD34^+$ fraction.

$CD34^+$ cells were then resuspended in StemSpan SFEM medium (StemCell Technologies, Vancouver, Canada) supplemented with 20 ng/ml of human Flt-3 ligand, stem cell factor and thrombopoietin (Peprtotech, NJ).

Inhibitors

The CysLT1R antagonist montelukast and all TKIs (imatinib, nilotinib, and dasatinib) were purchased from Selleck Chemicals (Houston, TX). The FLAP inhibitor licofelone, the LTB_4 receptor (BLT1) antagonist LY293111 and 5-LO inhibitor zileuton were purchased from Cayman Chemicals (Ann Arbor, MI). The 5-LO inhibitor BW4AC was purchased from Sigma Aldrich (St. Louis, MO).

Western blot

Cell lysates were prepared in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.5% Igepal, 5 mmol/L EDTA, and 0.1% SDS) containing 10% protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics AB). BCA Protein Assay Reagent (Interchim, Montluçon Cedex) was used to measure protein concentration and 50 mg of protein was loaded to Bis Tris 4% gel (NuPAGE, Invitrogen). Electrophoresis was performed in MES running buffer (NuPAGE, Invitrogen) at 180 V for 60 min and transferred to polyvinylidene fluoride membranes (Hybond-C Extra; Amersham Biosciences) in transfer buffer (NuPAGE, Invitrogen) and 10% methanol at 30 V for 90 min. Odyssey blocking buffer (Li-Cor Biosciences, Germany) was used to prevent nonspecific binding. Membranes were incubated overnight with following primary antibodies: rabbit polyclonal anti-human CysLTR1&2 (1:250) (Cayman Chemicals, MI), rabbit monoclonal anti-human BLT1(1:250) (Abcam, Cambridge, UK), rabbit polyclonal LTC4S (1:500) (Acris Antibodies, Herford, Germany),rabbit polyclonal anti-human 5-LO (1:500), and rabbit polyclonal anti-human FLAP (1:400) (both developed in-house). β-tubulin (Sigma-Aldrich) was used as loading control. In order to analyze bands membranes were incubated with IR-Dye-linked secondary antibodies (LI-COR Biosciences) for 1 h at room temperature.

Proliferation assays

Proliferation of the CML cell line cells was measured using the MTT cell proliferation assay, according to the manufacturer's instructions. Briefly, cells were seeded in triplicates in flat-bottomed 96-well plates at 30,000 cells/well and grown for 72 h alone or in the presence of leukotriene pathway inhibitors and/or TKIs (imatinib,

nilotinib, or dasatinib) in 37 °C and 5% CO_2. After incubation with MTT reagent for 4 h (Sigma-Aldrich, Germany) (0.5 mg/ml), the reaction was stopped with 10% SDS +0.01 mol/L HCl. After an overnight incubation in 37 °C, the absorption of the samples was measured at 595 nm. Values shown are the mean of three replicates in three independent experiments ± SEM.

The mononuclear $CD34^+$ primary cells from the peripheral blood of the CML patients, as well as from the normal bone marrow, were seeded in triplicates at a density of 15,000 cells/well in 96-well plates with or without presence of montelukast and/or imatinib. At repeated time points (up to 144 h), aliquots of the cells were then counted under microscope in hematocytometer chamber after adding Trypan blue solution (Sigma-Aldrich, Germany) to assess the number of viable cells.

Statistical analysis

All statistical analyses were performed in Microsoft Excel and Prism Software (GraphPad, Inc.) and the statistical significance of data was determined as $p < 0.05$ or less. For comparison between two groups unpaired *t* test (Student's *t* test) was used. All values are expressed as the mean ± standard error of the mean (SEM).

Results

Leukotriene pathway key proteins expressed in CML cells

Synthesis of and response to LT are dependent on key enzymes and protein receptors. We therefore analyzed the expression of LT-inducing proteins, as well as CysLT and BLT1 receptors, in the CML cell lines K562, KCL22 and KU812 by immunoblotting. Figure 2 shows a clear expression of each of seven studied key proteins in the respective cell lines. Thus, we can conclude that all three cell lines appear to have the prerequisites to produce LT and also to respond to LT stimulation. Inhibitors of these proteins would thus also be likely to affect the LT-signaling capacity in the studied cells.

Reduction of CML cell growth by tyrosine kinase inhibitors

In order to assess and calibrate the *in vitro* MTT cell growth model, and to relate the results to known clinical outcome data, CML cell lines were treated with each of the three TKIs imatinib, dasatinib, and nilotinib

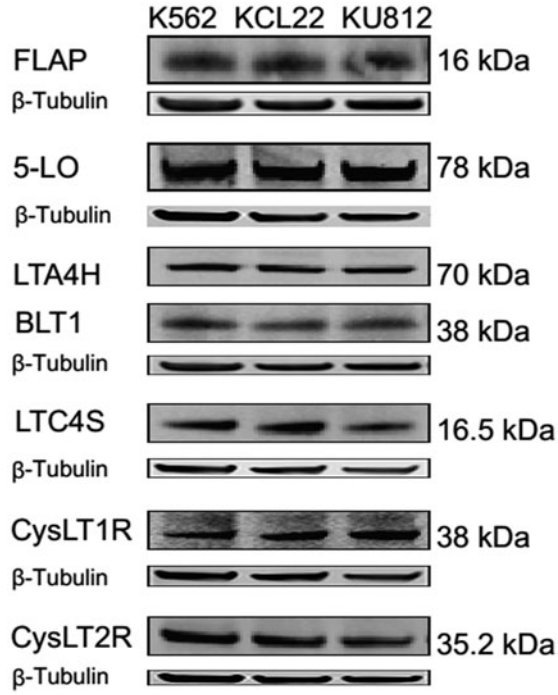

Figure 2. Expression of key proteins in the leukotriene pathway in the three human CML cell lines K562, KCL22 and KU812.

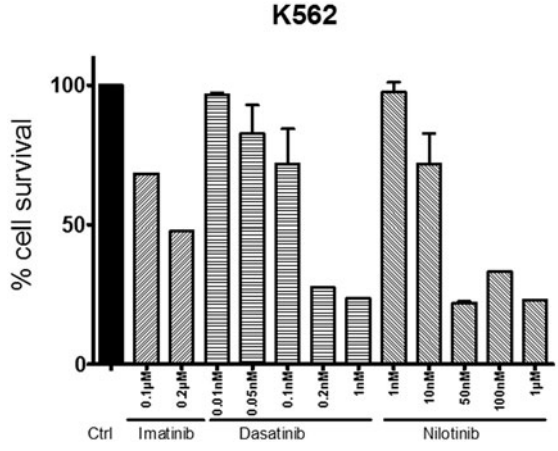

Figure 3. Growth inhibitory effects on CML cells by the TKIs imatinib, dasatinib and nilotinib.

in three day cultures. All three TKIs exerted clear, dose-dependent growth reductions of all three cell lines, when compared with the respective, untreated cells. In Figure 3, representative data for K562 cells are shown. Dasatinib provided the most potent growth inhibitory effects (with IC_{50} concentrations in the low nanomolar range), followed by nilotinib and imatinib (in the high nanomolar and micromolar range, respectively). Imatinib is the most widely used TKI in the treatment of CML. Therefore, for each CML cell line dose-response measurements were performed to

detect the respective IC_{50} level of imatinib (see Table 1). In subsequent combination experiments with LT-modulators imatinib was then employed at these concentration levels (see below).

The results are in line with those obtained in clinical settings utilizing these drugs in CML patients, with dasatinib considered as the most, and imatinib as the least potent of the three TKIs, drug concentration wise. The data also provide support for the MTT assay as a relevant model when studying CML cell proliferation *in vitro*.

In this study, we subsequently chose imatinib as a representative TKI.

Reduction of CML cell growth by the CysLT1 receptor antagonist montelukast

To investigate the role of the LT pathway in CML cell growth, we first subjected the three CML cell lines K562, KCL22 and Ku812 to montelukast, a CysLT1 receptor antagonist. In Figure 4(a), a dose-dependent, significant inhibitory effect of montelukast is demonstrated on all these three CML cell lines, in three day cultures. A pronounced, highly significant and reproducible inhibition was seen particularly at montelukast concentrations of 2–3 μM. Similarly, and as expected, imatinib also induced significant growth inhibition in all three cell lines, detectable already at a concentration of 0.3 μM (Figure 4(a)). The calculated IC_{50} values of montelukast, and other inhibitors of LT signaling (see below), as well as of imatinib, are presented in Table 1. For montelukast, the IC_{50} ranged from 1.2 to 1.8 μM, with KCL22 as the apparently most sensitive cells. Corresponding values for imatinib were 0.1–0.3 μM. When montelukast was added to imatinib a combined growth inhibition was observed, significantly more pronounced than with imatinib alone, in all three cell lines, in a dose-dependent fashion (Figure 4(a)).

We further assessed the effect of montelukast on the growth of primary CML cells, derived from the peripheral blood of two CML patients in chronic phase. Figure 4(c,d) depicts the number of viable cells in culture assessed at multiple time points, up to 144 h after plating.

Table 1. IC_{50} values of leukotriene pathway inhibitors and imatinib in different CML cell lines after 72 h treatment.

	IC_{50} (μM)				
Cell line	Montelukast	BWA4C	LY293111	Licofelone	Imatinib
K562	1.75	26.33	>40	8.53	0.3
KU812	1.33	4.25	15.21	5.26	0.1
KCL22	1.16	15.25	8.81	3.25	0.3

In Patient 1, the addition of montelukast (4 μM) reduced the number of cells, compared with the controls, at each time point scored with the most pronounced effect after 144 h (93% reduction, $p = 0.0004$; Figure 4(c)). As expected, imatinib (0.3 μM) also induced a growth inhibition which was consistent and significant from 96 h and onwards, with maximum effect after 144 h (89% reduction, $p = 0.0006$). When montelukast was added to imatinib a combined effect was achieved, with significant growth reduction, as compared with imatinib alone, at 72 h and 120 h (Figure 4(c)).

In Patient 2, a similar pattern was noted (Figure 4(d)). The inhibitory activity of montelukast alone was less prominent, with significant reduction of cell growth only at 120 h (22% reduction, $p = 0.015$). However, the additional growth inhibition when montelukast was added to imatinib was more evident, with significant reductions compared with imatinib alone, at four time points. A strong, consistent inhibition by imatinib alone vs. controls was noted from 48 h and onwards. At the 24 h time point, no significant growth modulatory effects were observed by either montelukast or imatinib or the combination montelukast plus imatinib (Suppl. Figure).

In control experiments, we examined normal bone marrow cells, colon cancer cells, and fibroblast. Unlike the CML cells, primary normal bone marrow cells appeared unaffected by co-incubation with montelukast. Thus, neither montelukast (1.7–5 μM) nor imatinib (0.3 and 1 μM) induced any significant reductions in cell numbers at the 48 h and 72 h time points (data not shown).The colon cancer cell line HCT116 was also assessed, in three day cultures. HCT116 is known to express CysLT1 receptor but does not express the *BCR-ABL* construct. As seen in Figure 4(b), montelukast added at micromolar concentrations exerted significant growth-inhibitory effects also on these cancer cells. In contrast, imatinib failed to exercise any influence, neither alone nor in combination with montelukast. Finally, we also investigated the growth-modulating capability of these two compounds on normal human fibroblasts (WI-38). Similar to the findings from normal bone marrow cells, we failed to detect any reduction in WI-38 cell growth in the presence of montelukast (0.2 and 2μM) or imatinib (1 and 5 μM) in 72 h cultures (data not shown).

Reduction of CML cell growth by the 5-LO inhibitor BWA4C, the FLAP antagonist licofelone and the BLT1 receptor antagonist LY293111

We next investigated the effect of inhibitors of the 5-lipoxygenase (5-LO) enzyme and the 5-LO activating

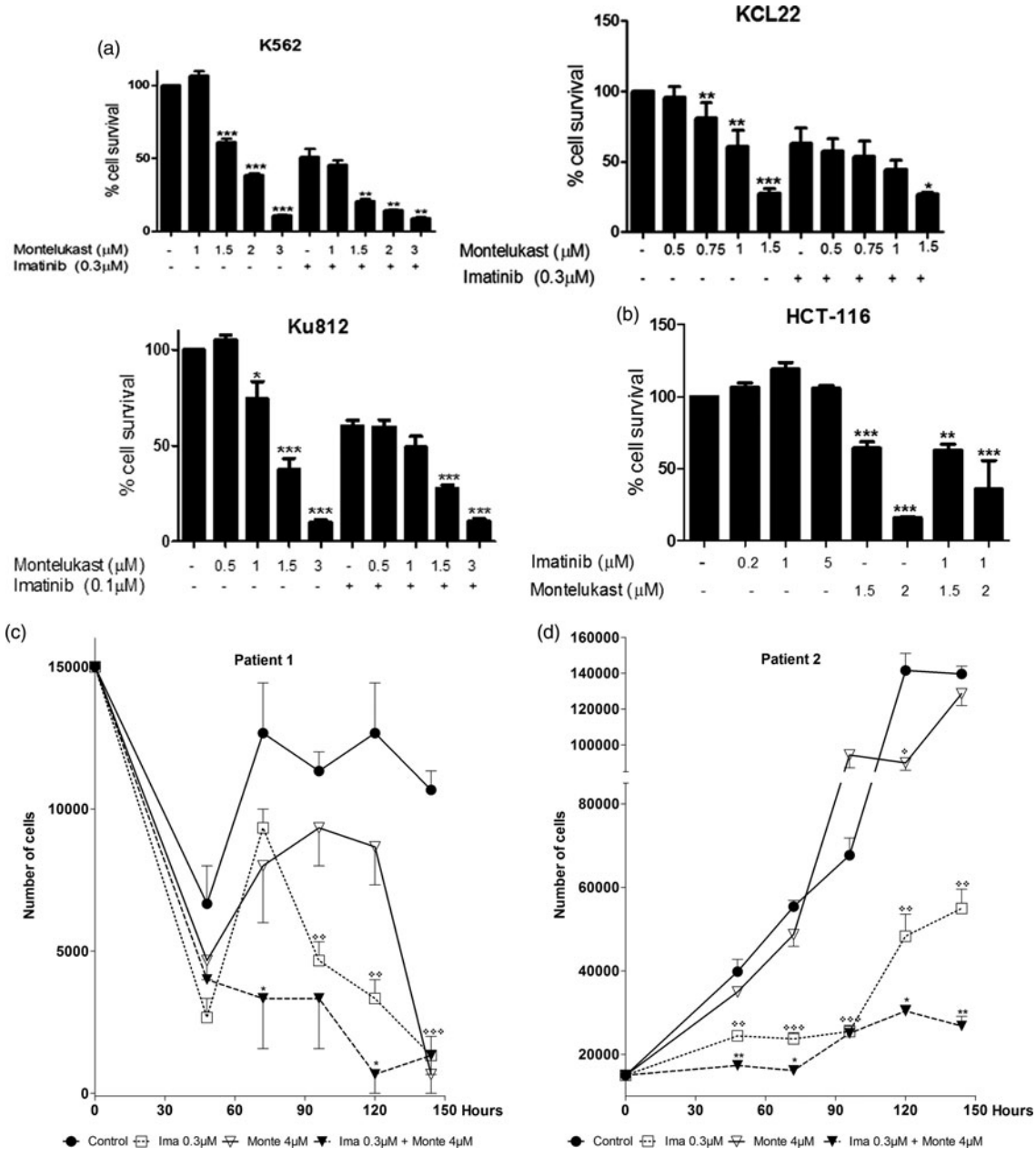

Figure 4. (a) Growth inhibitory effects on CML cells by imatinib and montelukast, administered as single drug or in combination. Data, mean of three independent experiments ± SEM. Treatment with montelukast was compared with untreated control, while combination treatment with imatinib and montelukast was compared with imatinib alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (b) Growth inhibitory effects on colon cancer HCT116 cells by imatinib and montelukast, administered as single drug or in combination. Data, mean of three independent experiments ± SEM. Treatment with montelukast was compared to untreated control, while combination treatment with imatinib and montelukast was compared with imatinib alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (c) and (d) Growth inhibitory effects on $CD34^+$ cells derived from the peripheral blood of two Philadelphia-positive CML patients in chronic phase by imatinib and montelukast, administered as single drug or in combination for up to 144 h. Data, mean ± SEM of three technical replicates. (❖) Reflects p values linked to inhibitory effects of imatinib or montelukast compared to untreated control. ❖, $p < 0.05$; ❖❖, $p < 0.01$; ❖❖❖, $p < 0.001$. (*) Reflects p values linked to inhibitory effects of the combination of imatinib and montelukast vs. imatinib alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

protein (FLAP), focusing on BWA4C and licofelone, respectively.

In all three cell lines, BWA4C induced a dose-dependent decrease in cell growth (Figure 5). There were, however, major differences between the cell lines with Ku812 being the most sensitive (IC_{50} 4 μM) and K562 the least sensitive (IC_{50} 26 μM, see Table 1). When combined with imatinib, BWA4C was also capable of inducing additional inhibitory growth effects, although within varying concentration ranges (Figure 5).

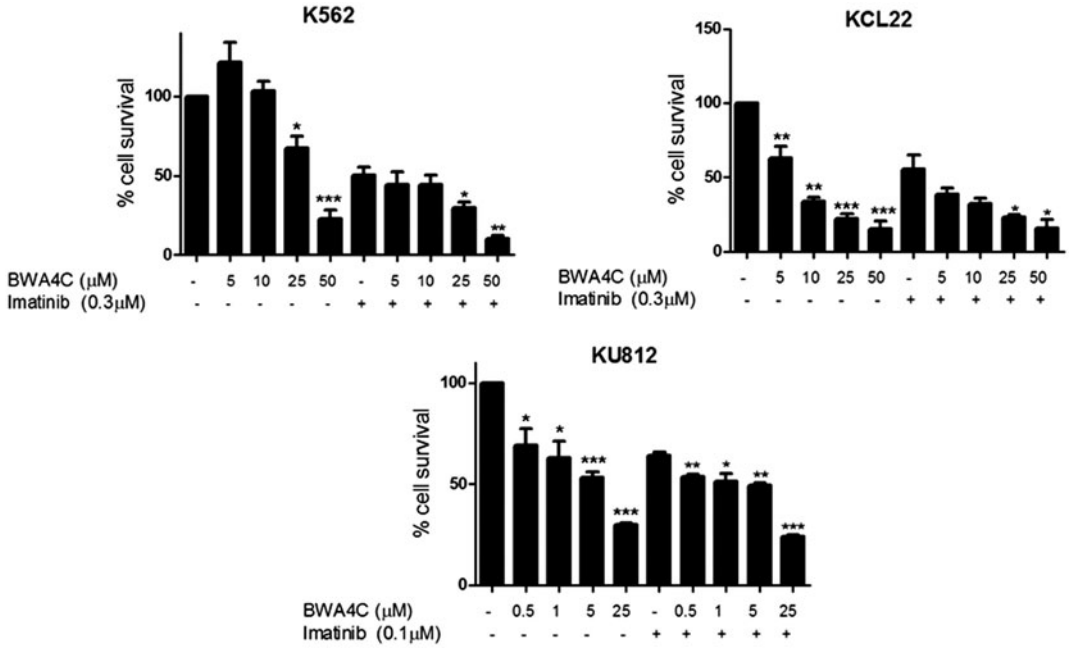

Figure 5. Growth inhibitory effects on CML cells by imatinib and BWA4C, administered as single drug or in combination. Data, mean of three independent experiments ± SEM. Treatment with BWA4C was compared to untreated control, while combination treatment with imatinib and BWA4C was compared with imatinib alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

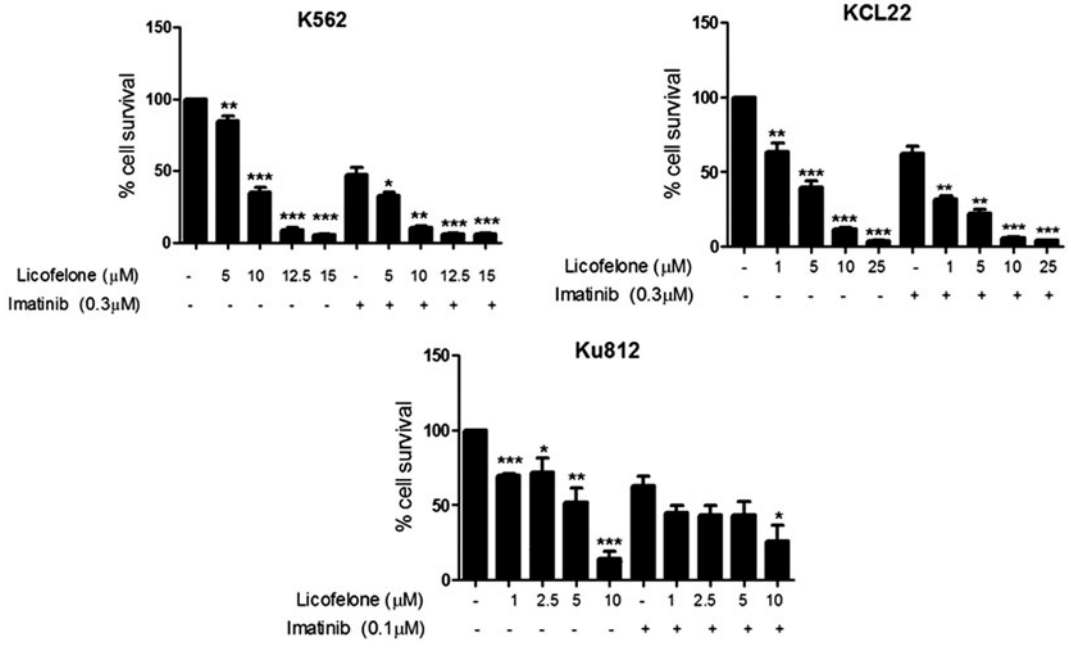

Figure 6. Growth inhibitory effects on CML cells by imatinib and licofelone, administered as single drug or in combination. Data, mean of three independent experiments ± SEM. Treatment with licofelone was compared with untreated control, while combination treatment with imatinib and licofelone was compared with imatinib alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

We also assessed the influence of zileuton, another 5-LO inhibitor, in the MTT model. Here we failed to detect any modulating effect on the growth of the tested CML cell lines, except at very high zileuton concentrations (above 80 μM), where moderate inhibition was seen (data not shown).

The FLAP antagonist licofelone was also found to affect CML cells. As shown in Figure 6, we obtained a concentration-dependent decrease in cell growth when adding this compound. Similarly to montelukast and BWA4C, the effect of licofelone was most pronounced on KCL22 and KU812 cells, while less

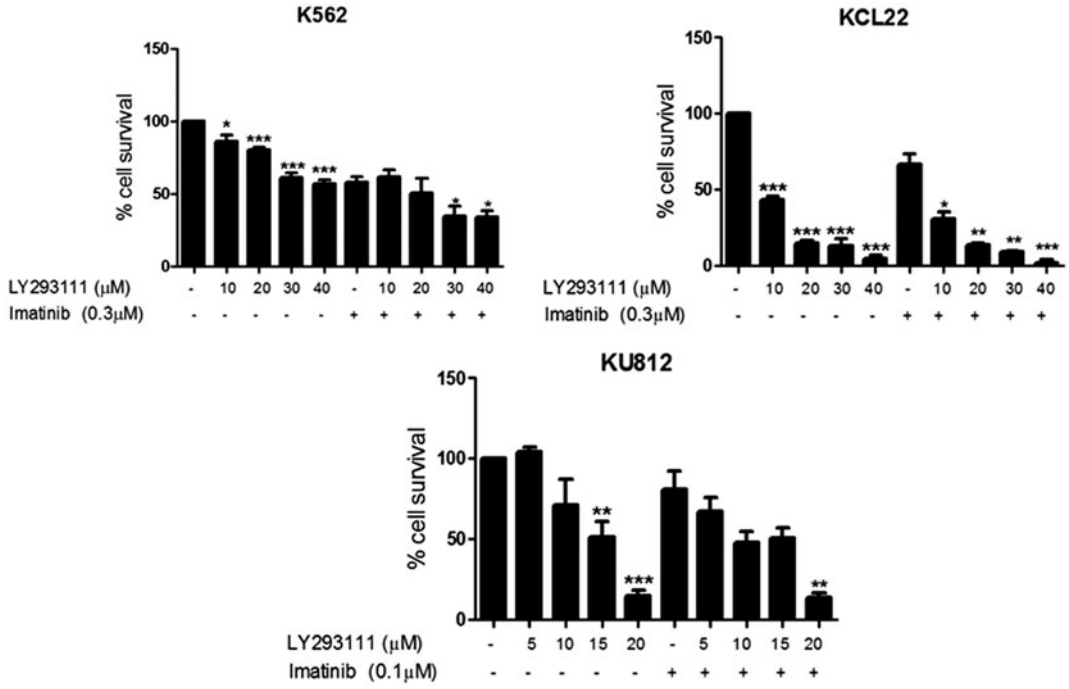

Figure 7. Growth inhibitory effects on CML cells by imatinib and LY293111, administered as single drug or in combination. Data, mean of three independent experiments ± SEM. Treatment with LY293111 was compared with untreated control, while combination treatment with imatinib and LY293111 was compared with imatinib alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

noticeable on K562 cells, with IC_{50} values ranging between approximately 3 and 9 μM (Table 1). As with the other described inhibitors, licofelone could induce an enhanced inhibition on cell growth when combined with imatinib (Figure 6).

Since LTB_4 receptor signaling is important for cell proliferation, and the expression of this protein was detected in our panel of CML cell lines (Figure 1), we also assessed whether antagonizing the BLT1 receptor could affect cell growth. As shown in Figure 7, a inhibitory effect of the BLT1 receptor antagonist LY293111 could be observed in two of the tested cell lines, KCL22 and KU812 ($p < 0.05$), while moderate influence was detected on the third (K562). Furthermore, additional inhibition was noted when LY293111 was added to any of the imatinib-challenged cell populations (Figure 7).

Discussion

In the present report, we have assessed the importance of the LT pathway on the growth and survival of human CML cells. The cells selected for study were derived from three different, well-characterized CML cell lines and from freshly drawn $CD34^+$ cells from peripheral blood collected from two CML chronic phase patients at diagnosis. We could observe a clear, dose-dependent reduction of cell growth after addition of various inhibitors of LT-signaling. In particular, the effects exerted by the CysLT1-receptor-antagonist montelukast were striking, but similar results were also gained with the 5-LO-inhibitor BWA4C and the FLAP-inhibitor licofelone. A less pronounced, and less consistent inhibition was observed with the BLT1 LTB_4 receptor antagonist LY293111. In contrast, the 5-LO-inhibitor zileuton did not affect cell growth when added at concentrations below 80 μM. As expected, we also observed a clear dose-dependent sensitivity to each of three tested TKIs (imatinib, dasatinib, and nilotinib) at *in vitro* drug concentrations mimicking the *in vivo*-situation.[22] When montelukast was added to imatinib a significant, combined growth inhibitory effect was noted on all tested CML cell populations, including the primary $CD34^+$ blood cells from the two studied CML patients. Similar combination results were also achieved when BWA4C or licofelone were added to imatinib in CML cell line experiments. Assessing the specificity of effects exerted by montelukast, we observed that it also inhibited the growth of the CysLT1 receptor expressing colon cancer cell line HCT-116, but left normal CD34-expressing bone marrow cells and normal fibroblasts (WI-38) unaffected. Furthermore, the biological effects observed by montelukast in our system were achieved with IC_{50} levels of approx. 2–4 μM, i.e. at concentrations readily assessable in the blood of patients clinically treated with the drug on the indication of bronchial asthma.

Although our data need to be confirmed in a larger group of CML patients, the results indicate that montelukast alone, or imatinib combined with montelukast, may improve treatment efficacy in CML. Whether individual patient expression patterns of LT-signaling (or in particular CysLT-signaling) can be linked to individual patient response to such therapeutic approaches needs to be evaluated in subsequent clinical trials.

A role for LT-signaling in tumor growth, as indicated by our data, does not seem to be restricted purely to CML. The influence of CysLT receptor signaling in non-hematologic malignancies has recently been highlighted. Montelukast was shown to inhibit the growth of six neuroblastoma cell lines with IC_{50} values in the range 2–10 μM.[23] Similarly, the growth of the colon cancer cell line HCT-116, including HCT-116 xenograft tumor, was also inhibited by montelukast.[24] Stimulation of human intestinal cell lines with LTD_4 enhanced their proliferation and survival.[25] Furthermore, an increased expression of CysLTR1 compared to CyLTR2 has been shown to mediate poor prognosis and a low level of differentiation in colorectal cancer, as well as in prostate cancer.[21,26] In the latter cancer form an overexpression of CysLTR1 has been reported, and also that CysLTR1 antagonists are capable of inhibiting prostate cancer cell growth.[20] Furthermore, CysLTR1 has also been demonstrated to mediate a chemokine-like effect improving survival of chronic lymphoid leukemia (CLL) cells.[27]

Down-stream signaling from CysLTR1 involves a number of pathways, such as Erk 1/2, CREB and PI3K-Akt-β-catenin.[10] Stimulation of these pathways leads to expression of proteins contributing to activation of proliferation and anti-apoptosis, such as cyklinD and BCL-xl.[10] Interestingly, it has been shown that signaling pathways down-stream from p210 *BCR-ABL* also include Raf-MEK-Erk and PI3K-Akt signaling.[28] The particular importance of increased β-catenin expression in CML, resulting in aberrant activation of pro-survival and self-renewal pathways presumably linked to the initiation and propagation of LSC, has recently been reviewed.[29]

Our data associated with blocking of BLT1 receptor signaling in CML also appears to be in concert with observations from other cancer systems. Thus, activation of this receptor has been reported to stimulate proliferation of pancreatic cancer cell lines and, similar to CysLTR, to induce a phosphorylation of Erk 1 and 2.[30] Conversely, blocking of the BLT1 receptor with LY293111 inhibited proliferation of pancreatic cancer cells, induced tumor cell apoptosis both *in vitro* and *in vivo*, and enhanced the anti-pancreatic cancer effect of gemcitabine, leading to clinical trials utilizing the LY293111 as a novel drug concept.[30,31] We noted that the BLT1 receptor protein was expressed by all three tested CML cell lines and that LY293111 did indeed cause an inhibition of their growth. However, the inhibition was modest and seen only at higher concentrations of LY293111 (from and above approximately 10 μM), i.e. above those previously reported necessary to reduce pancreatic cancer cell growth.[30]

We also observed that all three tested CML cell lines expressed 5-LO as well as FLAP. In addition, the LTA4H and LTC4S proteins were also detected, indicating that these CML cells possessed the enzymes required for endogenous synthesis of LTB_4 as well as the CysLT (Figure 1). The 5-LO inhibitor zileuton, that has proven effective in the clinical treatment of human asthma, was earlier shown to prolong survival of CML mice with an upregulated *ALOX5* expression.[18] However, when we added zileuton at clinically relevant concentrations to the human CML cell lines we failed to register any significant growth-modulating effect. This indicates that these CML cells, at the conditions present, were less dependent on an active 5-LO for their survival. This observation is in contrast to the findings made by Chen et al in the mouse system. In a recent report Lucas et al found that human CML cells expressed low levels of BLT1 receptor, and linked to this a downregulation of *ALOX5* mRNA levels.[32] The authors could also show that following imatinib treatment, *ALOX5* mRNA increased. These results suggest that the expression of 5-LO, and hence the 5-LO activity, may be less important for the survival of human CML cells. Interestingly, we noted that another 5-LO inhibitor, BWA4C, albeit at high concentration, induced an evident reduction of the growth of all our three cell lines tested. Furthermore, when inhibiting the 5-LO activating protein FLAP with licofelone we again registered a dose-dependent growth reduction in all three CML cell lines. In addition, licofelone induced an enhanced inhibition when administered together with imatinib. Recent data has demonstrated that BWA4C can exert cytotoxic effects on human cancer cells independent of the suppression of 5-LO activity.[33] Similarly, licofelone has been shown capable of inhibiting not only 5-LO but COX signaling as well.[34] It is also well documented that inhibition of COX can decrease tumor cell growth, e.g. in colon cancer.[35] We therefore need to consider the possibility of non-5-LO-mediated, 'off-target' effects contributing to our registered effects by BWA4C, and possibly by licofelone, on the CML cell growth. Another aspect worth considering in this context is the transcellular interactions described for LT formation. As an example, human platelets can express LTC4S, but not 5-LO,

making LT-formation from endogenous substrate impossible. Platelets can, however, produce CysLT after uptake of LTA_4 from neighboring myeloid cells.[36] Similar transcellular interplay may well be present, e.g. between leukemic cells and stromal cells in CML.

A conclusion that may be drawn from our present data is that the observed growth inhibition exerted through modulation of LT-signaling in our CML cells may be less dependent on 5-LO, but more clearly linked to CysLTR1, and that FLAP may play a key role in regulating 5-LO/COX signaling. This theory is supported by our previous data demonstrating a clear upregulation of LTC4S (on the levels of RNA, protein and activity) in Philadelphia-positive neutrophils (compared to normal neutrophils) drawn from CML patients at diagnosis.[17] We also detected an increased urinary excretion of CysLT from the CML patients, compared to controls,[17] suggesting an increased CysLT formation *in vivo* in these patients. Furthermore, when investigating bone marrow cells we have also shown that the expression of LTC4S was more pronounced in immature myeloid cells than in more differentiated myeloid cells (including neutrophils), both in cells derived from normal individuals and CML patients.[37] However, the expression of LTC4S was consistently higher in the CML patient-derived cells, as compared to corresponding normal cells.[37] An increased presence of CysLT in CML patients may thus contribute to an augmented myeloid progenitor cell growth, in a similar way as been shown in normal human myeloid progenitor cell cultures.[15]

A more extensive assessment of LT-signaling in various subtypes of *BCR-ABL*-positive cells from a larger cohort of CML patients, also relating abnormal expression patterns to clinical outcome, would be valuable. It would also be crucial to clarify, if possible at single-cell levels, the associations between LT-signaling and related intracellular pathways, such as Raf-MEK-Erk and PI3K-Akt. Present data suggest that an aberrant LT-signaling is of importance for CML pathogenesis and could thus constitute a novel therapeutic target for this disease. Some of the suitable drugs are already used today under other, non-hematological indications. LT-modulation may thus provide benefits in the clinical situation, providing an additional avenue to cure CML.

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