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Research paper

A kinase profile-adapted drug combination elicits synergistic cooperative effects on leukemic cells carrying *BCR-ABL1*^{T315I} in Ph+ CML

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ABSTRACT

In chronic myeloid leukemia (CML), resistance against second-generation tyrosine kinase inhibitors (TKI) remains a serious clinical challenge, especially in the context of multi-resistant BCR-ABL1 mutants, such as T315I. Treatment with ponatinib may suppress most of these mutants, including T315I, but is also associated with a high risk of clinically relevant side effects. We screened for alternative treatment options employing available tyrosine kinase inhibitors (TKI) in combination. Dasatinib and bosutinib are two second-generation TKI that bind to different, albeit partially overlapping, spectra of kinase targets in CML cells. This observation prompted us to explore anti-leukemic effects of the combination dasatinib + bosutinib in highly resistant primary CML cells, various CML cell lines (K562, K562R, KU812, KCL22) and Ba/F3 cells harboring various BCR-ABL1 mutant-forms. We found that bosutinib synergizes with dasatinib in inducing growth inhibition and apoptosis in all CML cell lines and in Ba/F3 cells exhibiting BCR-ABL1^{T315I}. Clear synergistic effects were also observed in primary CML cells in all patients tested (n = 20), including drug-resistant cells carrying BCR-ABL1^{T315I}. Moreover, the drug combination produced cooperative or even synergistic apoptosis-inducing effects on CD34$^+$/CD38$^-$ CML stem cells. Finally, we found that the drug combination is a potent approach to block the activity of major additional CML targets, including LYN, KIT and PDGFRα. Together, bosutinib and dasatinib synergize in producing anti-leukemic effects in drug-resistant CML cells. Whether such cooperative TKI effects also occur *in vivo* in patients with drug-resistant CML, remains to be determined in forthcoming studies.

1. Introduction

In most patients with chronic myeloid leukemia (CML), complete cytogenetic responses (CCyR) can be achieved with imatinib [1–4]. However, resistance against imatinib may occur [5–9]. In most of these cases, *BCR-ABL1* mutations are found [5–9]. Other mechanisms of drug-resistance are amplifications of *BCR-ABL1* or activation of additional pro-oncogenic signaling molecules [5–9]. In imatinib-resistant patients, alternative (second- or third-generation) BCR-ABL1 blockers, such as nilotinib, dasatinib, bosutinib and ponatinib [10–16], are usually prescribed. These agents are administered depending on the phase of CML, type of *BCR-ABL1* mutation(s), age and known comorbidities [13,11–16]. Compared to imatinib, the second- and third generation TKI bind to a larger number of target-kinases [17–20]. Such additional targets may explain superior drug effects but may also explain certain side effects, such as effusion formation in patients receiving dasatinib or vascular adverse events during treatment with nilotinib or ponatinib [21–24]. Some of these kinase-targets, such as KIT, LYN, AXL or FES may play a role in BCR-ABL1-independent survival and proliferation of CML (stem) cells, and inhibition of these targets may contribute to the superior anti-leukemic effects of these drugs [25–29].

Several patients with imatinib-resistant CML who are treated with a

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second- or third generation TKI enter long-term disease-free survival [11,14–16,24]. In other patients, however, CML cells develop resistance against novel TKI [5–9,14–16]. A special problem is the T315I mutant that confers resistance against most currently available BCR-ABL1 TKI [24,30,31]. Ponatinib is a third-generation TKI directed against various BCR-ABL1 mutant forms, including T315I. Several clinical studies have confirmed the efficacy of this TKI in patients with BCR-ABL1^{T315I}+ CML [17]. However, treatment with ponatinib is associated with clinically relevant (cardiovascular) side effects [23,24]. Another strategy for T315I + patients is hematopoietic stem cell transplantation (HSCT) [31–33]. However, HSCT can only be offered to young and fit patients [32,33]. Therefore, new strategies have been considered to overcome multi-drug resistance in CML. One attractive approach may be to combine BCR-ABL1 TKI with each other (directly or in rotation) in order to prevent the outgrowth of sub-clones bearing resistant BCR-ABL1 mutants [34–39]. Indeed, synergistic drug effects on CML cells have been described for certain TKI combinations such as ´imatinib + nilotinib´ [35].

Bosutinib is a multi-kinase inhibitor that has been described to target BCR-ABL1 and a broad spectrum of other kinase-targets [20,40]. The kinase spectra of bosutinib and dasatinib are overlapping but not identic. Indeed, some targets, such as SRC- and TEK-kinases, are bound by both bosutinib and dasatinib [18,20,40]. Other targets, however, such as PDGFRA or KIT, are only detected by dasatinib but are spared by bosutinib [20,40], or are only detected by bosutinib but are not recognized by dasatinib, such as FES, AXL or CAMK2G [18,20,40].

Bosutinib inhibits the survival of CML cells, including imatinib-resistant cells harboring various mutant forms of BCR-ABL1, but has only weak if any activity against BCR-ABL1^{T315I}+ cells [41]. Nevertheless, in contrast to other BCR-ABL1 TKI, bosutinib exhibited some residual activity against BCR-ABL1^{T315I} in a kinase assay [20].

Bosutinib has been tested successfully in clinical trials in patients with imatinib-resistant CML [42–45]. However, only little is known about potential cooperative effects between bosutinib and other drugs [46]. In the current study, we have tested the combination ´bosutinib + dasatinib´ on proliferation and survival of imatinib-sensitive and imatinib-resistant CML cells. We selected dasatinib as a drug-partner because of the large spectrum of additional target kinases recognized by this drug.

2. Patients and methods

2.1. Patients and isolation of primary CML cells

Primary CML cells were obtained from the peripheral blood (PB) of 23 patients with CML, including 12 patients with untreated chronic phase (CP) CML, two with relapsed CML after imatinib discontinuation (one in blast phase (BP)) and 9 with resistance against imatinib or multiple TKI at the time of sampling. In 7/9 patients with drug-resistant CML, *BCR-ABL1* mutations were detected. In 4 cases, the T315I mutation was identified. In one of these patients with *BCR-ABL1*^{T315I} + CML, a second (additional) mutation in *BCR-ABL1* (E255 K) was detected. In 3 other patients, *BCR-ABL1*^{F317L}, *BCR-ABL1*^{F359V}, or *BCR-ABL1*^{M244V} were detected (Table 1). Two *BCR-ABL1*^{T315I}+ patients were in CP and two *BCR-ABL1*^{T315I}+ patients were in BP. In one patient with drug-resistant CML, cells were obtained at two time points, one in CP before HSCT when CML cells exhibited BCR-ABL1^{T315I} and BCR-ABL1^{E255K}, and one after HSCT in BP where CML cells (blasts) were positive for BCR-ABL1^{E255K} but negative for BCR-ABL1^{T315I}. The patients´ characteristics are provided in Table 1. Control cells were obtained from the bone marrow (BM) of lymphoma patients without BM involvement or from acute leukemia patients in complete remission. All patient samples were collected during routine examinations. The study was approved by the ethics committee of the Medical University of Vienna. Informed consent was obtained in each case.

2.2. Reagents

Bosutinib was purchased from Selleck (Houston, TX) and dasatinib (BMS 354825) from Chemietek (Indianapolis, IN). Stock solutions of drugs were prepared by dissolving in dimethyl-sulfoxide (DMSO) (Merck, Darmstadt, Germany). RPMI 1640 medium, fetal calf serum (FCS) and antibiotics were purchased from PAA laboratories (Pasching, Austria), ^{3}H-thymidine from Amersham (Buckinghamshire, UK) and propidium iodide (PI) from Sigma (St. Louis, MO).

2.3. Cells and culture conditions

Primary CML cells and control BM cells were layered over Ficoll to isolate mononuclear cells (MNC). CML MNC and the CML cell lines K562, K562R, KU812 and KCL22 were maintained at 37 °C (5% CO_2) in RPMI 1640 medium with 10% FCS and antibiotics. Imatinib-resistant K562R cells [47] were maintained in imatinib (1 μM). KCL22 cells expressing *BCR-ABL1*^{T315I} (KCL22^{T315I}) were generated as described [48] and were kept in the presence of 5 μM imatinib. Ba/F3 cells expressing wild type *BCR-ABL1* (Ba/F3p210^{WT}) or various BCR-ABL1 mutant forms (Ba/F3p210^{T315I}, Ba/F3p210^{E255K}, Ba/F3p210^{M351T}, Ba/F3p210^{Y253F}, Ba/F3p210^{H396P}) [49] were kindly provided by Michael Deininger (Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA).

2.4. Measurement of ^{3}H-thymidine uptake

Cell lines and primary CML MNC were incubated in control medium (RPMI 1640 plus 10% FCS) or medium supplemented with various concentrations of bosutinib or dasatinib (each 0.1 nM to 25 μM) at 37 °C for 48 h. Then, ^{3}H-thymidine-uptake was determined as described [37,50]. To evaluate potential additive or synergistic drug effects, cells were exposed to bosutinib and dasatinib alone or in combination at a fixed ratio of drug concentrations essentially as reported [37,50].

2.5. Evaluation of survival and apoptosis

The effects of bosutinib and dasatinib (alone or in combination) on cell survival were analyzed by morphologic examination and flow cytometry. In typical experiments, cells were incubated with various concentrations of bosutinib or dasatinib (1 nM to 5 μM) as single drugs or in combination in 6-well culture plates at 37 °C (5% CO_2) for 48 h. Primary CML cells were cultured in the absence or presence of drugs for up to 120 h. The percentage of apoptotic cells was quantified on Wright-Giemsa-stained cytospin preparations. Apoptosis of drug-exposed CML cells (48 h) was also quantified by flow cytometry using AnnexinV-FITC (Alexis Biochemicals, San Diego, CA) on a FACScan (Becton Dickinson) essentially as reported [37,50]. In 4 patients (CML CP, n = 3; CML BP, n = 1) apoptosis was measured in drug-exposed CD34^{+}/CD38^{-} stem cells by multi-color flow cytometry as reported [50]. In these experiments, CML cells were incubated in control medium, dasatinib (0.1 or 1.0 μM), bosutinib (0.1 or 1.0 μM), or a combination of both drugs (each 0.1 μM or each 1.0 μM) for 48 h.

2.6. Western blot analysis

Western blot experiments were performed on cell lines and primary CML cells of patients with TKI-resistant disease including leukemic cells carrying BCR-ABL1^{T315I} following published techniques [37,50]. Cells were cultured in the absence or presence of bosutinib (1 μM) or/and dasatinib (1 μM) for 4 h (37 °C). Thereafter, cells were lysed in lysis buffer and analyzed by Western blotting as reported using antibodies against phospho-CRKL (p-CRKL), total CRKL, p-KIT, KIT, p-PDGFRA, PDGFR, p-LYN, or LYN. A list of antibodies used is provided in Supplemental Table S1.

Table 1
Patients´characteristics, *in vitro* response to bosutinib and effects of the drug combination ´bosutinib + dasatinib´. No., number; m, male; f, female; CP, chronic phase; AP, accelerated phase; BP, blast phase; ly, lymphatic; n.t., not tested; n.d., not detected; dis, discontinued; res, resistant; int, intolerant; nM, nanomolar; µM, micromolar; HSCT, allogenic hematopoietic stem cell transplantation; Syn, synergistic; Add, additive. IC_{50} values were determined by ^{3}H-thymidine uptake experiments. The *in vitro* response of leukemic cells to the combination ´bosutinib + dasatinib´ was assessed either by ^{3}H-thymidine uptake experiments (p), or by determination of apoptotic cells by light microscopy (a–m) or apoptosis by flow cytometry (a-fc).

patient no.	age	gender (m/f)	CML phase	BCR-ABL1 mutations	previous therapy	bosutinib IC_{50}	dasatinib IC_{50}	Combination effect ´bosutinib + dasatinib´
#1	54	M	CP	n.t.	none	n.t.	n.t.	Syn (a–m)
#2	60	F	CP	n.t.	none	n.t.	n.t.	Syn (a–m)
#3	64	M	CP	n.t.	none	> 1 µM	> 1 µM	Add/Syn (p)
#4	40	F	CP	n.t.	none	> 1 µM	> 1 µM	Syn (p)
#5	32	M	CP	n.t.	none	> 1 µM	> 1 µM	Add/Syn (p)
#6	81	F	CP	n.t.	none	n.t.	n.t.	Syn (a–m)
#7	22	M	CP	n.d.	imatinib (dis)	n.t.	n.t.	Syn (a–m)
#8	85	F	CP	n.t.	none	0.1-0.5 µM	> 1 µM	n.t.
#9	72	F	CP	n.d.	hydroxyurea imatinib (res) dasatinib (res)	< 10 nM	n.t.	Syn (a–m)
#10	27	F	CP	n.t.	none	> 1 µM	> 1 µM	Syn (p)
#11	46	F	CP	n.t.	none	n.t.	n.t.	Syn (a–m)
#12	45	F	CP	n.d.	hydroxyurea imatinib (int) dasatinib (res) nilotinib (res)	n.t.	n.t.	Syn (a–m)
#13	73	F	CP	F317L	hydroxyurea imatinib (res) dasatinib (res)	0.1-0.25 µM	< 0.01 µM	n.t.
#14	75	M	CP	F359V	hydroxyurea imatinib (res) dasatinib (int) nilotinib (res)	n.t.	> 1 µM	Syn (a–m)
#15	66	M	CP	M244V	hydroxyurea imatinib (res) dasatinib (res)	0.5-1 µM	0.5-1 µM	Syn (a–m,p)
#16	43	M	CP	E255K T315I	hydroxyurea imatinib (res) dasatinib (res)	0.1-0.5 µM	n.t.	n.t
#16 2nd time point	43	M	BP	E255K	relapse after HSCT	0.1-0.5 µM	0.01-0.05 µM	Syn (p)
#17	48	F	BP	T315I	hydroxyurea imatinib (res) dasatinib (res)	> 1 µM	> 1 µM	Syn (p)
#18	62	M	BP (ly)	T315I	hydroxyurea interferon-α fludarabine HSCT imatinib (res) dasatinib (res)	5-10 µM	5-10 µM	Syn (p)
#19	56	M	CP	T315I	imatinib (res)	0.5-1 µM	> 1 µM	Syn (p)
#20	21	F	CP	n.t.	none	n.t.	n.t.	Syn (a–m) $CD34^{+}/CD38^{-}$ (a-fc)
#21	34	F	CP	n.t.	none	0.01-0.05 µM	< 0.01 µM	Add (a–m) $CD34^{+}/CD38^{-}$ (a-fc)
#22	54	F	CP	n.t.	none	n.t.	n.t.	Syn (a–m) $CD34^{+}/CD38^{-}$ (a-fc)
#23	62	M	BP	n.d.	imatinib (dis)	0.01-0.05 µM	0.01-0.05 µM	Syn (p); Add (a–m) $CD34^{+}/CD38^{-}$ (a-fc)

2.7. Statistical analysis

To determine the significance levels in differences seen between drug-exposed cells and cells grown in control medium, the Student´s *t*-test for dependent samples was applied. Results were considered statistically significant when p was < 0.05. Drug-interactions (additive versus synergistic) were assessed by calculating combination index (CI) values employing Calcusyn software (Calcusyn; Biosoft, Ferguson, MO) as reported [37,50]. A CI of 1 indicates additive effects and CI values < 1 synergistic drug effects.

3. Results

3.1. Bosutinib exerts moderate anti-leukemic effects in primary CML cells expressing BCR-ABL1^{T315I} in patients with CP CML but not in those with BP

Bosutinib induced apoptosis in primary CML cells in most patients tested, including imatinib-sensitive cells (CP at diagnosis) and imatinib-resistant CML cells expressing BCR-ABL1^{WT}, BCR-ABL1^{F317L} or BCR-ABL1^{F359V} (Fig. 1A). No apoptosis-inducing effects of bosutinib were seen in normal BM cells (Fig. 1A). As shown in Fig. 1B and Table 1,

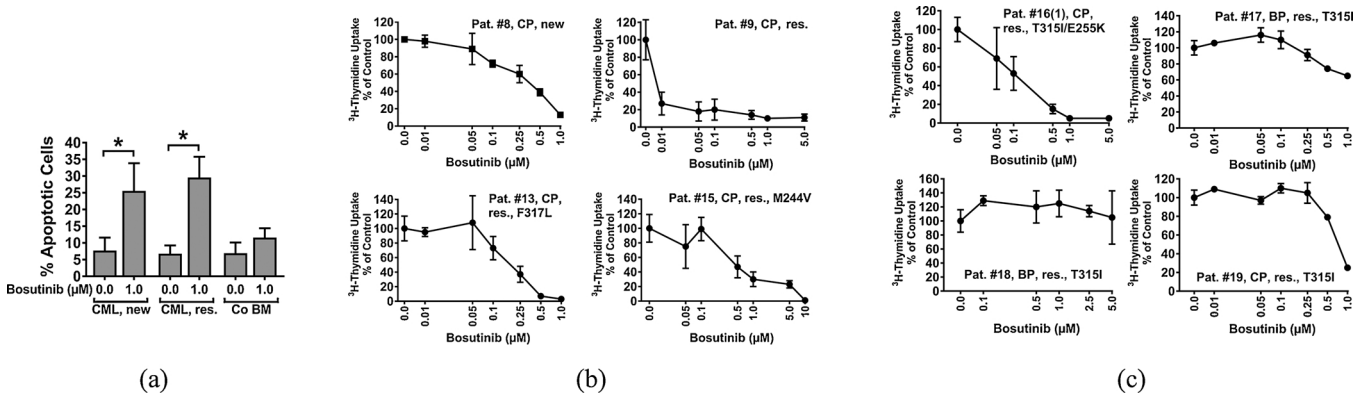


Fig. 1. Bosutinib exhibits anti-leukemic effects in primary CML cells isolated from imatinib-sensitive and imatinib-resistant patients.
(a) Mononuclear cells were isolated from the peripheral blood (PB) of 4 newly diagnosed CML patients (CML new; numbers #1, #2, #6, and #7) and from three imatinib- and dasatinb-resistant patients (CML res.; numbers #9, #12, and #14) as well as from the bone marrow of 3 normal donors (Co BM; lymphoma patients without BM involvement or BM from acute leukemia patients in complete remission). Cells were kept in the absence or presence of 1 μM bosutinib for 48 h at 37 °C. Then, cells were spun onto cytospin slides and stained by Wright-Giemsa. The number of apoptotic cells was determined by light microscopy. Results represent the mean ± S.D. of each patient group. Asterisk: $p < 0.05$ compared to control. (b,c) Leukemic cells isolated from the PB of one newly diagnosed CML patient (#8), 3 imatinib- and dasatinib-resistant patients (#9, #13 and #15) without detectable T315I mutation (b) and 4 BCR/ABL^{T315I}+ patients (#16, 1st time point, #17, #18 and #19) (c) were kept in various concentrations of bosutinib, as indicated, for 48 h before proliferation was quantified by ^{3}H-thymidine uptake. Results are expressed as percent of control (cells kept in control medium = 100%) and represent the mean ± S.D. of triplicates. Patient´ numbers (#) refer to Table 1.

bosutinib was also found to inhibit the proliferation of primary CML cells in most samples tested. Unexpectedly, this effect of bosutinib was even seen in cell samples of two patients with CML CP expressing BCR-ABL1^{T315I}, with IC_{50} values ranging between 0.1 and 1 μM (Fig. 1C). By contrast, bosutinib did not inhibit proliferation of leukemic cells in 2 patients with BCR-ABL1^{T315I}+ CML in (BP) (Fig. 1C). Dasatinib was also found to suppress proliferation of primary CML cells in most patients tested (Table 1). However, as expected, dasatinib did not block proliferation of CML cells exhibiting BCR-ABL1^{T315I} regardless of the phase of CML (Table 1).

3.2. Bosutinib and dasatinib exert anti-leukemic effects on various CML cell lines and on imatinib-resistant Ba/F3 sub-clones

We next compared the effects of bosutinib and dasatinib on various CML cell lines and Ba/F3 sub-clones exhibiting various mutant forms of BCR-ABL1. A summary of results is shown in Supplemental Table S2. By contrast, no major growth-inhibitory effects of bosutinib and dasatinib were seen in KCL22^{T315I} cells (Supplemental Table S2). Next, we examined the effects of bosutinib and dasatinib on imatinib-resistant Ba/F3 cells harboring various mutant-forms of *BCR-ABL1*. In most Ba/F3 clones, bosutinib and dasatinib were found to inhibit proliferation, with IC_{50} values of 10–100 nM, thereby confirming the available literature [41]. Between 0.01–1.0 μM, bosutinib failed to block growth of Ba/F3p210^{T315I} cells (Supplemental Table S2) which also confirmed the previous literature [41]. However, at higher concentrations (> 1 μM) bosutinib and dasatinib were found to counteract growth of Ba/F3 cells carrying BCR/ABL1^{T315I} (Supplemental Figure S1). Indeed, IC_{50} values ranged from 1 to 5 μM for bosutinib and from 5 to 10 μM for dasatinib in Ba/F3p210^{T315I} cells. Bosutinib and dasatinib showed no substantial effects on growth of IL-3-dependent non-transformed Ba/F3 cells (Supplemental Figure S1).

3.3. Bosutinib synergizes with dasatinib in inhibiting proliferation and survival in CML cell lines and Ba/F3 cells expressing TKI-resistant BCR-ABL1 mutants

Next, we examined drug combination effects. As visible in Fig. 2A, cooperative (additive or synergistic) effects between bosutinib and dasatinib on proliferation were seen in all human cell lines tested, including the imatinib-resistant cell lines K562R and KCL22^{T315I}. Additive or synergistic drug-interactions were confirmed by calculating CI values using Calcusyn software (Supplemental Figure S2). Furthermore, the combination bosutinib and dasatinib were found to synergize in inducing apoptosis in K562 and K562R cells (Figs. 2B and 2C, supplemental Figure S3). Finally, we were able to show that the combination `bosutinib + dasatinib´ induces synergistic apoptosis-inducing effects in Ba/F3p210^{T315I} cells (Fig. 2D, supplemental Figure S3).

3.4. Bosutinib synergizes with dasatinib in inducing growth-inhibition in imatinib-sensitive and imatinib-resistant primary CML cells

To confirm synergistic drug combination effects in primary CML cells, PB cells of 6 newly diagnosed CML patients and 4 imatinib- and dasatinib-resistant patients were used. Bosutinib was found to synergize with dasatinib in producing apoptosis in primary CML cells in all patients tested (Fig. 3A, supplemental Figure S3). In control experiments, normal BM MNC were tested. However, the drug combination failed to induce apoptosis in normal BM MNC (Fig. 3A). The anti-leukemic effects of the combination ´bosutinib + dasatinib´ were also confirmed in proliferation experiments. In fact, in all 10 patients tested, a cooperative or synergistic effect of the drug combination on proliferation was seen (Fig. 3B–C, Table 1). Synergistic drug interactions were confirmed by calculating CI values using Calcusyn software (Fig. 3C). In 4 of the 10 patients tested, *BCR-ABL1^{T315I}* was detected. As shown in Fig. 3C, synergistic drug effects were also seen in CML cells exhibiting *BCR-ABL1*^{T315I}. Remarkably, this synergistic effect was even seen in one patient with *BCR-ABL1*^{T315I}+ CML BP (Fig. 3C).

3.5. Effects of the drug combination ´dasatinib + bosutinib´ on survival of leukemic CD34^{+}/CD38^{-} stem cells (LSC) in patients with CML

CML LSC supposedly reside in a CD34^{+}/CD38^{-} fraction of the leukemic clone [9]. Since dasatinib and bosutinib produced apoptosis in primary CML cells, we were interested to learn whether the drug combination ´dasatinib + bosutinib´ would also induce apoptosis in patient-derived CD34^{+}/CD38^{-} LSC. Indeed, the drug combination ´dasatinib + bosutinib´ produced substantial apoptosis in CD34^{+}/CD38^{-} CML LSC after 48 h, whereas only weak effects on cell survival were seen after single-drug exposure (Fig. 4, supplemental

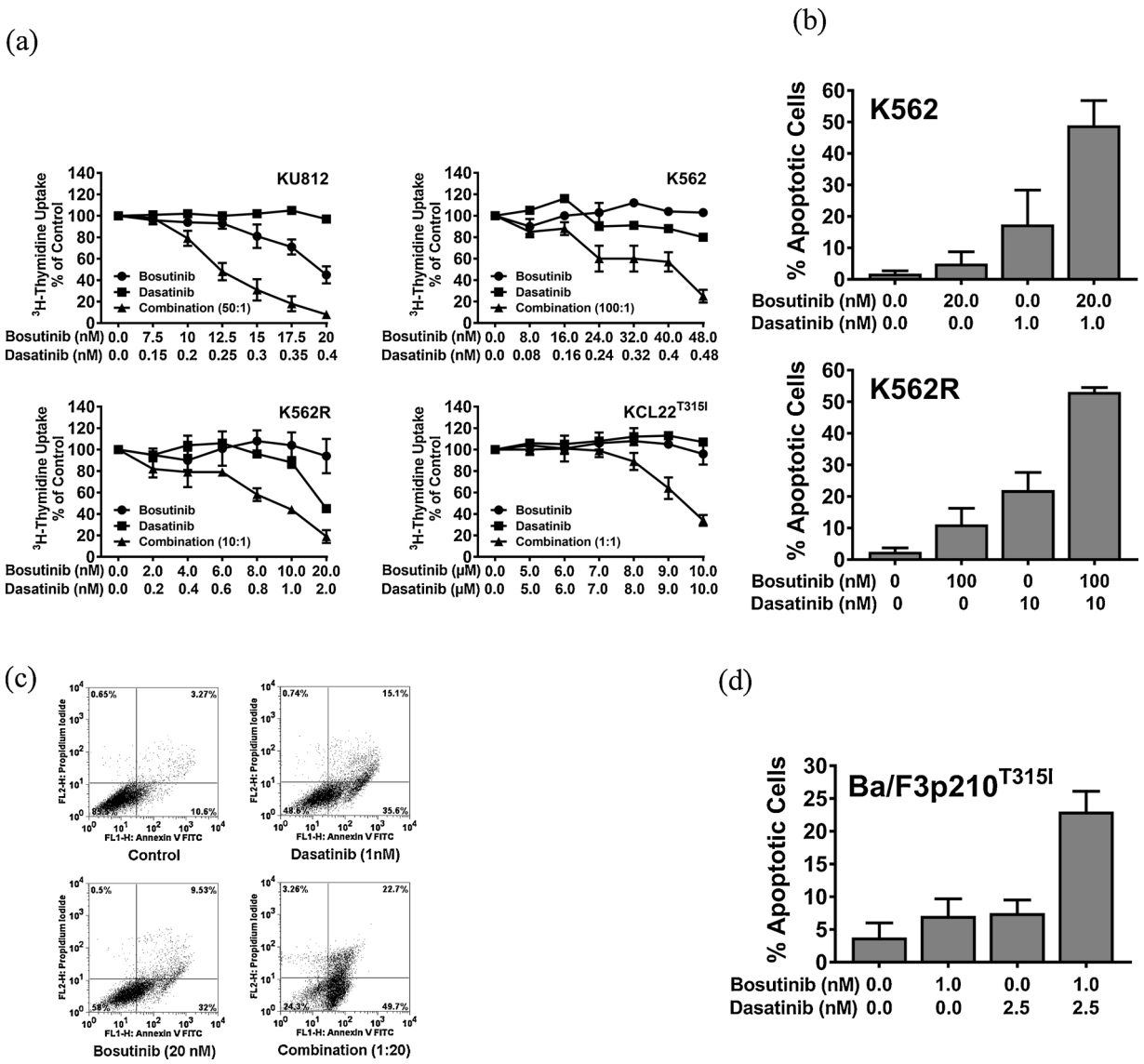
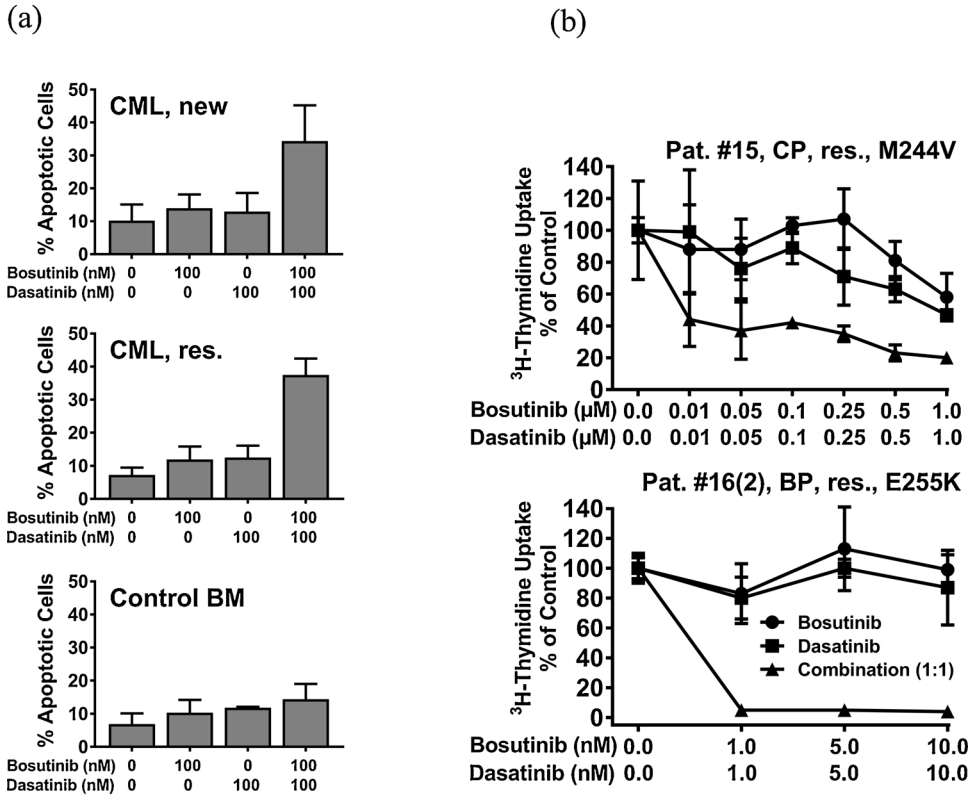


Fig. 2. Bosutinib synergizes with dasatinib in counteracting viability and proliferation of imatinib-sensitive and imatinib-resistant BCR-ABL1 + cell lines. (a) K562, K562R, KU812 and KCL22^{T315I} cells were incubated with bosutinib (●-●), dasatinib (■–■), or a combination of both drugs (fixed drug ratio, ▲ -▲) at 37 °C for 48 h. Then, ^{3}H-thymidine incorporation was measured. Results are expressed as percent of medium control (= 100%) and represent the mean ± S.D. of triplicates. (b) K562 and K562R cells were kept in control medium, or in bosutinib or dasatinib at the concentrations indicated either as single agents or in combination. After 48 h, the numbers of apoptotic cells were quantified on Giemsa-stained cytospin slides. Results represent the mean ± S.D. of three independent experiments. (c) In K562 cells, the percentage of apoptotic cells was also determined by combined AnnexinV/PI staining and flow cytometry. (d) Ba/F3p210^{T315I} were kept in control medium or various concentrations of bosutinib or dasatinib as indicated, either as single agents or in combination. After 48 h, the numbers of apoptotic cells were counted on Giemsa-stained cytospin slides. Results represent the mean ± S.D. of three independent experiments.

Figure S3). Overall, cooperative drug effects were seen in 4 patients examined, including 3 in CP and 1 in BP (Table 1). In most donors, the drug combination produced additive rather that synergistic effects on survival of CML LSC (Table 1).

3.6. Effects of bosutinib and dasatinib on additional kinase targets expressed in CML cells

Recent data suggest that dasatinib and bosutinib interact with a number of different kinase targets in CML cells [20] (Supplemental Table S3). However, the spectrum of recognized targets is partially overlapping [20] (Supplemental Table S3). We asked whether combination treatment would result in a complete and more potent inhibition of kinase targets in leukemic cells. As assessed by Western blotting, bosutinib, dasatinib and the combination ´dasatinib + bosutinib´ were found to block phosphorylation of CRKL (a marker of BCR-ABL1 activity) in KCL22 cells. As expected, these effects were not seen in KCL22^{T315I} or Ba/F3 cells exhibiting BCR-ABL1^{T315I} (Fig. 5). However, when exposed to a combination of bosutinib and dasatinib, several kinase targets, including the dasatinib-targets KIT and PDGFRa (not recognized by bosutinib) as well as LYN which is recognized by both drugs, were no longer detected in phosphorylated form in KCL22^{T315I} cells and Ba/F3 cells exhibiting BCR-ABL1^{T315I} (Fig. 5). In most instances, these combination effects may be explained by single drug activities. Although dasatinib was found to decrease phosphorylation of PDGFRa at 1 μM, no complete dephosphorylation was seen. However, phosphorylation of PDGFa was completely blocked when applying the drug combination `bosutinib + dasatinib´ (Fig. 5). These observations suggest, that the anti-leukemic effects exerted on Ph + cells expressing BCR-ABL1$^{T315I}$ by the combination `bosutinib + dasatinib´ may be



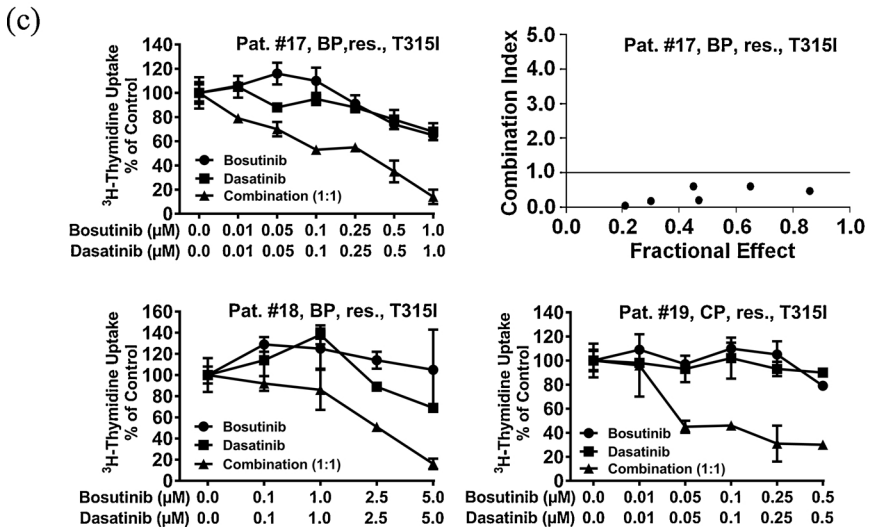


Fig. 3. Bosutinib synergizes with dasatinib in producing anti-leukemic effects in primary imatinib-sensitive and imatinib-resistant CML cells.

(a) Mononuclear cells (MNC) were isolated from the peripheral blood (PB) of 3 newly diagnosed CML patients ("CML, new"; #1, #6 and #11) (upper panel), of 4 imatinib- and dasatinib-resistant patients ("CML, res."; #9, #12, #14, and #15) (middle panel) as well as from the BM of 3 normal donors ("Control BM"; lower panel). Cells were kept in the absence or presence of dasatinb, bosutinib (100 nM each) or a combination of the two drugs for 48 h before cells were spun on cytospin slides. After Giemsa-staining, the number of apoptotic cells was determined by light microscopy. Results represent the mean ± S.D. of each patient group. (b,c) PB-MNC from 2 imatinib- and dasatinib-resistant patients (#15 and #16, 2nd time point) (b) and from 3 *BCR-ABL1*^{T315I}+ CML patients (#17, #18, and #19) (C) were incubated with bosutinib (●-●), dasatinib (■-■), or a combination of the two drugs (fixed drug ratio, ▲-▲) at 37 °C for 48 h. Then, ^{3}H-thymidine uptake was measured. Results are expressed as percent of medium control (= 100%) and represent the mean ± S.D. of triplicates. Patient´ numbers (#) refer to Table 1. Drug-interactions (additive, synergistic) were assessed by calculating combination index (CI) values using Calcusyn software as exemplified for patient #17 (c, upper right panel).

explained by inhibition of additional kinase targets.

4. Discussion

Resistance against first and second-line BCR-ABL1 TKI represents a major clinical challenge in Ph + CML [7–16]. In many of these patients, *BCR-ABL1* mutations are found. Among these, the T315I mutant is a special challenge because the mutation confers resistance against most currently available TKI except ponatinib [13,8–16]. In fact, by applying ponatinib, BCR-ABL1^{T315I}+ subclones can be suppressed in many patients [24]. However, long-term therapy with ponatinib is associated with clinically relevant and often severe side effects [23,24]. Another option is HSCT. However, HSCT cannot be offered to older or comorbid patients [32,33]. Therefore, novel treatment strategies need to be examined. One strategy is to combine effective TKI to overcome drug resistance in CML [35–39]. In the present study, we explored the anti-leukemic activity of the combination ´bosutinib + dasatinib´ in primary and TKI-resistant CML cells. The results of our study show that this drug combination exerts clear synergistic effects on CML cells in various phases of the disease, including TKI-resistant CML cells. Unexpectedly, the TKI combination was even found to suppress growth of leukemic cells exhibiting BCR-ABL1^{T315I} which may have clinical implications.

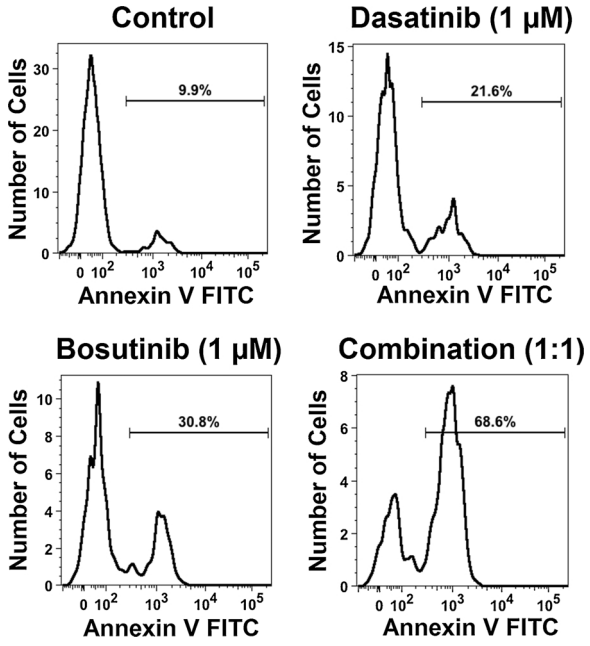


Fig. 4. Bosutinib synergizes with dasatinib in producing apoptosis in $CD34^+/CD38^-$ CML stem cells.

Peripheral blood leukocytes, obtained from patient #20, were exposed to control medium (Co), 1 μM bosutinib, 1 μM dasatinib, or a combination of both drugs (each 1 μM) for 48 h. Thereafter, $CD34^+/CD38^-$ stem cells were examined for signs of apoptosis by multicolor flow cytometry and staining for Annexin V-FITC and 4′,6-diamidino-2-phenylindole (DAPI). Results are expressed as percentage of Annexin V + cells (of all $CD34^+/CD38^-$ cells) after gating for DAPI − cells.

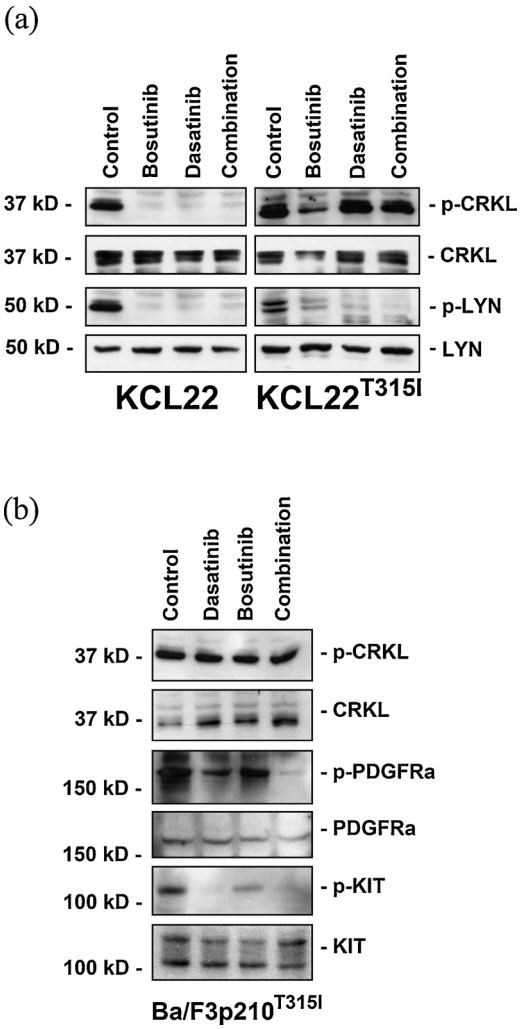


Fig. 5. Bosutinib and dasatinib block different kinases in BCR-ABL1^{T315I}+ cells.

KCL22, KCL22^{T315I} (a) and Ba/F3p210^{T315I} (b) cells were kept in control medium or in bosutinib or dasatinib (1 μM each) as single agents or in combination for 4 h. Then, cells were subjected to Western blot analysis using antibodies directed against p-CRKL, CRKL, p-LYN, LYN p-PDGFRa, PDGFRa, p-KIT or KIT as indicated.

Previous and more recent data suggest that the kinase target spectrum of dasatinib differs substantially from the target profile of bosutinib [20]. In fact, whereas dasatinib has been developed as a broadly acting kinase blocker, bosutinib was designed based on a more selective kinase spectrum that does not include PDGFR or KIT, with the hope to reduce side effects. In the present study, we exploited the different kinase interaction profiles of dasatinib and bosutinib by combining both drugs to achieve superior anti-leukemic effects in BCR/ABL1-transformed cells.

In a first step, we examined single drug effects in cell lines and primary CML cells. In all cell line models tested, we were able to confirm the superior anti-leukemic effects of bosutinib and dasatinib [10,14,15,34]. In fact, bosutinib and dasatinib were found to exert major anti-leukemic effects on imatinib-sensitive and imatinib-resistant K562 cells as well as in Ba/F3 cells expressing various BCR-ABL1 mutants except T315I. In primary CML cells, similar results were obtained. By contrast, in patients with *BCR-ABL1*^{T315I}+ CML in BP, leukemic cells did not respond to bosutinib or dasatinib. However, unexpectedly, bosutinib was found to exert growth-inhibitory effects on *BCR-ABL1*^{T315I}+ cells in two patients with CML CP, with reasonable IC_{50} values (< 1 μM) and dasatinib was found to produce also some growth-inhibitory effects in one of these donors. These data suggest that bosutinib may inhibit the growth of CP CML cells through blocking several additional drug targets (apart from BCR-ABL1) and some of these targets may also be recognized by dasatinib. This assumption would be supported by the hypothesis that the T315I mutant per se is a relatively weak oncoprotein [51,52], and leukemic proliferation of affected cells (subclones) thus require additional oncogenic driver-molecules. Alternatively, bosutinib retains some residual effects on BCR-ABL1, which would be in line with previous observations [20].

To explore the disease-modifying activity of the drug combination bosutinib + dasatinib we examined survival of drug-exposed cells. In all samples tested, the drug combination was found to produce additive or synergistic effects on proliferation and survival (apoptosis) in leukemic cells. However, the drug concentrations producing optimal synergistic effects varied among individual patients. Likewise, in the 3 patients with *BCR-ABL1*^{T315I}+ CML in whom the combination was tested, the optimal concentrations of each drug varied between 0.5 and 5 μM. One possible explanation for this phenomenon may be that higher drug concentrations are needed to achieve growth inhibition in cells derived from patients with advanced CML compared to cells obtained from patients in CP. Another explanation would be the different treatments that the patients received prior to developing a *BCR-ABL1*^{T315I}+ subclone. Indeed, some patients had received more treatment lines than others. Moreover, prior treatment with the same TKI that was later tested *in vitro* may have resulted in a reduced sensitivity of leukemic cells to these drugs in our *in vitro* experiments. To determine optimal concentrations for drug combination, further *in vivo* studies and clinical trials will be warranted.

The next important question was whether the drug combination would also block survival of CML stem- and progenitor cells known to exhibit multiple forms of TKI resistance [9,53,54]. To address this

question we extended our analyses to $CD34^+/CD38^-$ LSC and examined apoptosis by combined surface- and AnnexinV staining. In these experiments, we were able to show that the TKI combination ´bosutinib + dasatinib´ induces apoptosis in CML LSC, whereas less pronounced effects were seen when the two TKI were applied as single agents. With regard to dasatinib, our results confirm earlier reports suggesting some inhibitory effects of this TKI on putative CML LSC [54].

The most likely explanation for the superior anti-leukemic effects of the drug combination ´bosutinib + dasatinib´ compared to single drug effects may be additional drug targets that are recognized by these TKI. However, it remains unknown what targets or target combinations need (s) to be suppressed to contribute to synergistic drug interactions. To address this point, we performed Western blot experiments on CML cells exposed to suboptimal concentrations (where drug combination effects but no single drug effects on cell growth were seen) of bosutinib and dasatinib. We found that these suboptimal concentrations are indeed capable of blocking the kinase activity of several critical drug targets in CML cells, including KIT, LYN and PDGFRa.

In Ph+ CML, drug resistance remains a profound challenge despite the availability of novel, more effective TKI [7–16]. To overcome drug resistance, several different drug combinations have been proposed and some of them are currently tested in clinical trials. In the present study, we have combined two established BCR-ABL1 blockers, bosutinib and dasatinib, and show that the drug combination produces synergistic effects on TKI-resistant CML cells, including leukemic cells exhibiting $BCR\text{-}ABL1^{T315I}$. In our opinion, this observation may have clinical implications. First, both drugs are already used in clinical practice and it may be feasible and reasonable to test the drug combination *in vivo* in patients with TKI-resistant CML, either in rotation mode or in direct combination. Second, both drugs, when used as single agents at pharmacologically meaningful concentrations, are well known to produce certain side effects. Therefore, one could speculate that application of these drugs at lower doses in a combination approach may lead to reduced side effects *in vivo*. In a next step, *in vivo* studies and clinical trials are warranted to confirm the beneficial effects of the drug combination ´bosutinib + dasatinib´ and to determine the optimal drug concentrations and the mode of administration (i.e. simultaneous drug application vs. sequential application) to suppress the growth of CML cells.

In conclusion, our data show that the drug combination ´bosutinib + dasatinib´ represents a novel powerful approach to overcome drug resistance in Ph + CML. In addition, we show that this drug combination works in TKI-resistant clones, including CML cells harboring $BCR\text{-}ABL1^{T315I}$. Whether this approach can also overcome clinical resistance in patients with TKI-refractory CML *in vivo* remains to be determined in clinical trials.

Author contributions

Karoline V. Gleixner: performed cell culture, performed biologic assays, conducted Western blot analysis, planed the study, analyzed the data, wrote the draft; Irina Sadovink, Gregor Eisenwort and Harald Herrmann: performed flow cytometric experiments on primary CML cells and their precursors; Mathias Schneeweiss and Emir Hadzijusufoviv: performed cell culture, performed biologic assays; Konstantin Byrgazov: generated and provided new cell lines, performed cell culture, performed biologic assays, conducted Western blot analysis; Gabriele Stefanzl: performed biologic assays; Daniela Berger: conducted Western blot analysis; Thomas Lion: generated and provided new cell lines; Peter Valent: planed the study, analyzed the data, wrote the draft.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2018.12.013.

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