Activity of a novel Aurora kinase inhibitor against the T315I mutant form of BCR-ABL: *In vitro* and *in vivo* studies

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Despite promising results from clinical studies of ABL kinase inhibitors, a challenging problem that remains is the T315I mutation against which neither nilotinib nor dasatinib show significant activity. In the present study, we investigated the activity of a novel Aurora kinase inhibitor, VE-465, against leukemia cells expressing wild-type BCR-ABL or the T315I mutant form of BCR-ABL. We observed a dose-dependent reduction in the level of BCR-ABL autophosphorylation in VE-465-treated cells. Exposure to the combination of VE-465 and imatinib exerted an enhanced apoptotic effect in K562 cells. Combined treatment with VE-465 and imatinib caused more attenuation of the levels of phospho-AKT and c-Myc in K562 cells. Further, the isobologram indicated the synergistic effect of simultaneous exposure to VE-465 and imatinib in K562 cells. To assess the *in vivo* efficacy of VE-465, athymic nude mice were injected intravenously with BaF3 cells expressing wild-type BCR-ABL or the T315I mutant form. The vehicle-treated mice died of a condition resembling acute leukemia by 28 days; however, nearly all mice treated with VE-465 (75 mg/kg, twice daily; intraperitoneally for 14 days) survived for more than 56 days. Histopathological analysis of vehicle-treated mice revealed infiltration of the spleen. In contrast, histopathological analysis of organs from VE-465-treated mice demonstrated normal tissue architecture. Taken together, the present study shows that VE-465 exhibits a desirable therapeutic index that can reduce the *in vivo* growth of T315I mutant form and wild-type BCR-ABL-expressing cells in an efficacious manner. (*Cancer Sci* 2008; 99: 1251–1257)

The development of imatinib has redefined the management of chronic myeloid leukemia (CML).[1,2] Most newly diagnosed patients with chronic-phase disease, treated with imatinib, achieve durable complete cytogenetic responses.[3] However, a small percentage of these patients and most advanced-phase patients relapse on imatinib therapy.[3–5] Furthermore, only a minority of patients achieve undetectable levels of BCR-ABL transcript.[6] The leading cause of acquired resistance to imatinib is reactivation of BCR-ABL kinase activity via kinase domain mutations that decrease the sensitivity of the kinase to imatinib by 3- to >100-fold.[7–9] Structural analysis has revealed that imatinib binds to a unique, inactive conformation of the ABL kinase domain in which the activation loop is in a closed position that precludes substrate binding.[10] Mutations that confer resistance to imatinib either affect residues involved directly in drug binding, impair the ability of the ABL kinase to undergo the extensive conformational changes required for imatinib binding, or favor the active conformation of the kinase to which imatinib is unable to bind.[10–12] Thus, the need for alternative or additional treatments for imatinib-resistant BCR-ABL-positive leukemia has led to the design of a second generation of targeted therapies, and these efforts have resulted mainly in the development of clinically active small-molecule inhibitors such as nilotinib and dasatinib.[13] However, the challenging problem that remains is the T315I mutation, against which neither nilotinib nor dasatinib show significant activity.

The T315I mutation is one of the most common mutations found in patients undergoing imatinib therapy, and this mutation accounts for approximately 20% of imatinib-resistant cases. Thr315 is located in the center of the imatinib-binding site in ABL.[12,14] The residue at this position is referred to as the 'gatekeeper' residue because it separates the ATP-binding site from an internal cavity that is of variable size in different protein kinases, and the nature of the gatekeeper residue is an important determinant of inhibitory specificity.[15] The compound MK-0457, originally developed as an Aurora kinase inhibitor and currently in clinical trials, has been shown to bind a number of recurring imatinib-resistant mutant forms of BCR-ABL, including those with mutations at the gatekeeper position.[16–18] In contrast to imatinib, which penetrates deeply into the ABL kinase domain, MK-0457 is not fully buried within the kinase domain, and is anchored to it by four hydrogens bound to sequence-invariant elements.[19] Comparison with the structures of the Aurora kinases reveals that all of the essential contacts between MK-0457 and the protein involve highly conserved elements, explaining the broad specificity of this compound.[19]

In the present study, we investigated the activity of the novel Aurora kinase inhibitor VE-465, a compound related to MK-0457, against leukemia cells expressing wild-type or the T315I mutant form of BCR-ABL. We observed a dose-dependent reduction in the level of T315I mutant form and wild-type BCR-ABL kinase activity in VE-465-treated cells. Further, we evaluated the effect of VE-465 on the growth of BaF3 cells expressing wild-type or T315I mutant BCR-ABL *in vivo*. Systemic administration of VE-465 prolonged the survival in mice injected with BaF3 cells expressing wild-type or T315I mutant BCR-ABL. These results demonstrate that VE-465 exhibits a desirable therapeutic index that can reduce the *in vivo* growth of wild-type and T315I mutant BCR-ABL-expressing cells in an efficacious manner.

Materials and Methods

Antibodies and reagents. Anti-ABL antibody[11,20] was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phosphotyrosine monoclonal antibody (PY20) was purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Anti-Akt and antiphospho-Akt (Ser473) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Anti-c-Myc antibody was purchased from Novus (Littleton, CO, USA). VE-465 was kindly provided by Merck (Blue Bell, PA, USA).

Cells and cell culture. K562 cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

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TF-1BCR-ABL cells were described previously.[21] BaF3 p185 BCR-ABL cells and BaF3 T315I BCR-ABL cells were provided by Dr Martin Ruthardt (Frankfurt University, Frankfurt, Germany). These cell lines were cultured in RPMI-1640 (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA).

Apoptosis assay. The incidence of apoptosis was determined by flow cytometric analysis with the fluorescein isothiocyanate-conjugated APO2.7 monoclonal antibody (clone 2.7), which was raised against a 38-kDa mitochondrial membrane protein (7A6 antigen) expressed by cells undergoing apoptosis.[22]

Immunoblotting and immunoprecipitation. Immunoblotting and immunoprecipitation were carried out as described previously.[23]

Colony-forming assay. K562 cells, BaF3 p185 BCR-ABL cells, primary leukemia cells expressing T315I, and primary bone marrow mononuclear cells were treated with each agent and seeded in triplicate in conditioned MethoCult GF H4434 medium (Stem Cell Technologies, Vancouver, Canada). The leukemic colonies (>50 cells) were scored on day 14. Macroscopic colonies were counted in triplicate dishes on day 14. Primary leukemia cells expressing T315I and primary bone marrow mononuclear cells were obtained with informed consent prior to the study.

Isobologram method. The theoretical basis of the isobologram method and the procedure for making isobolograms have been described previously in detail.[24] K562 cells were suspended to a final concentration of 1×10^5 cells/mL in fresh medium, plated in 24-well dishes, and incubated with VE-465 or imatinib or a combination of the two at 37°C for 72 h. The number of cells in each well was counted by flow cytometry, and the cell numbers were normalized by dividing the number of cells.[20]

Cell cycle analysis. Logarithmically growing K562 cells were incubated with either VE-465 or dimethyl sulfoxide for 48 h. Single-cell suspensions were fixed in 70% ethanol for 15 min, incubated with RNase (1 mg/mL) at 37°C for 30 min, and labeled with 400 μL propidium iodide (50 μg/mL) for 15 min at room temperature. Cell cycle profiles were determined by flow cytometric analysis.[25]

***In vivo* efficacy of VE-465 in a mouse model of BCR-ABL-induced leukemia.** For the *in vivo* assessment of VE-465, 6-week-old female nude mice were injected with 10^6 BaF3 p185 BCR-ABL cells or BaF3 T315I BCR-ABL cells and then assigned randomly to either the vehicle alone or VE-465 treatment groups. At 24 h after the injection, these mice were treated with either vehicle or VE-465 (75 mg/kg b.i.d.; intraperitoneally for 14 days; resting for 14 days). Mice were observed daily, and bodyweight as well as signs of stress (e.g. lethargy, ruffled coat, or ataxia) were used to detect possible toxicities. The average tumor weight per mouse was calculated and used to analyze the group mean tumor weight ± SE ($n = 5$ mice).

Tumor and tissue processing. Tumors were collected at the selected times and fixed in paraformaldehyde. Paraffin-embedded tissues were sectioned and processed for gross histopathology by hematoxylin–eosin staining.

Results

VE-465 inhibits the proliferation of wild-type and T315I mutant BCR-ABL-expressing leukemia cells and inhibits BCR-ABL autophosphorylation. VE-465 is a synthetic small-molecule inhibitor of Aurora kinase (Fig. 1a) with apparent inhibition constant (Ki, app) values of 1.0, 26.0, and 8.7 nM for Aurora-A, Aurora-B, and Aurora-C, respectively. VE-465 inhibited the proliferation of K562 cells, BaF3 p185BCR-ABL cells, and BaF3 T315IBCR-ABL cells with inhibitory concentration (IC_{50}) values ranging from 50 to 500 nM (Fig. 1b–d). We also evaluated its growth-inhibitory effects on several human leukemia cell lines, primary leukemia cells, and primary bone marrow mononuclear cells (Table 1; Fig. 1e). VE-465 inhibited the human leukemia cell lines and primary leukemia cells with IC_{50} values ranging from 50 to 150 nM (Table 1; Fig. 1e). Because the Aurora kinases are essential for proliferation, BaF3 T315IBCR-ABL cells were incubated with the indicated concentrations of VE-465 for 48 h, after which, we examined the effects of VE-465 on the cell cycle profile by flow cytometry (Fig. 1f). VE-465 caused accumulation of cells in G_2/M arrest (Fig. 1f). To assess the inhibition of BCR-ABL kinase activity, BCR-ABL-expressing cells were cultured with the indicated concentrations of VE-465 for 24 h, and autophosphorylation of BCR-ABL was analyzed by immunoblotting (Fig. 2a–c). VE-465 inhibited wild-type and T315I mutant BCR-ABL autophosphorylation with IC_{50} values ranging from 2.0 to 5.0 μM, significantly higher than the results of the cell-proliferation assay (Figs 1,2). These results suggest that the growth-inhibitory effect of VE-465 on BCR-ABL-expressing cells is caused by Aurora kinase inhibition.

Table 1. VE-465 blocks leukemia cell proliferation

Cell line	IC_{50} (nM)
HL-60	50.2
NB4	38.6
U937	110.5
HAL-01	148.2
OM9; 22	98.4
TF-1	112.2
TF-1 BCR-ABL	152.4

IC_{50}, inhibitory concentration.

Cotreatment of VE-465 and imatinib enhances the induction of apoptosis in BCR-ABL-transformed cells. K562 and TF-1 BCR-ABL cells were cultured with the indicated concentrations of VE-465 and imatinib for 72 h, after which the percentage of apoptotic cells was determined using APO2.7 (Fig. 3a,b). When 50 nM VE-465 was combined with imatinib in K562 cells, the increase in apoptotic cells was virtually complete for imatinib concentrations higher than 1 μM (Fig. 3a). Cotreatment of VE-465 and imatinib also enhanced the induction of apoptosis in TF-1 BCR-ABL cells (Fig. 3b). Next, we determined the effect of cotreatment with VE-465 and imatinib on the level of signaling proteins downstream of BCR-ABL (Fig. 4a). Compared with treatment with either agent alone, relative low concentrations of VE-465 (50 nM) and imatinib (100 nM) for 24 h caused more attenuation of the levels of phospho-Akt and c-Myc (Fig. 4a). Corresponding immunoblotting analysis also revealed that the combination of 50 nM VE-465 and 100 nM imatinib resulted in increased caspase-3 and poly (ADP-ribose) polymerase (PARP) degradation in K562 cells (Fig. 4b). Together, these findings indicate that a combination of minimally toxic concentrations of VE-465 and imatinib is effective in inducing apoptosis in wild-type BCR-ABL-expressing cells.

Combined effects of VE-465 and imatinib in BCR-ABL-transformed cells. We determined the colony growth of K562 and BaF3 cells expressing p185 BCR-ABL (Fig. 5a,b). Cotreatment with VE-465 and imatinib caused significantly more inhibition of colony growth than treatment with either agent alone in K562 and BaF3 p185BCR-ABL cells (Fig. 5a,b). Further, we used the isobologram method to determine whether the combined effect of VE-465 and imatinib was additive or synergistic. Figure 5c showed the dose–response curve for VE-465 in combination with imatinib in K562 cells. The isobologram was generated on the dose–response curve (Fig. 5d). The observed data from the isobologram indicated the synergistic effect of simultaneous exposure to VE-465 and imatinib in K562 cells (Fig. 5d).

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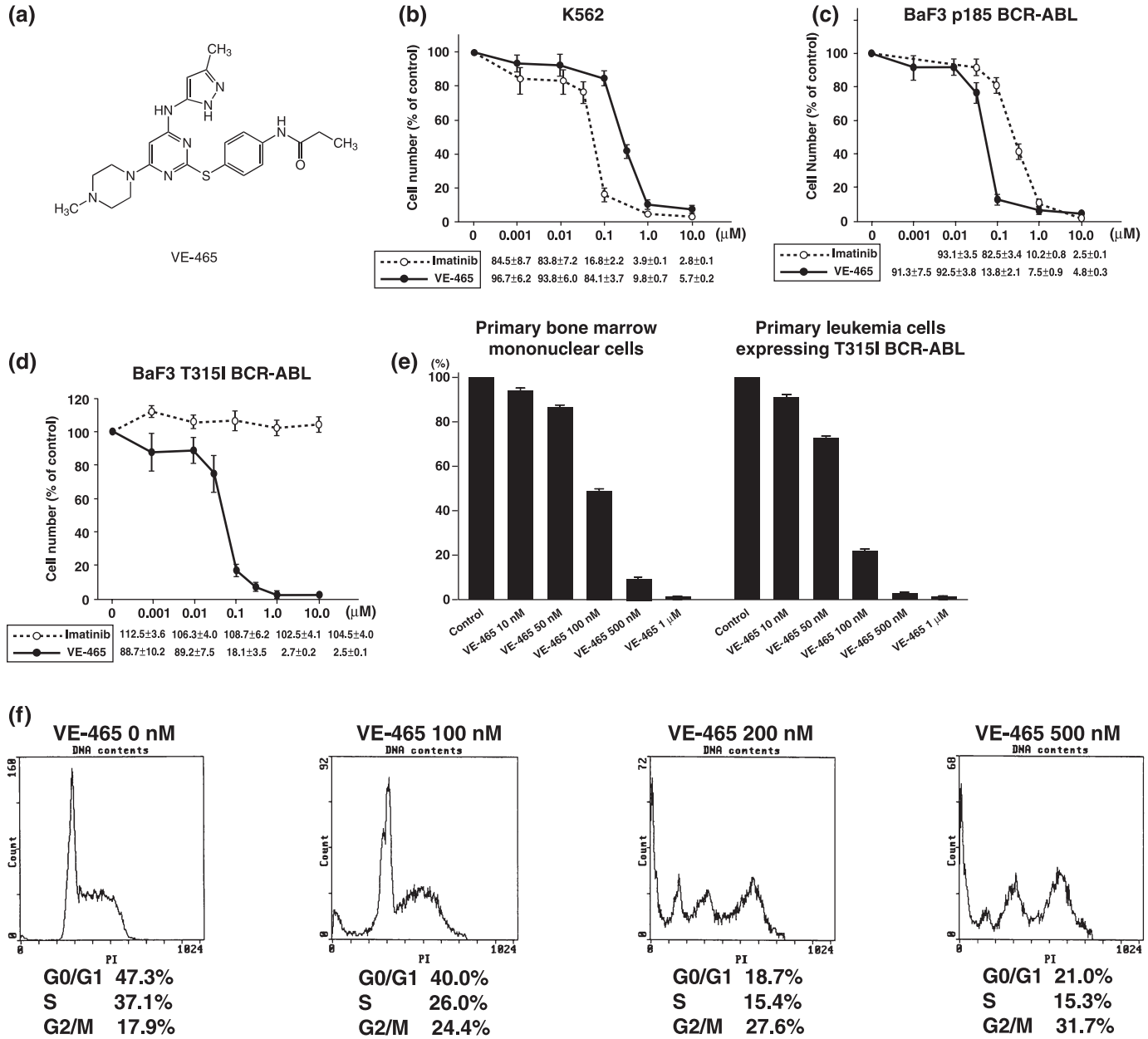

Fig. 1. VE-465 inhibits the growth of wild-type BCR-ABL or T315I mutant form of BCR-ABL-expressing leukemia cells. (a) Chemical structure of VE-465. (b) K562, (c) BaF3 p185BCR-ABL, and (d) BaF3 T315IBCR-ABL cells were cultured with the indicated concentrations of imatinib or VE-465 for 48 h. Bars = SEM, $n = 3$. In all dose-dependent curves, viable cell counts are represented as the percentage of control cells for each compound's dose. (e) Primary bone marrow mononuclear cells and primary leukemia cells expressing T315T obtained with informed consent prior to conducting studies were grown in methylcellulose containing the indicated concentrations of VE-465. Colony counts were assessed on each individual sample at least twice, and results are presented as average ± SD for colonies counted from triplicate plates under each condition. (f) BaF3 T315IBCR-ABL cells were incubated with the indicated concentrations of VE-465 for 48 h, after which we examined the effects of VE-465 on cell-cycle profile of BaF3 T315I BCR-ABL by flow cytometry.

Systemic VE-465 treatment decreases T315I BCR-ABL kinase activity *in vivo* and prolongs survival in a mouse model of BCR-ABL-induced leukemia. To further study the activity of VE-465 on tumor growth *in vivo*, we tested a mouse model of BCR-ABL-induced leukemia (Fig. 6). Intravenous injection of BaF3 cells expressing p185BCR-ABL or T315IBCR-ABL into nude mice resulted in an aggressive malignancy resembling acute leukemia, characterized by splenomegaly, circulating blasts, and invasion of leukemia cells into hematopoietic and non-hematopoietic tissue (Fig. 6a). At 24 h after the injection, the mice were divided into two groups (five mice per group), with each group receiving either vehicle or VE-465 (75 mg/kg, b.i.d.; intraperitoneally for 14 days; resting for 14 days). The vehicle-treated mice died of a condition resembling acute leukemia by 28 days; however, nearly all of the mice treated with VE-465 survived for more than 56 days (Fig. 6b). All VE-465-treated mice demonstrated modest weight loss (less than 10% initial bodyweight). Histopathological analysis of vehicle-treated mice revealed infiltration of the spleen and bone marrow with leukemic blasts (Fig. 6a). In contrast, histopathological analysis of organs from VE-465-treated mice demonstrated normal tissue architecture and no evidence of residual leukemia (Fig. 6a). BCR-ABL kinase

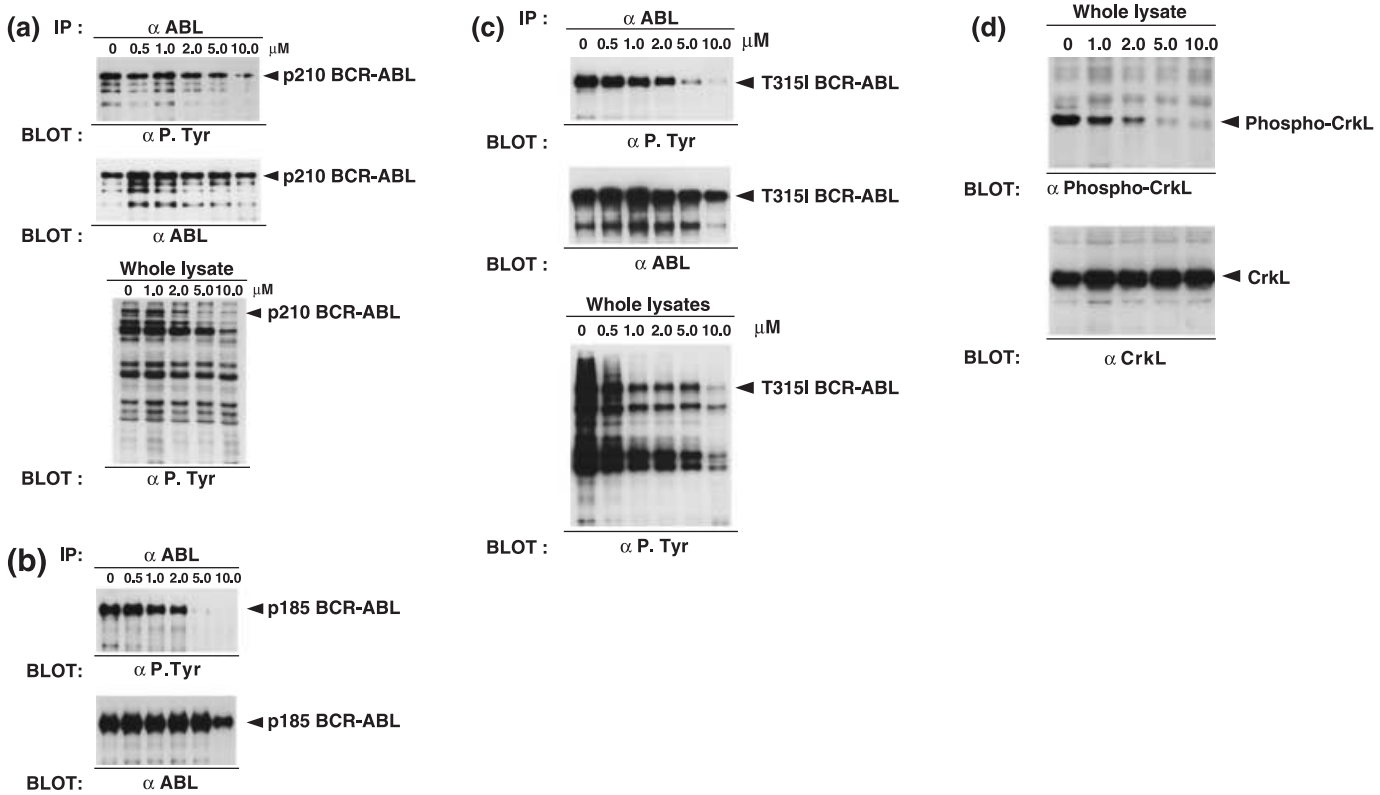

Fig. 2. Inhibition of BCR-ABL autophosphorylation by VE-465 in wild-type BCR-ABL- or T315I BCR-ABL-expressing leukemia cells. (a) K562, (b) BaF3 p185 BCR-ABL, and (c) BaF3 T315I BCR-ABL cells were cultured with the indicated concentrations of VE-465 for 24 h. The cell lysates were immunoprecipitated with anti-ABL antibody, and then the immunoprecipitates were immunoblotted with antiphosphotyrosine (P.Tyr) antibody (PY20), or anti-ABL antibody. (a,c lower panels) Whole-cell lysates were immunoblotted with anti-P.Tyr antibody. (d) BaF3 T315IBCR-ABL cells were cultured with the indicated concentrations of VE-465 for 24 h, and whole-cell lysates were immunoblotted with antiphospho-CrkL or anti-CrkL antibodies.

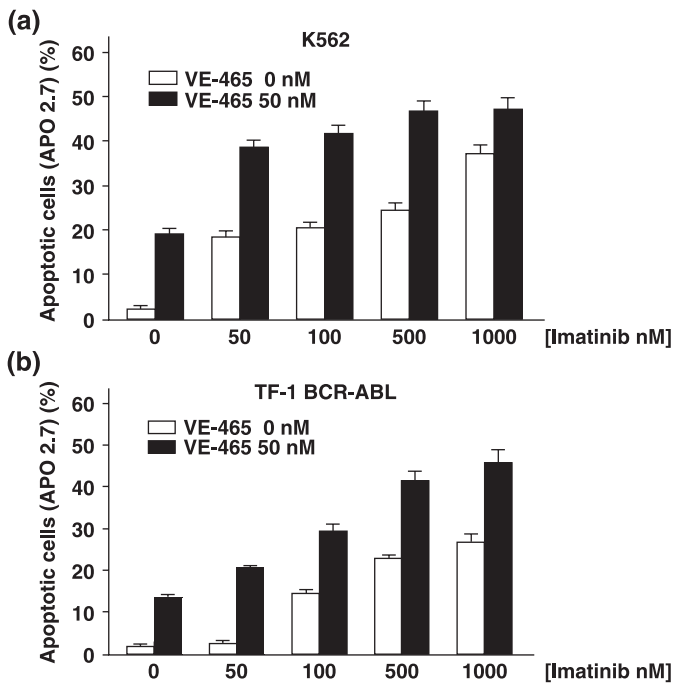

Fig. 3. Cotreatment of VE-465 and imatinib enhances the induction of apoptosis in BCR-ABL-transformed cells. (a) K562 and (b) TF-1BCR-ABL cells were cultured with the indicated concentrations of imatinib and dasatinib for 72 h, after which the percentage of apoptotic cells were determined using APO2.7 monoclonal antibody.

activity from leukemia cells expressing T315I BCR-ABL was also examined by immunoprecipitation after 6 h of treatment (Fig. 6c). Systemic administration of 50, 100, and 200 mg/kg VE-465 decreased the T315I BCR-ABL kinase activity compared to the control (Fig. 6c). These results demonstrate that VE-465 exhibits a desirable therapeutic index that can reduce the *in vivo* growth of wild-type and T315I mutant BCR-ABL-expressing cells in an efficacious manner.

Discussion

T315 serves as a gatekeeper residue that controls access to a hydrophobic region of the BCR-ABL enzymatic active site that is not contacted by ATP.[10,26] The T670I mutation in KIT and the T790M mutation in epidermal growth factor receptor are homologous to the T315I mutation in BCR-ABL, and all three mutations confer resistance to ATP-competitive kinase inhibitors.[27,28] Mutations of this residue can have profound effects on small-molecule binding in the context of different kinases, yet the mutations do not inactivate kinase function.[29] The T315I mutation in BCR-ABL confers resistance not only to imatinib, but also to all other second-generation ATP-competitive BCR-ABL inhibitors described so far, including dasatinib and nilotinib.[12,30] Clearly, the T315I mutation in BCR-ABL is predicted to be highly resistant to these agents and may drive the majority of acquired-resistance cases in the near future.

The Aurora kinase inhibitor MK-0457 is able to bind both wild-type and mutated BCR-ABL and has been reported to inhibit T315I BCR-ABL in primary patient cells at low micromolar concentrations.[31] Additionally, recent cocrystal studies have shown that this Y-shaped molecule engages the ABL kinase

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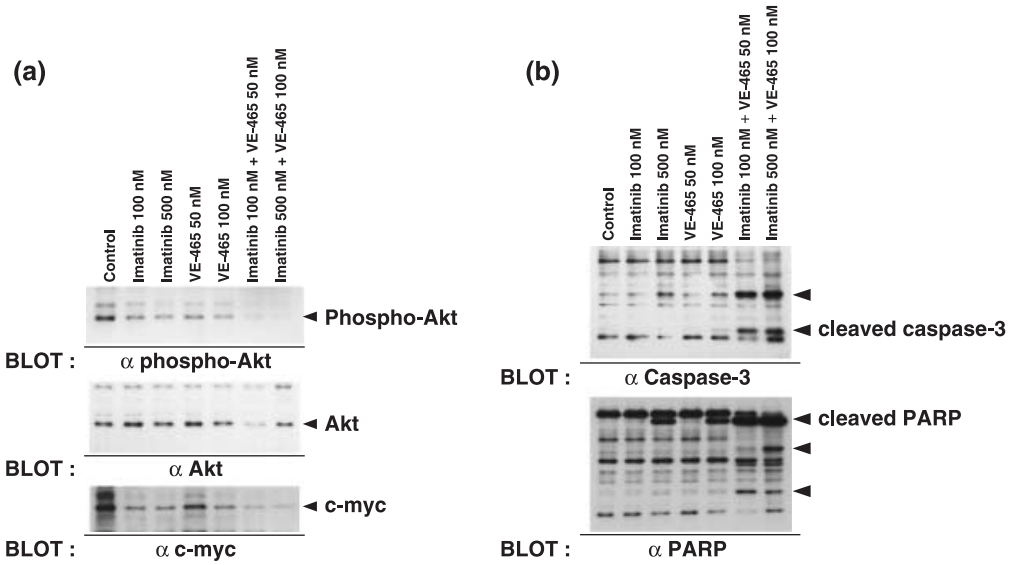

Fig. 4. Cotreatment with VE-465 and imatinib causes greater attenuation of phosphor-AKT and c-Myc, and induces caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavage. (a) K562 cells were cultured with the indicated concentrations of VE-465 or imatinib for 24 h, and the cell lysates were immunoblotted with antiphospho-Akt, anti-Akt, or c-Myc antibodies. (b) K562 cells were cultured with the indicated concentrations of VE-465 or imatinib for 24 h, and the cell lysates were immunoblotted with anticaspase-3 or anti-PARP antibodies.

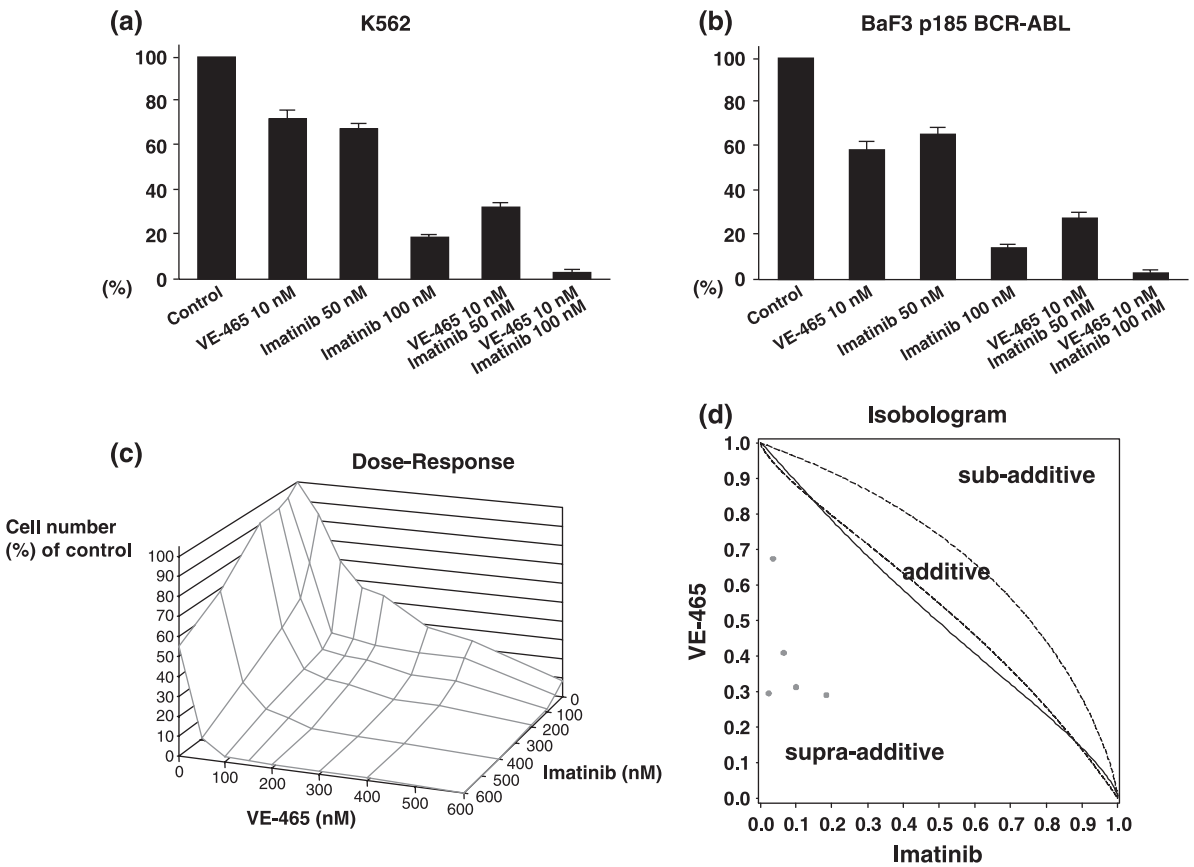

Fig. 5. Combination effects of VE-465 and imatinib in BCR-ABL-transformed cells. (a) K562 and (b) BaF3 p185BCR-ABL cells were grown in methylcellulose containing the indicated concentrations of VE-465 and imatinib. Colony counts were assessed on each individual sample at least twice, and the results are presented as average ± SD for colonies counted from triplicate plates under each condition. (c) The dose–response curve for VE-465 in combination with imatinib in K562 cells. (d) The isobologram shows the synergistic effect of simultaneous exposure to VE-465 and imatinib in K562 cells.

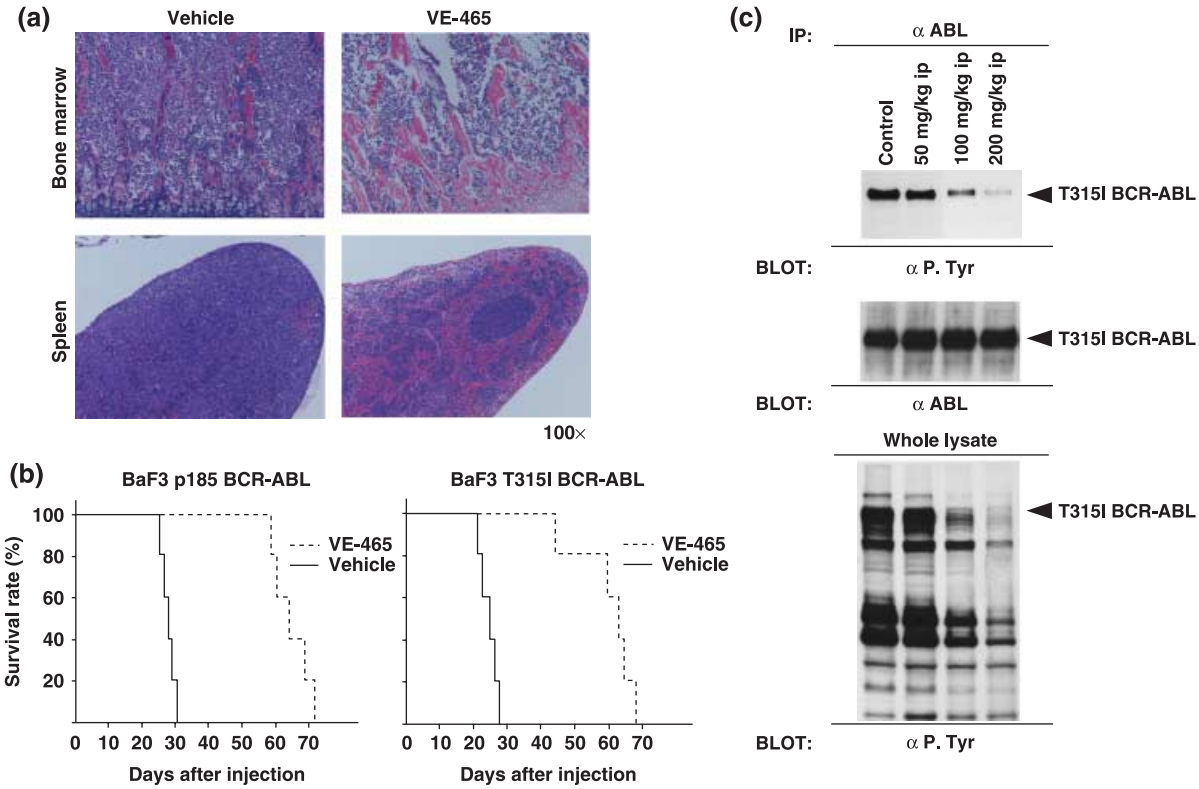

Fig. 6. *In vivo* efficacy of VE-465 in a mouse model of BCR-ABL-induced leukemia. (a) Histopathological analysis of bone marrow and spleen of mice injected with 10^6 BaF3 p185 BCR-ABL cells and treated with either VE-465 (75 mg/kg twice daily (b.i.d.); intraperitoneally (ip) for 14 days; 14 days resting) or vehicle alone. Magnification of photographs is indicated. (b) Survival of mice injected with 10^6 BaF3 p185 BCR-ABL cells or BaF3 T315I BCR-ABL cells and treated with either vehicle alone or VE-465 (75 mg/kg b.i.d.; ip for 14 days; 14 days resting). All mice that did not receive VE-465 died of a condition resembling acute leukemia by 28 days; nearly all mice treated with VE-465 survived for more than 56 days. (c) VE-465 inhibited T315I BCR-ABL kinase activity *in vivo*. Mice injected with BaF3 T315I BCR-ABL cells on day 20 with vehicle alone exhibited acute leukemia, characterized by splenomegaly and circulating blasts. The T315I BCR-ABL kinase activity of leukemia cells from the spleen was examined by immunoprecipitation after 6 h VE-465 treatment. Systemic administration of 100 or 200 mg/kg VE-465 decreased T315I BCR-ABL kinase activity compared to the control. P. Tyr, phosphotyrosine.

domain in such a way that a close encounter with the gatekeeper residue is avoided, explaining why the compound is able to accommodate the substitution of threonine with isoleucine without any significant decrease in binding affinity.[31] MK-0457 is currently undergoing a phase I clinical trial in BCR-ABL-positive leukemias, and encouraging responses in patients harboring the T315I mutation have been reported.[18] However, a phase II clinical trial in the specific setting of T315I-positive leukemia revealed several non-hematological toxicities. Therefore, the development of related compounds will be important for further clinical studies.

In the present study, we investigated the activity of a novel Aurora kinase inhibitor, VE-465, against wild-type and T315I mutant BCR-ABL-expressing leukemia cells. VE-465 inhibited the proliferation of K562 cells, BaF3 p185BCR-ABL cells, and BaF3 T315IBCR-ABL cells with IC_{50} values ranging from 50 to 500 nM (Fig. 1b–d). Further, VE-465 ranging from 200 to 500 nM caused accumulation of cells in G_2/M arrest (Fig. 1f). These results suggest that the growth-inhibitory effect of VE-465 on BCR-ABL-expressing cells is caused by Aurora kinase inhibition. VE-465 inhibited wild-type and T315I mutant BCR-ABL autophosphorylation with IC_{50} values ranging from 2.0 to 5.0 μM (Figs 1,2). However, it is important to note that systemic administration of 100 and 200 mg/kg VE-465 decreased T315I BCR-ABL autophosphorylation compared with the control (Fig. 6c). These results suggest that systemic administration of 100 or 200 mg/kg VE-465 in mice may achieve low micromolar plasma VE-465 concentrations.

We have also demonstrated that combined treatment with VE-465 and imatinib is significantly more active than either agent alone against K562 and BaF3 p185BCR-ABL cells (Figs 3–5). Indeed, the isobologram indicated the synergistic effect of simultaneous exposure to VE-465 and imatinib in K562 cells (Fig. 5d). VE-465 and imatinib and the combination of these compounds suppress the expression of c-Myc protein differently (Fig. 4a). Recently, Samanta *et al*. demonstrated that signal transduction by the BCR-ABL–Jak2 network results in phosphorylation of Akt, which leads to stabilization of c-Myc and activates nuclear factor (NF)-κB to cause elevation of c-Myc transcripts.[32] Compared with treatment with either agent alone, cotreatment with VE-465 and imatinib caused more attenuation of the levels of phosho-Akt and c-Myc (Fig. 4a). A recent study also showed that Aurora kinase inhibition by MK-0457 downregulated NF-κB in the human lung cell line A549.[33] Further data are required to resolve the combined effect of VE-465 and imatinib in BCR-ABL transformed cells.

Combinations of different BCR-ABL kinase inhibitors, including imatinib, dasatinib, and VE-465, might be effective in reducing the occurrence of drug-resistant mutants. Interestingly, new evidence has shown that sequential ABL kinase inhibitor therapy in CML selects for compound drug-resistant BCR-ABL mutations, demonstrating the potential hazards of sequential kinase inhibitor therapy and suggesting a beneficial effect of combination therapy.[34] In the case of imatinib-resistant leukemia, because of the nature of resistant mechanisms, such as the emergence of BCR-ABL kinase domain point mutations, it will

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likely be of significant clinical benefit to simultaneously administer more than one BCR-ABL inhibitor to patients as a way to suppress the development of drug-resistant mutants.

In conclusion, strategies to effectively control resistance mediated by the T315I mutation represent the next major frontier in the targeted treatment of CML. Agents such as VE-465, coupled with existing ABL kinase inhibitors, may help improve survival in advanced-phase CML. Strategies aimed at greater frontline disease eradication and suppression of resistance are needed, most of which depend on further research into combination chemotherapy and next-generation BCR-ABL kinase inhibitors that are active against the T315I BCR-ABL mutant.

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