Clinical Cancer Research

Axl Blockade by BGB324 Inhibits BCR-ABL Tyrosine Kinase Inhibitor–Sensitive and -Resistant Chronic Myeloid Leukemia

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Abstract

Purpose: BCR-ABL kinase inhibitors are employed successfully for chronic myeloid leukemia (CML) treatment. However, resistant disease and persistence of BCR-ABL1–independent leukemia stem and progenitor cells (LSPC) remain clinical challenges. The receptor tyrosine kinase Axl can mediate survival and therapy resistance of different cancer cells. We investigated the therapeutic potential of Axl inhibition in CML.

Experimental Design: We used primary cells from patients with CML and TKI-sensitive and -resistant BCR-ABL1$^+$ CML cell lines and a novel ponatinib-resistant cell line KCL-22 PonR. We analyzed the effects of genetic and pharmacologic Axl blockade by the small-molecule Axl inhibitor BGB324 *in vitro* and *in vivo*. In BCR-ABL1–unmutated cells, we also investigated BGB324 in combination with imatinib.

Results: We demonstrate overexpression of Axl receptor tyrosine kinase in primary cells of patients with CML compared with healthy individuals and a further increase of Axl expression in BCR-ABL TKI-resistant patients. We show that Axl blockage decreased growth of BCR-ABL TKI-sensitive CML cells including CD34$^+$ cells and exerts additive effects with imatinib via inhibition of Stat5 activation. BGB324 also inhibits BCR-ABL TKI-resistant cells, including T315I-mutated and ponatinib-resistant primary cells. BGB324 exerted therapeutic effects in BCR-ABL1 T315I-mutated and ponatinib-resistant preclinical mouse models. Notably, BGB324 does not inhibit BCR-ABL1 and consequently inhibits CML independent of BCR-ABL1 mutational status.

Conclusions: Our data show that Axl inhibition has therapeutic potential in BCR-ABL TKI-sensitive as well as -resistant CML and support the need for clinical trials. *Clin Cancer Res; 23(9); 2289–300. ©2016 AACR.*

Introduction

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia (Ph) chromosome resulting from a reciprocal translocation between the chromosomes 9 and 22, which is present at least in 90% of CML cases (1, 2). This translocation results in *BCR-ABL1*, an oncogenic fusion protein. BCR-ABL1 is a constitutively activated tyrosine kinase (TK) triggering several prosurvival signaling pathways in CML cells, conferring a proliferative advantage and resistance to apoptosis (3, 4). Therefore, BCR-ABL tyrosine kinase inhibitors (TKI)

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Translational Relevance

ABL tyrosine kinase inhibitors (TKI) are employed for chronic myeloid leukemia (CML) treatment and induce durable remissions in the majority of patients. However, resistant disease and persistence of BCR-ABL1–independent leukemia stem and progenitor cells remain clinical challenges. Furthermore, ABL-TKIs are associated with the risk of severe side effects in long-term use. Therefore, novel therapeutic strategies are warranted to target persisting stem cells and resistant clones alone or in combination with ABL-TKI.

We demonstrate here the activity of an Axl inhibitor, BGB324, against TKI-sensitive and -resistant BCR-ABL1 positive cells, including T315I-mutated and ponatinib-resistant primary cells. Inhibition of Axl with BGB324 exerted therapeutic effects in complementary T315I-mutated and ponatinib-resistant preclinical mouse models. BGB324 was well-tolerated in phase I clinical trials in healthy volunteers and patients with acute myeloid leukemia (BGBC001, BGBC003 ongoing). Thus, our findings can be rapidly translated to the clinics and BGB324 holds promise to improve treatment of patients with CML.

were developed, which became the standard-of-care in CML. Today, 5 different BCR-ABL TKIs, imatinib, dasatinib, nilotinib, bosutinib, and ponatinib, are approved in different clinical scenarios leading to durable responses in most CML patients treated (5–8). Nevertheless, resistance to BCR-ABL TKI represents a clinical challenge in a substantial fraction of patients with CML. Well-described mechanisms of resistance include mutations in the BCR-ABL1 tyrosine kinase domain, including the gatekeeper T315I mutation mediating resistance to all approved agents with the exception of ponatinib. Furthermore, BCR-ABL1–independent mechanisms of CML stem cell persistence and resistance hamper treatment of patients with CML (9–11). BCR-ABL TKIs are associated with side effects in long-term use (12, 13). Despite the dramatic clinical progress upon treatment with these drugs and the possibility to discontinue treatment in approximately 50% of patients (14), allogeneic stem cell transplantation represents the only proven long-term curative treatment (15–17). Therefore, novel therapeutic strategies are warranted to target persisting stem cells and resistant clones alone or in combination with BCR-ABL TKI.

An increased activation of several tyrosine kinases in imatinib- and nilotinib-resistant cell lines and patients with CML treated with these drugs were reported (18, 19). Therefore, targeting additional kinases besides BCR-ABL1 could be a pertinent option to treat BCR-ABL TKI-refractory patients. Axl, a receptor tyrosine kinase of the Tyro3, Axl, Mer (TAM) family, is implicated in oncogenesis and exhibits transforming potential in CML (20). Growth arrest–specific gene 6 (Gas6) is the only identified activating ligand for Axl (21, 22). It is known that Axl phosphorylation mediates intracellular signaling through PI3K/Akt, Erk, and PLC pathways, affecting diverse cellular functions including enhanced cell survival and proliferation (reviewed in refs. 23, 24). Overexpression, but not mutation, of Axl contributes to drug resistance in other cancers including gastrointestinal stromal tumor (GIST; ref. 25), non–small cell lung carcinoma (NSCLC; ref. 26), and acute myeloid leukemia (AML; ref. 27). The clinically applicable small-molecule BGB324 represents a selective and potent Axl inhibitor (28). A phase I trial of BGB324 in healthy volunteers indicated that the drug is well-tolerated (29). Furthermore, safety, pharmacokinetics, and clinical activity of BGB324 are currently being investigated in a phase Ib trial in patients with AML and lung cancer (BGBC003, BGBC004; NCT02488408, NCT02424617). Recent data show that BGB324 is well-tolerated and exerts antileukemic activity in patients with AML. Pharmacodynamic analyses indicate that BGB324 inhibits Axl phosphorylation in AML patients (28).

Thus far, Axl expression has only been investigated in less than 10 patients with CML, yielding the result that BCR-ABL TKI-resistant unmutated BCR-ABL1 CML cells express higher levels of Axl than BCR-ABL TKI-sensitive cells (30, 31). Expression of Axl in BCR-ABL1 T315I-mutated and ponatinib-resistant CML has not yet been investigated, even though these situations currently represent the most challenging clinical scenarios. Here, we investigated whether Axl might be a suitable target in CML and determined the efficacy of genetic and pharmacologic Axl inhibition in BCR-ABL TKI-sensitive and -resistant BCR-ABL1–positive cells, including ABL T315I-mutated and ponatinib-resistant primary cells and animal models. In light of the tolerability of BGB324 in humans, this therapeutic approach could be rapidly translated to the clinic.

Materials and Methods

Animals

Six to 8-week-old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were purchased from Jackson laboratory. All animal experiments were carried out in concordance with the institutional guidelines for the welfare of animals in experimental neoplasia and were approved by the local licensing authority (Behörde für Soziales, Gesundheit, Familie, Verbraucherschutz; Amt für Gesundheit und Verbraucherschutz, project number G53/12).

Patient samples

Axl expression levels were analyzed in bone marrow mononuclear cells (BMMNC) from healthy donors and from patients with newly diagnosed CML at the University Hospital Hamburg-Eppendorf. This analysis was carried out with approval of the local medical ethics committee (approval numbers MC-220/13). In addition, patients with CML at primary diagnosis were consented in Glasgow for biobanking of their stem/progenitor cells and MNCs harvested from peripheral blood samples provided during the course of their routine clinical care. The research tissue bank had approval from the West of Scotland Research Ethics Committee 4 (REC reference 10/S0704/2). Gas6 levels were determined in the peripheral blood plasma of patients with chronic phase and blast crisis. qPCR analysis of Axl mRNA was performed using samples of chronic phase patients with CML. They were investigated at the Jena University Hospital after 6 months of therapy with nilotinib. Furthermore, samples from 17 patients with failure or suboptimal response toward BCR-ABL TKI treatment according to the current European LeukemiaNet (ELN) recommendations were analyzed at the same timepoint. Patients were treated with imatinib ($n = 11$) or nilotinib ($n = 6$). Four patients showed mutations within the BCR-ABL1 kinase domain (G250E, E255K, T315I, F359V, $n = 1$ each). These analyses were approved by the local ethics committee. All studies with human

samples were carried out in accordance with the Declaration of Helsinki.

Isolation and culture of MNC and CD34$^+$ cells

MNCs were isolated from blood or bone marrow using Ficoll-Paque. Primitive (CD34$^+$) cells were enriched by positive selection using CliniMACS technology and stored frozen in the vapor phase of liquid nitrogen until required.

Drug treatment conditions

Treatments were carried out in serum-free medium containing cytokines with different concentrations of BGB324. Imatinib was used at different concentrations alone or in combination with BGB324 for 3 or 48 hours as indicated in the figure legends. In the combinatory experiment, the IC_{50} dose levels of 1.5 μmol/L BGB324 and 500 nmol/L imatinib, respectively, were used. Primary MNCs or CD34$^+$ isolated from healthy donors or CML cells were treated as specified. After 48 hours, cell viability was assessed by WST-1 assay (Roche Applied Science). Primary cells from a BCR-ABL TKI-resistant patient and a patient with a BCR-ABL1 T315I mutation were treated for 72 hours with 100 nmol/L ponatinib, 150 nmol/L dasatinib, and 3 μmol/L of BGB324 prior to apoptosis and colony assays.

Flow cytometry

Proliferation and apoptosis levels were measured by flow cytometry using BrdUrd incorporation and 7-aminoactinomycin D (7-AAD) staining (to exclude dead cells; BD) and Annexin V-FITC/propidium iodide, respectively. Events were captured using BD FACS Calibur.

Colony-forming assay

Clonogenic potential of cells surviving *in vitro* drug treatments was determined by colony-forming cell assay whereby cells were aliquoted in duplicate in semisolid culture medium (Methylcellulose H4034; Stem Cell Technologies), cultured for 12 days and colonies scored by light microscopy.

Cloning of LeGO vectors and production of lentiviral particles

Cloning was performed using a LeGO vector for silencing human Axl (for detailed protocols and vector maps, refer to http://www.lentigo-vectors.de). For silencing, Axl pLKO.1 vector containing shRNA Axl sequence 5′-ccggcgaaatcctctatgtcaacatctcgagatgttgacatagaggatttcgttttt-3′ was purchased from Sigma MISSION. Only cells with a minimum of 70% knockdown compared with controls were used for experiments.

ELISA and qPCR

Human or murine Gas6 levels were determined in cell culture supernatants and peripheral blood plasma using ELISA kits according to the manufacturer's instructions (R&D Systems).

Quantitative RT-PCR of human Axl was performed with cDNA from peripheral blood MNCs (PBMNC) BCR-ABL TKI-resistant or -sensitive patients with CML using premade Gene-Expression Assays from Applied Biosystems (human primers for *Axl* Hs01064444_m1 and *GAPDH* Hs99999905_m1) and the Eppendorf MasterCycler technology (Eppendorf).

Western blot analysis

Western blotting was carried out as described (27, 32). The pAkt, Akt, p-p44/42 MAPK (pErk1/2), p44/42 MAPK (Erk1/2), pBCR-ABL, BCR-ABL, pCrkl, Crkl, pStat5, and Stat5 antibodies were purchased from Cell Signaling (distributed through New England Biolabs GmbH). β-Actin antibody was purchased from Santa Cruz Biotechnology. pAxl antibody (Y799) was from R&D Systems. The Axl antibody was a gift from Björn Dahlbäck.

CML mouse models and treatments

For the BaF3/p210, BaF3/T315I, KCL-22 T315I, and KCL-22 PonR xenograft model, 1×10^7 cells were injected subcutaneously into the flank of 6- to 8-week-old NSG mice. Mice were randomized and treated twice daily with placebo, 25 or 50 mg/kg BGB324 by oral gavage (BID). Mice were sacrificed because of ethical regulations. For the systemic model, 6-week-old female NSG mice were sublethally irradiated and intravenously transplanted the next day with KCL-22 PonR cells. Three days later, mice were treated with placebo or 25 mg/kg BGB324 as described previously. Animals were considered end-stage and sacrificed according to ethical regulations when they showed symptoms of hind limb paralysis or developed multiple chloromas.

Immunohistochemistry of phospho-histone H3

Tumors were resected and fixed. Image analysis was performed using AxioVision imaging software (Carl Zeiss Microscopy). Phospho-histone H3 (pHH3)-positive cells were calculated as the number of counted pHH3$^+$ cells per square millimeter of analyzed tumor tissue.

Statistical analysis

Data represent mean ± SEM of representative experiments, unless otherwise stated. Statistical significance was calculated by the Student *t* test unless otherwise stated. To study dependence of numerical-dependent parameters of $n > 2$ categorical variables, ANOVA was used where indicated. Survival analysis was carried out using the Kaplan–Meier method (log-rank test). All statistical analyses have been performed with GraphPad Prism 5.0 (GraphPad Software) software.

Supplementary information

Detailed protocols are provided as Supplementary Information.

Results

Axl signaling is activated in CML cell lines and patient samples

We previously discovered the role of the Gas6-Axl axis in AML, promoting proliferation of leukemia cells and establishing a chemoprotective niche, which can be abrogated by Axl-targeting approaches (27). On the basis of these data, we investigated whether Axl might play a role in CML pathobiology. To address this question, Axl protein expression was evaluated in BMMNCs from healthy donors and newly diagnosed chronic phase CML patient samples. Immunoblotting revealed presence of Axl in all the CML patient samples analyzed and showed that Axl expression levels were enhanced in BMMNCs from 6 of 7 patients with CML compared with healthy BMMNCs. In addition, Axl phosphorylation was evaluated indicating active signaling of the receptor in 6 of 7 patients with CML (Fig. 1A).

Axl Western blotting in different CML cell lines showed that the receptor was expressed in all human BCR-ABL1$^+$ KCL-22, K562, BV-173 and murine BaF3/p210 cell lines, similar to our observations in patients with CML. Interestingly, Axl phosphorylation was increased in serum-deprived growth conditions indicating its increased activity in challenging conditions (Fig. 1B).

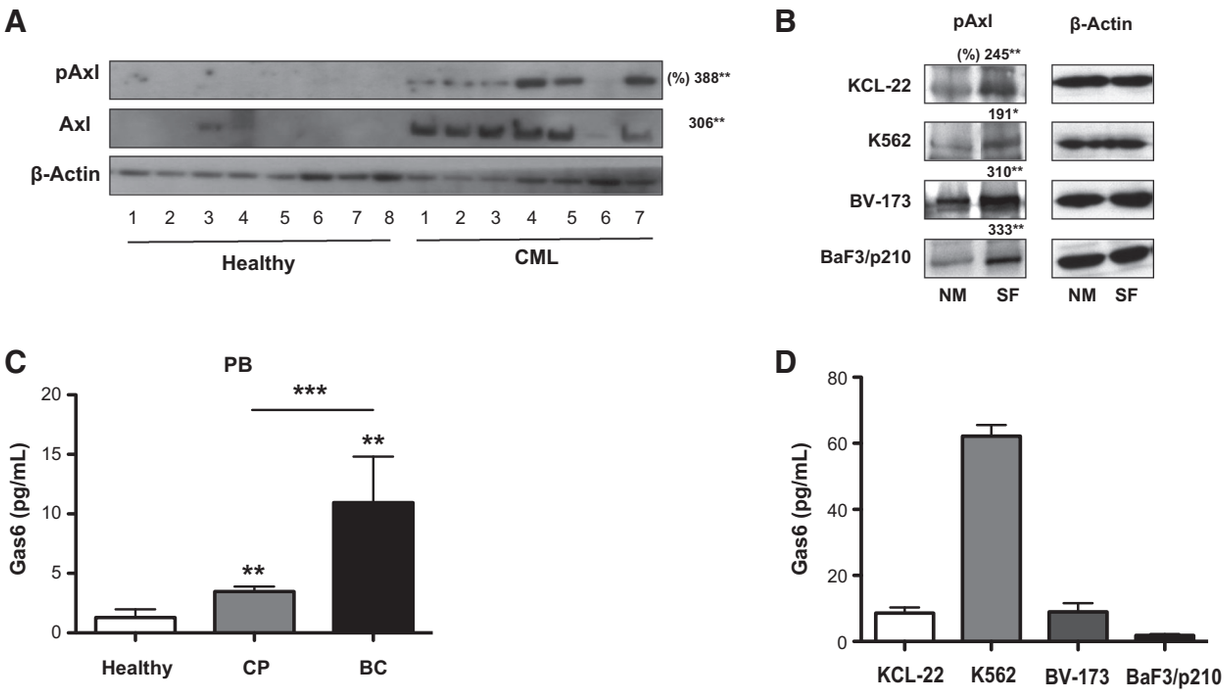

Figure 1.

Axl signaling is activated in patients with CML and cell lines. **A,** Western blot analysis of Axl expression and phosphorylation (pAxl) showing lower Axl and pAxl levels in BMMNCs from healthy donors compared with newly diagnosed patients with chronic phase CML ($n = 8/7$). Densitometric quantification of (pAxl/β-actin)/(Axl/β-actin) from CML was normalized to healthy samples. Data are presented as percentage of healthy donors. **B,** Western blot analysis of pAxl expression in different human BCR-ABL1$^+$ KCL-22, K562, BV-173 and murine BaF3/p210 cell lines. pAxl expression was compared after 24 hours of culturing the cells in normal medium [NM, containing 10% FCS or in serum free (SF, containing 0.1% FCS) conditions]. Densitometric quantification of (pAxl/β-actin)/(total protein expression/β-actin) was normalized to NM cultured cells. Data are presented as percentage of pAxl as detected in NM cells ($n = 3$). **C,** Gas6 ELISA from peripheral blood plasma samples indicating upregulation of hGas6 in chronic phase and blast crisis CML compared with healthy donors ($n = 14/50/7$). **D,** Gas6 ELISA determining secreted Gas6 protein levels in supernatants from different human BCR-ABL1$^+$ KCL-22, K562, BV-173 and murine BaF3/p210 cell lines. Values are presented as the mean of picogram per milliliter (pg/mL) from 3 replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student t test.

Next, we quantified the levels of secreted Gas6, the main ligand of Axl, in serum samples from healthy donors and patients with CML in chronic phase and blast crisis. Gas6 protein levels were measured by ELISA. The results showed that Gas6 serum levels were 2.7-fold and 8.5-fold increased in chronic phase and blast crisis, respectively, compared with healthy donors (healthy: 1,290 ± 684 pg/mL; chronic phase, 3,465 ± 405 pg/mL; blast crisis, 10,940 ± 3,868 pg/mL; Fig. 1C). Hence, increased amounts of Gas6 are present in the circulation of patients with CML compared with healthy subjects.

Similarly, secreted Gas6 levels were quantified in the different CML cell lines in which Axl expression was previously detected, revealing that Gas6 is expressed at different levels in these cell lines (Fig. 1D).

Thus, Axl signaling is activated in MNCs from patients with CML and cell lines. Furthermore, Gas6 serum levels are increased in CML. Therefore, the Gas6-Axl axis might represent a potential therapeutic target in CML and we tested whether its genetic or pharmacological inhibition would impair CML cell survival.

Genetic knockdown of Axl inhibits CML cell growth

To investigate whether Axl represents a therapeutic target in CML, we knocked down its expression by means of shRNA in KCL-22 and K562 cells (Supplementary Fig. S1A). Silencing of Axl in CML cells decreased their viability compared with control transduced cells (Fig. 2A). Knockdown of Axl inhibited phosphorylation of pStat5, pAkt, and pErk, important signaling intermediates for CML survival and proliferation (Fig. 2B and Supplementary Fig. S1A).

BGB324 decreases viability of CML cell lines and primary CML cells

To investigate the therapeutic potential of pharmacologic Axl inhibition, we used the selective clinical-stage small-molecule Axl kinase inhibitor BGB324 (28, 33). KCL-22 and K562 cells were incubated with different doses of BGB324 for 3 hours. Western blot analysis indicated that Axl phosphorylation was inhibited in a dose-dependent manner in both cell lines. Thus, BGB324 blocks its target in CML cells. Beyond that, we were interested in elucidating whether treatment with BGB324 affects similar pathways compared with the genetic knockdown of Axl. Immunoblot analyses suggested in concordance with the genetic blockade phospho(p)Stat5, pAkt, and pErk were inhibited upon treatment with BGB324 (Fig. 2C).

Consequently, incubation of KCL-22, K562, BV-173, and murine BaF3/p210 cell lines with different concentrations of

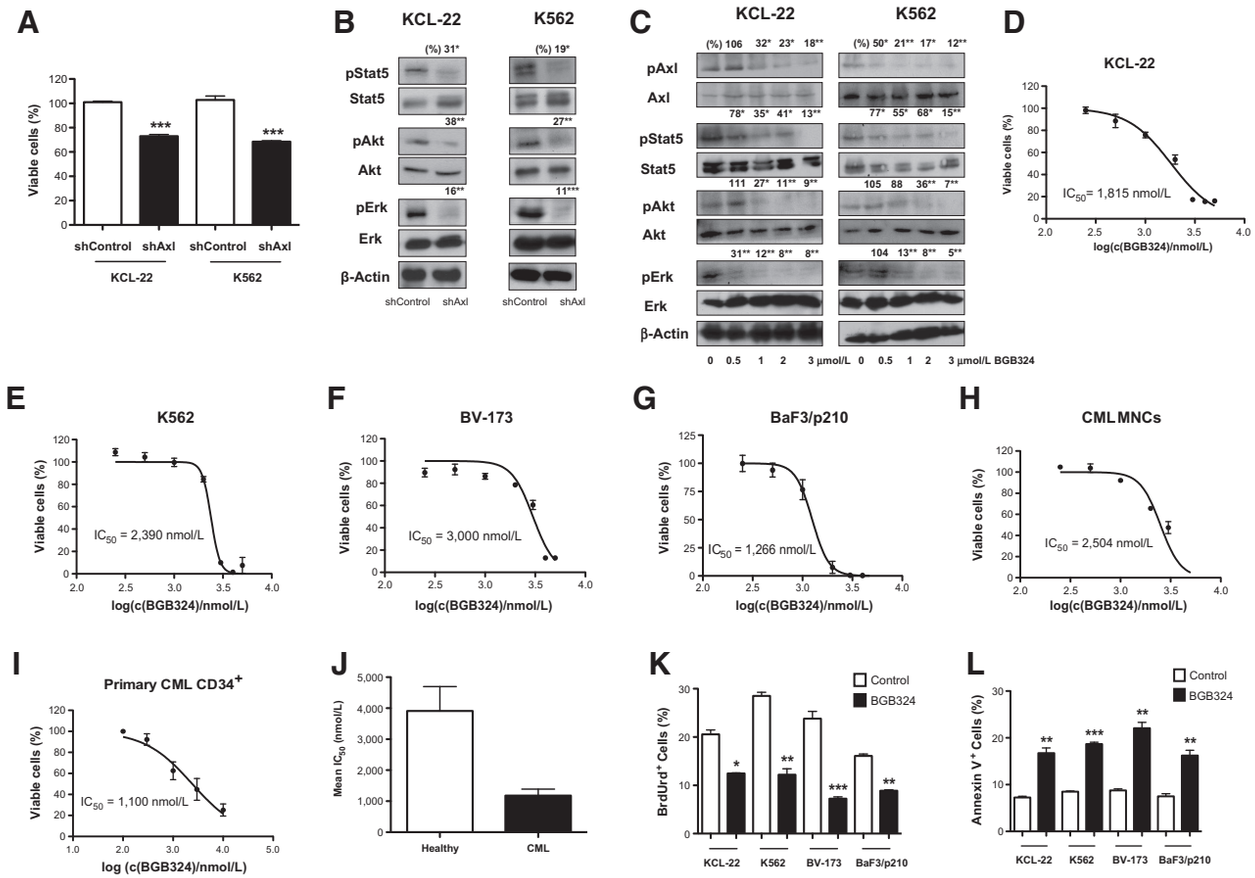

Figure 2.

BGB324 decreases viability of CML cell lines and primary CML cells. **A,** Silencing of Axl expression by shRNA reduced the number of viable KCL-22 and K562 cells as shown by WST-1 assay. Percentage of viable cells was normalized to control-transduced cells ($n = 3$). Western blot analyses of pStat5, pAkt, and pErk (**B**) after knockdown of Axl expression or (**C**) after incubation with different concentrations of BGB324 (as indicated) for 3 hours indicating downregulation of these signaling pathways activity in KCL-22 and K562 cells ($n = 3$). Densitometric quantification of (phosphorylated protein/β-actin)/(total protein expression/β-actin) was normalized to control-transduced cells and represented as percentage. **D–G,** Assessment of half maximal inhibitory concentration (IC_{50}) for BGB324. Treatments were performed with increasing concentrations (0, 1, 2, 3, 4, and 5 μmol/L) of BGB324 (expressed as logarithm) for 48 h in human KCL-22 (**D**), K562 (**E**), BV-173 (**F**) and murine BaF3/p210 (**G**) cell lines ($n = 3$). IC_{50} of BGB324 in (**H**) MNCs and (**I**) in $CD34^+$ cells isolated from bone marrow of patients with CML ($n = 17/6$). **J,** Mean of the IC_{50} of $CD34^+$ cells from healthy donors versus patients with CML ($n = 6/3$; $P = 0.05$). **K** and **L,** FACS quantification of proliferation ($BrdUrd^+$ staining) and apoptosis (Annexin V^+ staining) in different human BCR-ABL1$^+$ KCL-22, K562, BV-173 and murine BaF3/p210 cell lines. Proliferation was decreased (**K**) and apoptosis was increased (**L**) with 1.5 μmol/L of BGB324 after 24 hours ($n = 3$). Data are presented as percentage of mean $BrdUrd^+$ or Annexin V^+ cells from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student t test.

BGB324 reduced viability of these cell lines with an IC_{50} of 1.8 ± 0.2, 2.4 ± 0.1, 3.0 ± 0.3, and 1.3 ± 0.1 μmol/L, respectively (Fig. 2D–G). In addition, MNCs isolated from the bone marrow of patients with chronic phase CML were inhibited with an IC_{50} of 2.5 ± 0.3 μmol/L (Fig. 2H). In contrast, MNCs isolated from bone marrow of healthy donors were almost completely resistant to BGB324 (IC_{50}: 9.8 ± 1.9 μmol/L, $n = 6$). BGB324 also had activity *in vitro* against primary chronic phase CML $CD34^+$ cells (Fig. 2I). BGB324 inhibited *in vitro* growth of BCR-ABL1$^+$ $CD34^+$ cells with an IC_{50} of 1100 nmol/L, more than 3-fold lower than for $CD34^+$ cells from healthy donors (Fig. 2J and Supplementary Fig. S2A).

Subsequently, we investigated whether the observed reduction of cell viability was due to reduced proliferation and/or increased apoptosis. After incubation of CML cell lines with 1.5 μmol/L of BGB324 for 24 hours, we quantified $BrdUrd^+$ and Annexin V^+ cells by means of flow cytometry. These experiments indicated that BGB324 treatment inhibited proliferation and inducted apoptosis in CML cell lines (Fig. 2K and L and Supplementary Fig. S3A).

Altogether, these experiments show that Axl inhibition by BGB324 blocks proliferation and induces apoptosis of CML cells.

BGB324 does not inhibit BCR-ABL1–induced signaling

The affinity of BGB324 for Abl was 51.88 nmol/L in BIAcore assays. The selectivity of BGB324 for Axl over Abl was more than 9-fold and more than 100-fold in biochemical assays and in cellular assays, respectively (33). However, we wished to rule out that the observed biologic effects might be off-target effects of BGB324 via inhibition of BCR-ABL1. To address this question, KCL-22 and K562 cells were treated

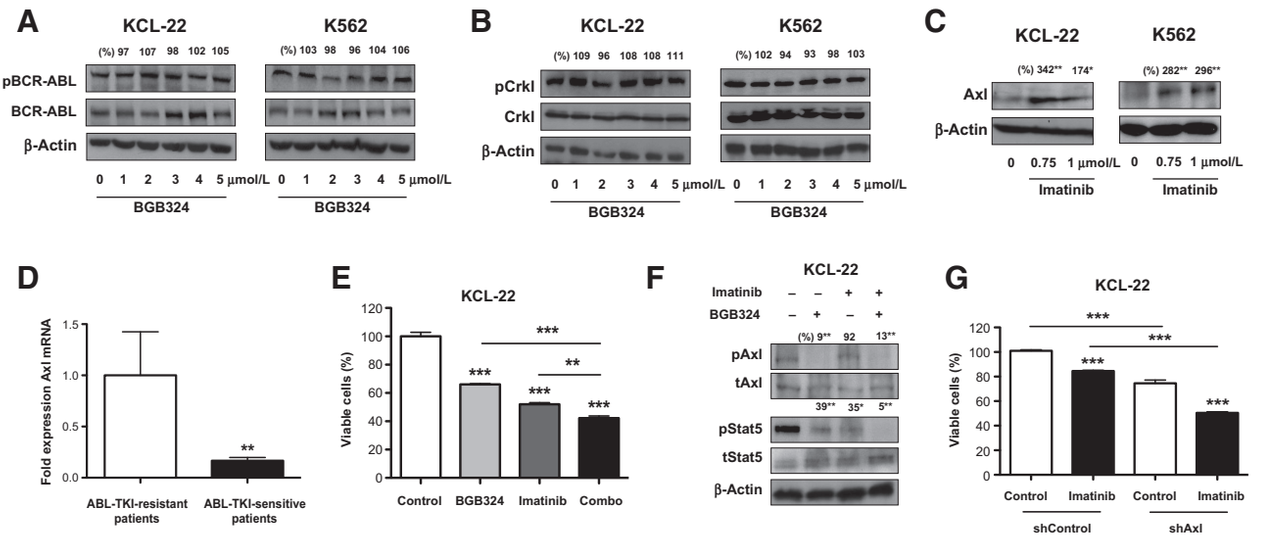

Figure 3.

Axl inhibition exerts additive therapeutic effects with BCR-ABL inhibitors. **A–C,** Western blot analysis of BCR-ABL1 (**A**) and Crkl (**B**) phosphorylation in KCL-22 and K562 cells, showing that is not affected by different doses of BGB324 (as indicated) after 3 hours of incubation ($n = 3$). Densitometric quantification of (phosphorylated protein/β-actin)/(total protein expression/β-actin) normalized to control-treated cells and represented as percentage. **C,** Upregulation of Axl expression in KCL-22 and K562 cells after treatment with imatinib (0.75 and 1 μmol/L) for 48 hours ($n = 3$). Densitometric quantification of (Axl/β-actin) normalized to control-treated cells and represented as percentage. **D,** Axl mRNA levels in CML MNCs measured by qPCR, showing increased Axl expression in cells from patients who developed resistance to nilotinib ($n = 17$) after 6 months of treatment compared with sensitive patients ($n = 20$). GAPDH was used as a housekeeping gene. **E,** KCL-22 cells were treated with BGB324 (1.5 μmol/L) or imatinib (500 nmol/L) as single therapy or in combination for 48 hours, and cell viability was measured by WST-1. BGB324 and imatinib elicited additive antileukemic effects in KCL-22 cells ($n = 3$). Percentage of viable cells was normalized to control-treated cells. **F,** Western blot analysis of phospho-Axl and phospho-Stat5 levels after BGB324 (1.5 μmol/L) and imatinib (500 nmol/L) treatment for 3 hours ($n = 3$). Densitometric quantification of (phosphorylated protein/β-actin)/(total protein expression/β-actin) normalized to control-treated cells and represented as percentage. **G,** WST-1 assay measuring cell viability in shcontrol- or shAxl-transduced cells upon imatinib treatment (500 nmol/L) for 48 hours. Blocking Axl expression increased CML cell sensitivity toward imatinib compared with control-transduced cells ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student t test.

with imatinib or with BGB324. After 3 hours, BCR-ABL1 and Crkl phosphorylation were measured by Western blotting. As expected, phosphorylation of BCR-ABL1 and Crkl was inhibited after imatinib treatment (Supplementary Fig. S4A and S4B). However, BGB324 did not inhibit BCR-ABL1 signaling because pBCR-ABL1 and pCrkL levels were unaffected by a broad dose range of BGB324 (Fig. 3A and B).

Axl expression is upregulated after BCR-ABL TKI treatment in CML

Axl overexpression has been implicated in resistance against BCR-ABL TKI in imatinib- and nilotinib-insensitive CML cell lines (30, 31). Furthermore, upregulation of Axl can confer resistance toward treatment with chemotherapy or targeted therapies in different cancers (25, 27). Therefore, to investigate whether Axl overexpression occurs upon treatment with BCR-ABL TKI, KCL-22 and K562 cells were incubated with increasing concentrations of imatinib for 48 hours and Axl expression was measured. Upon treatment with imatinib, CML cell lines showed upregulation of Axl at the protein level, in line with published data showing upregulation of Axl in BCR-ABL TKI-resistant cell lines (Fig. 3C; ref. 31).

Moreover, Axl expression was evaluated in samples of patients with chronic phase CML after 6 months of therapy with imatinib or nilotinib. Patient samples were grouped depending on whether they were responding according to ELN guidelines (34). In concordance with the results observed before, Axl mRNA levels were higher in PBMNCs derived from BCR-ABL TKI-resistant patients compared with those who were BCR-ABL TKI-sensitive (Fig. 3D). Altogether, the data show that upregulation of Axl might be involved in resistance toward BCR-ABL TKI in CML.

Blockade of Axl exerts additive therapeutic effects with imatinib in CML cell lines

In a next step, we wished to determine the efficacy of simultaneous inhibition of Axl and BCR-ABL1 in impairing viability of CML cells. Concomitant inhibition of Axl by BGB324 (1.5 μmol/L) and BCR-ABL1 by imatinib (500 nmol/L) both used at their IC_{50} dose level resulted in an additive effect on growth inhibition upon combined treatment (Fig. 3E).

Moreover, to clarify which signaling pathways are involved in the biologic effects observed after the combined therapy, key mediators potentially affected by both Axl- and BCR-ABL1-induced pathways were investigated. An additive effect of inhibition of Stat5 phosphorylation was detected when imatinib and BGB324 were combined, indicating that Axl induces phosphorylation of Stat5 by BCR-ABL1–independent pathways (Fig. 3F). An additive inhibitory effect on phosphorylation of Erk and Akt was not detected (Supplementary Fig. S5A). Of note, we could not see an upregulation of Axl in these experiments because analysis of phosphorylation of signal transduction intermediates was performed after 3 hours, which is too early for detecting differences in protein translation.

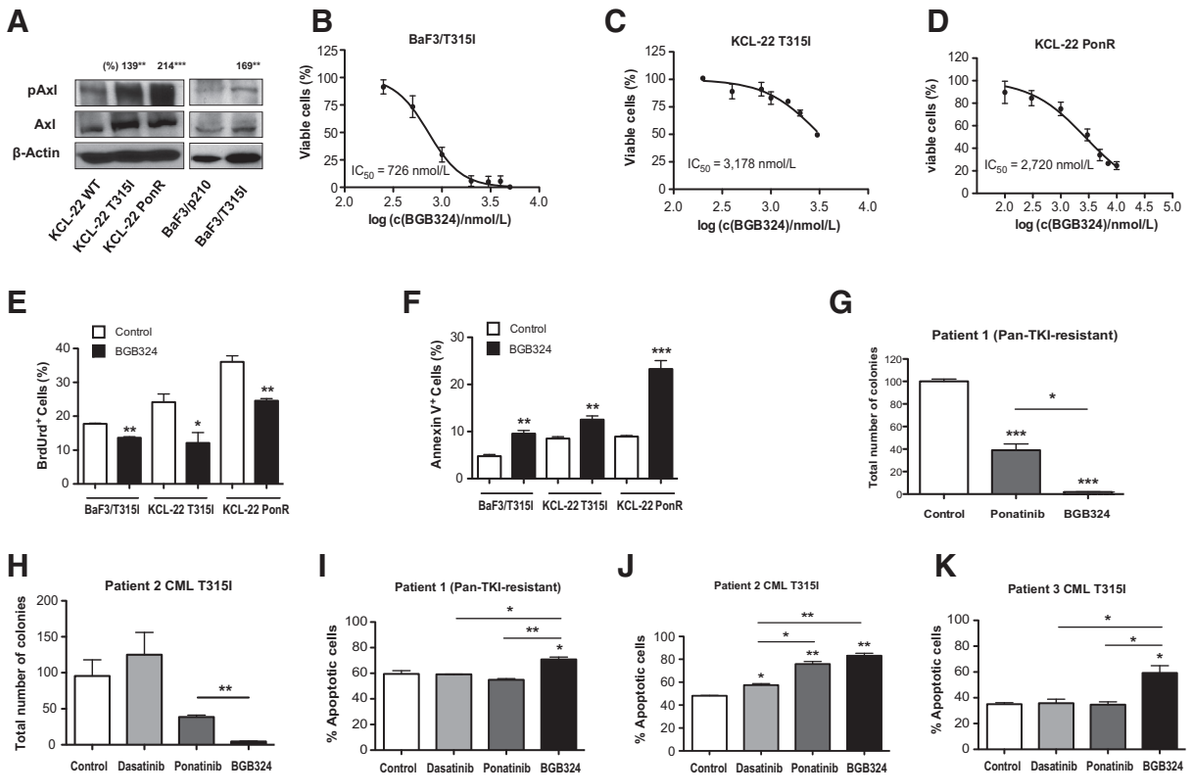

Figure 4.

Blocking Axl inhibits TKI-insensitive BCR-ABL1$^+$ cell lines and primary CML cells. **A,** Western blot analysis of total and phosphorylated Axl in human KCL-22 T315I, KCL-22 PonR and murine BaF3/T315I BCR-ABL TKI-resistant cell lines indicating higher pAxl expression levels compared with human KCL-22 WT or murine BaF3/p210 cell lines. Densitometric quantification of (pAxl/β-actin)/(Axl/β-actin) was normalized to the corresponding control and represented as percentage ($n = 3$). **B–D,** Determination of the IC_{50} of BGB324. Treatments were performed with increased concentrations (0, 1, 2, 3, 4, and 5 μmol/L) of BGB324 (expressed as logarithm) for 48 hours in the murine BaF3/p210 (**B**) and in the human KCL-22 T315I (**C**) and KCL-22 PonR (**D**) cell lines ($n = 3$). **E** and **F,** FACS quantification of proliferation (BrdUrd$^+$ staining) and apoptosis (Annexin V$^+$ staining) in the murine BaF3/p210 and in the human KCL-22 T315I and KCL-22 PonR cell lines. Proliferation was decreased (**E**) and apoptosis increased (**F**) with 1.5 μmol/L of BGB324 after 24 hours ($n = 3$). Data are presented as percentage of mean BrdUrd$^+$ or Annexin V$^+$ cells. **G–K,** Colony-forming and apoptosis cell assays performed using primary cells treated for 72 hours with 100 nmol/L ponatinib, 150 nmol/L dasatinib, and 3 μmol/L of BGB324. BGB324 induced greater reduction of colony growth using cells from a BCR-ABL TKI-resistant patient (including ponatinib) without kinase domain mutation (Patient 1, performed in duplicates; **G**) and from a patient with a BCR-ABL1-T315I mutation (Patient 2, performed in duplicates; **H**), compared with 100 nmol/L ponatinib. **I–K,** BGB324 induced apoptosis in CML MNCs from Patient 1 (performed in triplicates; **I**) and 2 patients harboring a BCR-ABL1-T315I mutation (Patients 2 and 3, performed in duplicates; **J** and **K**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, Student t test.

To validate the efficacy of simultaneous inhibition of Axl and BCR-ABL1, shcontrol-, and shAxl-transduced KCL-22 cells were treated with imatinib (500 nmol/L) and their viability was determined after 48 hours. These experiments demonstrated an additive effect of combined BCR-ABL1 and Axl blockade (Fig. 3G).

Altogether, our data indicate that Axl represents a therapeutic target in BCR-ABL1–unmutated CML. Inhibition of Axl exerts therapeutic efficacy alone and has an additive therapeutic effect when combined with imatinib. We next investigated whether Axl blockade might be of use in BCR-ABL TKI-resistant CML.

BGB324 reduces viability of BCR-ABL1 T315I-mutated and ponatinib-resistant CML cell lines

We investigated different BCR-ABL1$^+$ cell lines harboring the ABL T315I mutation and a novel ponatinib-resistant cell line. The cell line KCL-22 PonR was generated by subcloning parental KCL-22 in increasing concentrations of ponatinib (35). BCR-ABL1 is unmutated in these cells; however, cell death is not induced with 100 nmol/L ponatinib, a dose commonly leading to cell death in ponatinib-sensitive cells. First, Axl expression was evaluated in base line in different BCR-ABL TKI-resistant human KCL-22 and murine BaF3 cell lines. Axl phosphorylation was found to be higher in the BCR-ABL TKI-resistant cell lines BaF3/T315I, KCL-22 T315I, and KCL-22 PonR, when compared with the respective parental cell lines (Fig. 4A).

Thus, Axl could be a therapeutic target in BCR-ABL TKI-resistant CML. Next, we treated BaF3/T315I, KCL-22 T315I, and KCL-22 PonR cells with increasing concentrations of BGB324, and after 48 hours, cell viability was measured. Treatment with BGB324 inhibited cell growth with an IC_{50} of 0.72 ± 0.1, 3.2 ± 0.3, and 2.7 ± 0.2 μmol/L in BaF3/T315I, KCL-22 T315I, and KCL-22 PonR cells, respectively (Fig. 4B–D). When BCR-ABL1 and Crkl phosphorylation were measured by Western blotting in KCL-22 T315I-mutated cells, phosphorylation of BCR-ABL1 and Crkl was not

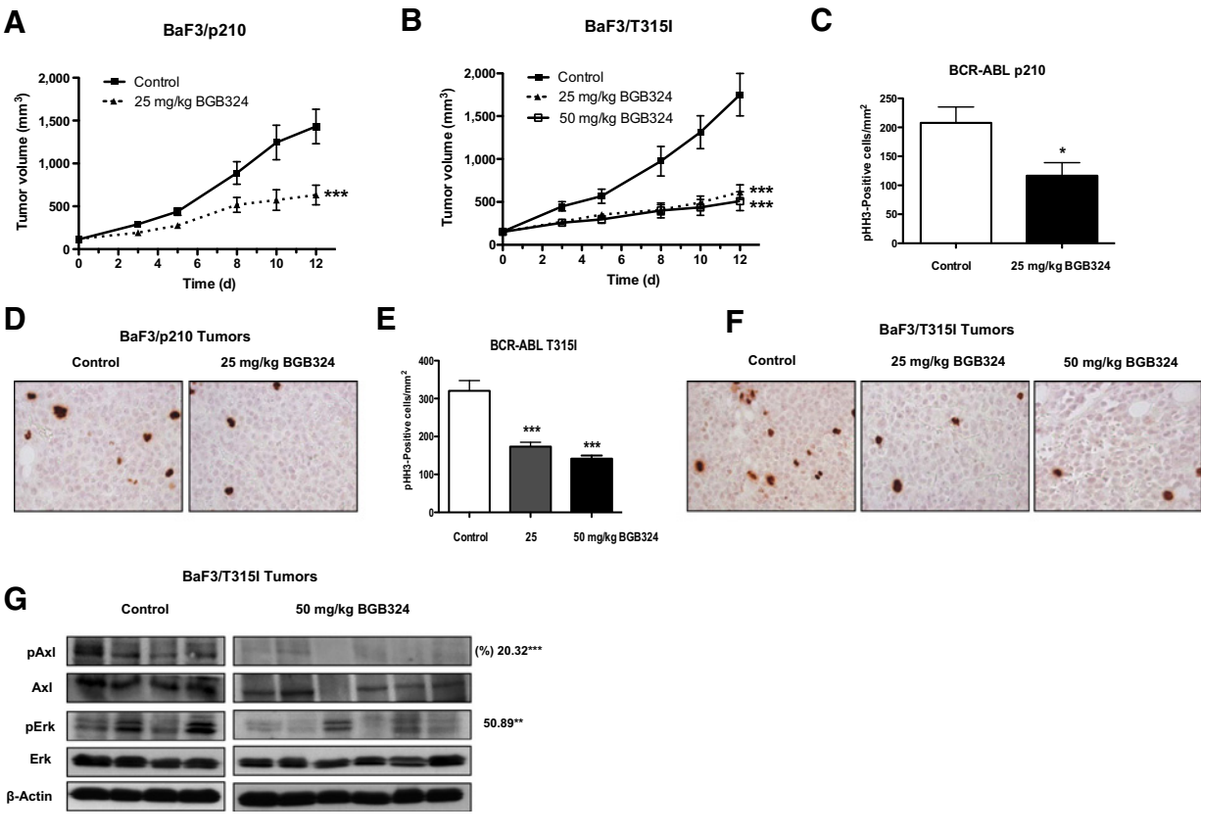

Figure 5.

Axl inhibition reduces growth of BCR-ABL1$^+$ cells *in vivo*. **A** and **B,** Tumor growth curves in mice injected subcutaneously with BaF3/p210 (**A**) or BaF/T315I (**B**) cells, revealing reduced tumor volume in animals treated with 25 mg/kg (**A** and **B**) and 50 mg/kg (**B**) of BGB324 compared with tumor-bearing control mice ($n = 7$, P values are calculated by 2-way ANOVA). **C** and **E,** Morphometric analysis of pHH3$^+$ cells performed in tumor tissue from BaF3/p210 (**C**) and BaF3/T315I (**E**) *in vivo* models indicating inhibition of proliferation in tumors treated with 25 mg/kg (**C** and **E**) and 50 mg/kg (**E**) of BGB324 compared with control-treated mice ($n = 7$). **D** and **F,** Representative immunohistochemical pictures of pHH3 staining on sections from BaF3/p210 (**D**) or BaF3/T315I (**F**) control- and BGB324-treated tumors. **G,** Immunoblots for Erk and Axl (phosphorylated and total protein) from BaF3/T315I tumor protein extracts, demonstrating reduced activation of these signaling pathways after treatment with 50 mg/kg of BGB324. Densitometric quantification of (phosphorylated protein/β-actin)/(total protein expression/β-actin) was normalized to control-treated tumors ($n = 4$ of 7) and represented as percentage. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

modified after treatment with different doses of BGB324 or imatinib for 3 hours (Supplementary Fig. S6A and S6B). Thus, also in T315I-mutated cells, BGB324 does not inhibit BCR-ABL1–induced signaling.

Subsequently, proliferation and apoptosis assays were carried out. BaF3/T315I, KCL-22 T315I, and KCL-22 PonR cells were incubated with 1.5 μmol/L of BGB324 for 24 hours, and BrdUrd and Annexin V assays were performed. We found that BGB324 inhibited proliferation in all the 3 cell lines studied. In addition, BGB324 exerted a proapoptotic effect on the BCR-ABL TKI-resistant cell lines shown by increased number of Annexin V$^+$ cells upon treatment (Fig. 4E and F and Supplementary Fig. S3A). Thus, similar to BCR-ABL1–unmutated cells, BGB324 reduces proliferation and induces apoptosis in BCR-ABL1 T315I-mutated and ponatinib-resistant cell lines.

BGB324 induces apoptosis of BCR-ABL1 T315I-mutated and BCR-ABL TKI-resistant primary CML cells

Furthermore, we assessed whether treatment with BGB324 would also have biologic effects on BCR-ABL1 T315I-mutated and BCR-ABL panTKI-resistant primary cells including cells unresponsive to ponatinib. PBMNCs from panBCR-ABL TKI-resistant ($n = 1$) and BCR-ABL1 T315I-mutated patients ($n = 2$) were isolated and treated for 72 hours with ponatinib, dasatinib, and BGB324 prior to colony and apoptosis assays. The colony formation assays indicated that BGB324 significantly reduced the colony formation of cells from BCR-ABL TKI-resistant and BCR-ABL1 T315I-mutated patients compared with control or BCR-ABL TKI treatment (Fig. 4G and H). The inhibitory effect of 3 μmol/L BGB324 on colony growth was 2.9-fold more pronounced in BCR-ABL TKI-resistant primary CML cells than in CD34$^+$ cells from healthy donors indicating a therapeutic window for the drug (Supplementary Fig. S7A). In line with the previous results, BGB324 was able to significantly induce apoptosis in cells from all the 3 studied patients (Fig. 4I–K and Supplementary Fig. S8A). Therefore, BGB324 blocked growth of colonies and induced apoptosis of BCR-ABL1 T315I-mutated and BCR-ABL TKI-resistant (including ponatinib) primary cells.

Altogether, our data indicate that BGB324 inhibits also CML cells that are resistant against current therapy including ponatinib.

BGB324 inhibits T315I-mutated CML *in vivo*

With the evidence that Axl inhibition by BGB324 showed therapeutic potential in drug-resistant CML *in vitro*, we proceeded to investigate its effects *in vivo*. For this purpose, 2 approaches were adopted. In a first step, ectopic models were used, whereby murine BaF3/p210 and BaF3/T315I cells were inoculated subcutaneously into non-obese diabetic (NOD)/severe combined immunodeficiency gamma (NSG) mice. After 12 days of treatment with BGB324, significant inhibition of tumor growth was observed in mice bearing p210 BCR-ABL1$^+$-unmutated tumors (control: 1,432 $\pm$ 403 mm^3; BGB324: 632 $\pm$ 229 mm^3) and in mice bearing BCR-ABL1 T315I-mutated tumors (control: 1,751 $\pm$ 606 mm^3, BGB324: 614 $\pm$ 224 mm^3; Fig. 5A and B). Therefore, BGB324 exerted a reduction in tumor growth of BCR-ABL1$^+$ and T315I-mutated cells *in vivo*.

To further elucidate the effects of Axl inhibition on CML tumor cells *in vivo*, we analyzed the proliferation ratio in tissue from BaF3/p210 and BaF3/T315I tumors treated with BGB324 compared with control treatment. Cell proliferation as determined by histomorphometric analysis of pHH3 revealed reduced proliferation of CML cells in BGB324-treated tumors compared with controls in both cell lines (Fig. 5C–F). Hence, BGB324 inhibits proliferation of BCR-ABL1–unmutated and T315I-mutated CML *in vivo* corroborating our *in vitro* data.

To investigate whether BGB324 affected the kinase activity of its direct target, the phosphorylation status of Axl was determined in control-treated CML tissue compared with BGB324-treated CML tumor tissue. This analysis demonstrated inhibition of Axl phosphorylation by BGB324 treatment, indicating target inhibition by the compound. Importantly, Axl inhibition reduced also the level of phosphorylated Erk (Fig. 5G). Thus, diminished Erk phosphorylation by BGB324 could explain the observed reduced proliferation of CML cells *in vivo*.

Axl blockade exerts therapeutic effects in human BCR-ABL TKI-resistant CML *in vivo*

In a next step, we determined whether treatment with BGB324 could also reduce tumor growth of BCR-ABL TKI-resistant human CML cells. Therefore, T315I-mutated and KCL-22 PonR cells were injected subcutaneously into NSG mice and treated by BGB324 or control. These experiments showed a significant tumor growth inhibition upon treatment with BGB324 compared with control treatment in both models, leading to 65% and 58% regression of tumor volume of KCL-22 T315I and KCL-22 PonR tumors, respectively (Fig. 6A and B).

Subsequently, to determine whether reduced proliferation might be responsible for decreased growth of BCR-ABL1 T315I-mutated and PonR tumors upon treatment with BGB324, cell proliferation was quantified by pHH3 analysis. The results indicated reduced proliferation of BGB324-treated KCL-22 T315I and KCL-22 PonR tumor cells compared with controls (Fig. 6C–F). Furthermore, in concordance with the previous results and with our *in vitro* data, Axl phosphorylation was decreased in BGB324-treated tumors. In addition, we found that pErk was significantly reduced in lysates from tumor tissues after treatment with BGB324 (Fig. 6G).

BGB324 prolongs survival in a ponatinib-resistant systemic mouse model

In addition to the ectopic models we also investigated the therapeutic effect of BGB324 in a systemic model. For the second *in vivo* approach, the human KCL-22 PonR cells were intravenously injected into sublethally irradiated NSG mice (36). Three days later, mice were divided in 2 groups and treated with placebo or 25 mg/kg BGB324 as described previously. In this aggressive model, treatment with BGB324 resulted in significant prolongation of overall survival by 7 days (Fig. 6H).

Altogether, BGB324 inhibits ABL TKI-resistant and -sensitive CML *in vitro* and *in vivo* independent of BCR-ABL1 mutational status.

Discussion

BCR-ABL TKIs have revolutionized CML treatment, but primary and acquired resistance as well as their toxicity continue to pose significant clinical challenges. Thus, there is the necessity of searching for new targets and personalized therapies (16, 37, 38).

The most important findings from our study are: (i) Axl signaling is activated in BCR-ABL1–unmutated, T315I-mutated, and ponatinib-resistant CML cell lines; (ii) genetic blockade of Axl or treatment with the selective small-molecule Axl inhibitor BGB324 inhibits proliferation and induces apoptosis of these cell lines; (iii) BGB324 inhibits primary CD34$^+$ CML cells and inhibits colony formation of T315I-mutated and ponatinib-resistant primary CML cells; and (iv) BGB324 does not inhibit BCR-ABL1 and exerts additive effects with imatinib via inhibition of STAT5 signaling. BGB324 inhibits CML growth in different mouse models including a systemic-ponatinib resistant model.

Altogether, our data indicate that the clinical-stage Axl inhibitor BGB324 might represent a novel treatment option in CML, potentially useful in combination with approved BCR-ABL TKI based on the observed additive effect which might lead to a more rapid and/or deeper response. The achievement of a deeper response would be clinically meaningful because the time until which a response is achieved and its depth has a pronounced prognostic impact in CML (39). Furthermore, a major molecular response is currently an inclusion criterion in clinical trials of BCR-ABL TKI discontinuation (40).

In addition, our data indicate that BGB324 is active in BCR-ABL TKI-resistant CML cells and mouse models, including T315I-mutated and ponatinib-resistant systemic mouse models and primary cells. Especially in patients harboring the T315I gatekeeper mutation and in ponatinib-resistant CML, novel treatment options are warranted. Our data show that BGB324 is active in CML resistant to all approved treatments which is consistent with the role of Axl in promoting drug resistance in different cancers (41). Importantly, we also demonstrate that there is a therapeutic window for the drug because the effect of BGB324 on survival and colony growth is much less pronounced in healthy CD34$^+$ stem cells than in CML cells. This conclusion is also supported by our experience in the AML trial BGB324 where the treatment has been overall well-tolerated in 25 patients (28).

We also observed an inhibitory effect of BGB324 on BV-173 cells, which resemble acute lymphocytic leukemia (ALL) cells (42), suggesting a therapeutic benefit of BGB324 in this subgroup of ALL carrying the Ph chromosome. Nevertheless, further studies are necessary to elucidate the therapeutic potential of BGB324 in BCR-ABL1$^+$ ALL.

Upon investigation of important signal transduction pathways promoting CML growth and survival, pronounced reduction of phosphorylated MAPK, Stat5, and Akt was found after Axl

inhibition. Previous data showed that BGB324 (formerly known as R428) has more than 9-fold and more than 100-fold higher affinity for Axl versus Abl in biochemical versus cellular assays, respectively (33). Biochemical BIAcore data indicate that BGB324 has higher affinity to mutated BCR-ABL1 including T315I, M351T, and E255K mutations (43). Therefore, we wished to investigate whether BGB324 might block BCR-ABL1 signaling in addition to Axl downstream signaling. Our data indicate that BGB324 does not inhibit BCR-ABL1–mediated signaling because its phosphorylation and phosphorylation of Crkl (one of the main mediators of BCR-ABL1 signaling) were not inhibited with BGB324 in BCR-ABL1–unmutated and T315I-mutated cells, even at the highest doses used. Thus, the biologic effect of BGB324 is due to Axl- and not BCR-ABL1 inhibition. It is well-established in literature that biochemical selectivity does not always reflect the activity of an inhibitor in a living cell (44, 45).

Recently, an allosteric inhibitor of BCR-ABL1 (ABL001) was discovered, which in contrast to the approved BCR-ABL TKI does not bind to the ATP site of the kinase domain but induces negative regulation of the kinase activity by an allosteric mechanism (46). Thereby, emergence of resistance due to mutations in the ATP site can be circumvented in preclinical models. ABL001 is currently being investigated in a phase I study. The mechanism of action of ABL001 is distinct from BGB324, thus it might be interesting to combine both drugs. However, further studies are necessary to investigate this combinatory approach.

Interestingly, higher levels of Axl expression were found after imatinib treatment in CML cell lines and in patients who were BCR-ABL TKI-resistant compared with TKI-sensitive patients. Overexpression, but not mutation, of Axl contributes to drug resistance in other cancers including GIST (25), NSCLC (26), and in AML (27). These data are consistent with our results suggesting that upregulation of Axl upon treatment could be involved in resistance toward BCR-ABL TKI in CML. Nevertheless, further studies are needed to dissect the functional role of Axl in mediating BCR-ABL TKI resistance in CML.

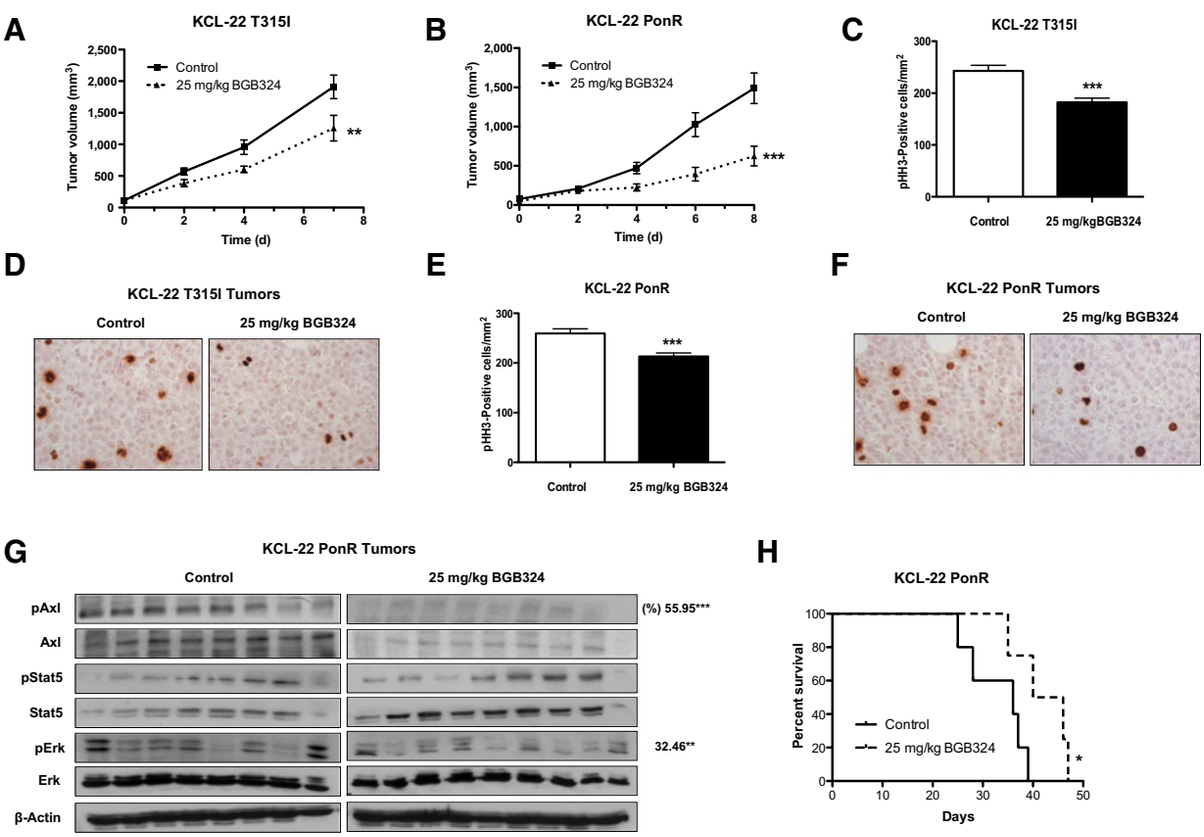

Figure 6.

Axl blockade exerts therapeutic effects in human BCR-ABL TKI-resistant CML *in vivo*. **A** and **B,** Tumor growth curves in mice injected subcutaneously with KCL-22 T315I (**A**) or KCL22 PonR (**B**) cells, showing reduced tumor volume in animals treated with 25 mg/kg of BGB324 compared with tumor-bearing control mice [$n = 8$ (**A**), $n = 9$ (**B**); P values are calculated by 2-way ANOVA]. **C** and **E,** Quantification of pHH3$^+$ cells performed in tumor tissue from KCL-22 T315I (**C**) and KCL-22 PonR tumors (**E**) indicating inhibition of proliferation in tumors treated with 25 mg/kg of BGB324 compared with control-treated mice [$n = 8$ (**C**), $n = 9$ (**E**)]. **D** and **F,** Representative immunohistochemical pictures of pHH3 staining of sections from KCL-22 T315I (**D**) or KCL-22 PonR (**F**) control- and BGB324-treated tumors. **G,** Western blot analysis of Erk, Stat5, and Axl (phosphorylated and total protein) from KCL-22 PonR tumor protein extracts, showing a downregulation of these signaling pathways after treatment with 25 mg/kg of BGB324. Densitometric quantification of (phosphorylated protein/β-actin)/(total protein expression/β-actin) was normalized to control-treated tumors ($n = 8/9$) and represented as percentage. **H,** Kaplan–Meier curve showing the overall survival (OS) *in vivo* in the systemic KCL-22 PonR mouse model revealing a benefit of Axl inhibition with 25 mg/kg of BGB324 [median overall survival (OS), 36 days (control) vs. 43 days (BGB324), $n = 5$]. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Precedents exist for multi-kinase inhibitor prescribed in CML, for example, dasatinib with its predominant activity against both Src and Abl, having the advantage of reaching deeper into the stem cell pool (47), but this carries the potential disadvantage of inducing dose-limiting toxicities, such as pulmonary hypertension (48), and pleural effusion (49), owing to off-target effects. Of the clinically available BCR-ABL TKI routinely in use in CML, only bosutinib targets Src/Abl and Axl but at low potency against Axl (174 times higher IC_{50} for Axl vs. unmutated BCR-ABL1) (50). Bosutinib is less effective against BCR-ABL1 mutants, including E255K/V, than against unmutated BCR-ABL1. BGB324 with its Axl kinase inhibition profile and activity in unmutated and mutated BCR-ABL TKI-resistant and -sensitive cells, including T315I-mutated and ponatinib-resistant CML, and its safety in healthy volunteers and in patients with AML (28, 29) may represent a novel treatment option in CML, alone and in combination with BCR-ABL TKI. Optimal treatment of patients with CML would involve a well-tolerated, orally bioavailable, targeted inhibitor that simultaneously suppresses BCR-ABL1 unmutated and evolving mutant clones.

In summary, our data highlight the advantage to be gained from inhibition of Axl even in the most resistant CML cells and support the need for human clinical trials of the novel inhibitor BGB324 at all stages of this disease.

Disclosure of Potential Conflicts of Interest

G. von Amsberg reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Astellas, Bristol-Myers Squibb, Novartis, Roche, and Sanofi. R.E. Clark reports receiving commercial research grants from and is a consultant/advisory board member for Bristol-Myers Squibb, Novartis, and Pfizer and also reports receiving speakers bureau honoraria from Ariad, Bristol-Myers Squibb, Novartis, and Pfizer. S. Koschmieder reports receiving commercial research grants from Bristol-Myers Squibb and Novartis and is a consultant/advisory board member for and reports receiving speaker fees for talks on chronic myeloid leukemia from Ariad, Bristol-Myers Squibb, and Novartis. T.H. Brümmendorf reports receiving commercial research grants from Novartis and Pfizer and is a consultant/advisory board member for Ariad, Bristol-Myers Squibb, Novartis, and Pfizer. A. Hochhaus reports receiving commercial research grants from and is a consultant/advisory board member for Ariad, Bristol-Myers Squibb, Novartis, and Pfizer. T.L. Holyoake reports receiving speakers bureau honoraria from and is a consultant/advisory board member for Bristol-Myers Squibb and Novartis. S. Loges reports receiving a commercial research grant from and is a consultant/advisory board member for BerGenBio. No potential conflicts of interest were disclosed by the other authors.

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