Inhibition of heat shock protein 90 prolongs survival of mice with BCR-ABL-T315I–induced leukemia and suppresses leukemic stem cells

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Development of kinase domain mutations is a major drug-resistance mechanism for tyrosine kinase inhibitors (TKIs) in cancer therapy. A particularly challenging example is found in Philadelphia chromosome–positive chronic myelogenous leukemia (CML) where all available kinase inhibitors in clinic are ineffective against the BCR-ABL mutant, T315I. As an alternative approach to kinase inhibition, an orally administered heat shock protein 90 (Hsp90) inhibitor, IPI-504, was evaluated in a murine model of CML. Treatment with IPI-504 resulted in BCR-ABL protein degradation, decreased numbers of leukemia stem cells, and prolonged survival of leukemic mice bearing the T315I mutation. Hsp90 inhibition more potently suppressed T315I-expressing leukemia clones relative to the wild-type (WT) clones in mice. Combination treatment with IPI-504 and imatinib was more effective than either treatment alone in prolonging survival of mice simultaneously bearing both WT and T315I leukemic cells. These results provide a rationale for use of an Hsp90 inhibitor as a first-line treatment in CML by inhibiting leukemia stem cells and preventing the emergence of imatinib-resistant clones in patients. Rather than inhibiting kinase activity, elimination of mutant kinases provides a new therapeutic strategy for treating BCR-ABL–induced leukemia as well as other cancers resistant to treatment with tyrosine kinase inhibitors. (Blood. 2007; 110:678-685)

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Introduction

The human Philadelphia chromosome (Ph) arises from a translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)].1 The resulting chimeric BCR-ABL oncogene encodes a constitutively activated, oncogenic tyrosine kinase that induces chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL). The BCR-ABL TKI, imatinib mesylate, induces a complete remission in CML patients,2 but is unable to completely eradicate BCR-ABL–expressing leukemic stem cells.3,4 Over time, patients frequently become drug resistant and develop progressive disease despite continued treatment.5-11 Resistance is predominantly due to emergence of kinase domain mutations. Three newly developed BCR-ABL kinase inhibitors—dasatinib,12 AP23464,13 and AMN10714—are effective against the BCR-ABL-T315I mutant.15,16 New approaches are needed to treat drug-resistant forms of CML as well as BCR-ABL–induced B-ALL, a leukemia that does not respond well to available TKIs.15,16

Heat shock protein 90 (Hsp90) is a highly conserved, constitutively expressed molecular chaperone that facilitates folding of client proteins such as BCR-ABL, and affects the stability of these proteins.17-21 When BCR-ABL contains resistance-conferring mutations, it becomes even more dependent on Hsp90 in vitro.20 We therefore evaluated the therapeutic effect of Hsp90 inhibition by using a novel water-soluble inhibitor, IPI-504,22 in drug-resistant animal models of leukemia induced by BCR-ABL–WT and T315I.

Materials and methods

Cell lines

The 32D myeloid cell line was grown in RPMI 1640 medium containing 10% FCS and 10% WEHI medium. The BaF/3 pre-B-cell line was grown in RPMI 1640 medium containing 10% FCS, 10% WEHI medium, and 50 µM 2-mercaptoethanol. To generate the BCR-ABL–expressing 32D or BaF/3 line, the cells were transduced with the BCR-ABL-WT- or BCR-ABL-T315I-IRES-GFP-MSCV retrovirus, and the BCR-ABL–expressing cells were selected by GFP sorting by fluorescence-activated cell sorter (FACS).

Histology

The lungs from the placebo- or drug-treated mice were fixed in Bouin fixative (Fisher Scientific, Pittsburgh, PA) for 24 hours at room temperature, followed by an overnight rinse in water. Ten-µm sections were stained with hematoxylin and eosin (H&E) and observed by a model DMRE compound microscope (Leica, Heidelberg, Germany). All sections were imaged with a 2.5 × PH1 objective (NPLan, NA 0.25) and 10 × PH1 objective (NPLan, NA 0.40). All images were imported into MetaMorph software (Molecular Devices, Downingtown, PA) as a series of tagged image files. All images were then constructed in Adobe Photoshop 6.0 (Adobe, San Jose, CA).

Antibodies and Western blot analysis

Antibodies against c-ABL, Hsp90, Hsp70, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein lysates were prepared by lysing cells in radioimmunoprecipitation (RIPA) buffer, and immunoprecipitation and Western blotting were carried out as described previously.23
Bone marrow transplantation/transplantation

The retroviral vector MSCV-ires-eGFP carrying the p210 BCR-ABL cDNA was used to make high-titer, helper-free, replication-defective ecotropic virus stock by transient transfection of 293T cells using the kat system, as previously described. Six- to 10-week-old wild-type BALB/c or C57BL/6 mice (The Jackson Laboratory) were used for leukemogenesis experiments. Induction of CML and B-ALL was as described previously. Briefly, to model CML, bone marrow from 5-FU-treated (200 mg/kg) donor mice was transduced twice with BCR-ABL retrovirus by coxenodation in the presence of IL-3, IL-6, and SCF. To model B-ALL, bone marrow from non-5-FU-treated donors was transduced without cytokines. Wild-type recipient mice were prepared by 900 cGy (for BALB/c) or 1150 cGy (for C57BL/6) gamma irradiation and a dose of 0.5 × 10^6 (CML) or 1.0 × 10^6 (B-ALL) cells transplanted via tail vein injection. Diseased mice were analyzed by histopathological and biochemical analyses as described previously.

Flow cytometry

Hematopoietic cells were collected from peripheral blood and bone marrow of the diseased mice, and red blood cells were lysed with NH₄Cl red blood cell lysis buffer (pH 7.4). The cells were washed with PBS, and stained with B220-PE for B cells, Gr1-APC for neutrophils, and Sca1-APC/c-kit-PE for hematopoietic stem cells. After staining, the cells were washed once with PBS and subjected to FACS analysis.

Culture of leukemia stem cells

Bone marrow cells isolated from CML mice were cultured in vitro in the presence of stemspan SFEM, SCF, IGF-2, TPO, heparin, and α-FGF as reported previously for culture of hematopoietic stem cells.

Drug treatment

IPI-504 was dissolved in a solution containing 50 mM citrate, 50 mM ascorbate, 2.44 mM EDTA, pH 3.3. Imatinib was dissolved in water. The drugs were given orally in a volume of less than 0.5 mL by gavage (50 or 100 mg/kg, every other day for IPI-504, and 100 mg/kg, twice a day for imatinib) beginning at 8 days after bone marrow transplantation, and continuing until the morbidity or death of the leukemic mice. Placebo is a solution containing 50 mM citrate, 50 mM ascorbate, 2.44 mM EDTA, pH 3.3.

Results

Inhibition of Hsp90 by IPI-504 causes BCR-ABL protein degradation

IPI-504 is the hydroquinone hydrochloride derivative of the well-described Hsp90 inhibitor, 17-AAG; the chemical structure of IPI-504 is shown in Figure 1A. To examine the effects of IPI-504 on stability of BCR-ABL protein and to test whether the degradation of BCR-ABL protein is initiated through IPI-504–mediated disassociation of BCR-ABL from Hsp90, T315I–expressing 32D cells were treated with IPI-504 (2 μM) for 30 minutes and 4 hours, respectively. Protein lysates were analyzed by Western blotting using antibodies indicated. WCL indicates whole cell lysate; IP, immunoprecipitation; and IB, immunoblotting. (C) The proteasome inhibitor PS-341 restored IPI-504–mediated depletion of BCR-ABL protein. BCR-ABL-T315I–expressing 32D cells were treated with IPI-504 (2 μM) alone or IPI-504 plus PS-341 (100 nM) for 4 or 8 hours, respectively. Protein lysates were analyzed by Western blotting using antibodies indicated. The well-described Hsp90 client, Akt, was evaluated as a positive control. Note that the cells were pretreated with PS-341 for 30 minutes prior to the cotreatment with IPI-504 and PS-341. The black lines indicate that the lanes that were not adjacent on the same original Western blotting gel were brought together to generate this figure.

Hsp90 is a therapeutic target for BCR-ABL–induced CML

An investigation of whether Hsp90 is an effective target for the treatment of CML in vivo was conducted in the bone marrow transplantation (BMT) mouse model of CML, in which bone marrow cells from BALB/c donor mice pretreated with 5-fluorouracil (5-FU) and transduced with BCR-ABL results in development of CML in BALB/c recipient mice. Mice with WT or T315I–transduced bone marrow from 5-FU–treated WT BALB/c donor mice were treated with a placebo, the Hsp90 inhibitor IPI-504, or imatinib alone, or the 2 agents in combination. All placebo-treated mice developed and died of CML within 3 weeks after BMT (Figure 2A). As expected, imatinib treatment was effective in treating WT-induced CML but not CML induced by T315I (Figure 2A). In a dose-dependent manner, treatment with IPI-504 alone significantly prolonged survival of mice with WT CML, but even more markedly prolonged survival of mice with T315I-induced CML (Figure 2A, P < .001). Inhibition of Hsp90 by IPI-504 appears to be more effective in treating CML induced by T315I than by WT BCR-ABL, consistent with results in Figure 1A and in line with previously reported results with the Hsp90 inhibitor, 17-AAG. In both cases, inhibition of Hsp90 results in degradation of mutant BCR-ABL more readily than WT. Treatment of mice with WT CML with both IPI-504 and imatinib was slightly more effective (but statistically insignificant) than with imatinib alone in prolonging survival of the mice (Figure 2A), while treatment of mice with BCR-ABL–induced CML with these 2 drugs did not further prolong survival of the mice compared with the mice treated with IPI-504 alone (Figure 2A). Prolonged survival of IPI-504–treated CML mice correlated with decreased peripheral blood BCR-ABL–expressing (GFP-positive) leukemia cells during therapy (Figure 2B, P < .001) and less splenomegaly at necropsy.
As lung hemorrhage caused by infiltration of mature myeloid leukemia cells is a major cause of death of CML mice, we further evaluated the therapeutic effect of IPI-504 on CML by examining the severity of lung hemorrhages at day 15 after BMT. Compared with placebo-treated mice, fewer hemorrhages were observed in the lungs of IPI-504–treated mice with BCR-ABL-T315I–induced CML (Figure 2D). Western blot analysis of spleen-cell lysates from the treated CML mice showed that IPI-504 reduced the levels of BCR-ABL protein in CML mice (Figure 2E).

**Hsp90 is a therapeutic target for B-ALL induced by BCR-ABL-T315I**

CML often initiates in a chronic phase and eventually progresses to a terminal blastic phase, in which either acute myeloid or acute B-lymphoid leukemia develops. Some Ph+ leukemia patients have B-ALL as their first clinical appearance. B-ALL is similar pathologically to acute B-lymphoid leukemia in the blast phase of CML. Notably, both forms of acute leukemia do not respond well to available BCR-ABL kinase inhibitors. To model B-ALL in mice, BCR-ABL–transduced bone marrow cells from donor mice that are not pretreated with 5-FU are transplanted into BALB/c mice. In this model, the malignant pre-B cells express the cell surface markers B220 and CD19, and phenotypically resemble de novo Ph+ B-ALL and lymphoid blast crisis of CML. To determine whether inhibition of Hsp90 is effective in treating WT or T315I-induced B-ALL, these mice were treated with a placebo, IPI-504 alone, imatinib alone, or the 2 agents in combination (Figure 3). All placebo-treated recipients of WT or T315I-transduced bone marrow developed and died of B-ALL within 5 to 6 weeks after BMT (Figure 3A). IPI-504 treatment did not prolong survival of CML mice with BCR-ABL-WT–induced CML (comparing between left and right panels).
Hsp90 inhibition has differential effects on BCR-ABL degradation and Hsp70 induction in myeloid and lymphoid cells in vitro

To investigate why inhibition of Hsp90 is more effective in treating CML than B-ALL (Figures 2-3), we compared the effects of treatment with IPI-504 on BCR-ABL-WT or BCR-ABL-T315I at protein level in a mouse myeloid cell line (32D) and a mouse lymphoid cell line (BaF/3) (Figure 4). BCR-ABL-expressing 32D and BaF/3 cells were treated with different concentrations of IPI-504. After treatment, levels of BCR-ABL-WT protein were dramatically decreased in 32D cells (Figure 4A), but only slightly in BaF/3 cells (Figure 4B). Compared with BCR-ABL-WT, BCR-ABL-T315I was more sensitive to IPI-504-induced degradation in both 32D and BaF/3 cells, but levels of BCR-ABL protein were decreased much more markedly in 32D cells than in BaF/3 cells (Figure 4A-B). These results indicate that inhibition of Hsp90 by IPI-504 affects BCR-ABL stability more strongly in myeloid cells than in lymphoid cells. It has been shown that the Hsp90 antagonists geldanamycin and 17-AAG alter chaperone association in CML (Figure 2A), IPI-504 treatment significantly prolonged survival of mice treated with placebo (n = 8 for BCR-ABL-WT; n = 10 for BCR-ABL-T315I), IPI-504 (n = 13 for BCR-ABL-WT; n = 8 for BCR-ABL-T315I), and combination of imatinib and IPI-504 (n = 10 for BCR-ABL-WT; n = 8 for BCR-ABL-T315I) (Figure 3A). Flow cytometric evaluation of the leukemic process in IPI-504– or imatinib-treated mice with B-ALL induced by BCR-ABL-WT (left panel) or BCR-ABL-T315I (right panel). The number of circulating leukemic cells (calculated as percentage of B220+ GFP+ cells × white blood cell count) in B-ALL mice treated with placebo, imatinib, IPI-504, or the combination of imatinib and IPI-504. (Left panel) BCR-ABL-WT. (Right panel) BCR-ABL-T315I.

CML mice is higher than that in leukemic cells from B-ALL mice (Figure 4C-D). Thus, Hsp70 is not an explanation for the decreased sensitivity of B-ALL compared with CML upon IPI-504 treatment (Figures 2-3).

Inhibition of Hsp90 suppresses CML stem cells

In the BMT CML model, imatinib prolongs survival of mice with BCR-ABL–induced CML,33,39 but does not stop progression of the disease,13 partially due to the inability of imatinib to completely eradicate leukemia stem cells.40 Hematopoietic stem cells (HSCs) have been identified in the CML model by showing that the Lin–c-Kit+ Sca-1+ population is sufficient to confer leukemia in recipient mice.41 To investigate whether inhibition of Hsp90 has an...
showed that there was no change in levels of Lin−c-Kit−Sca-1+ cells, representing leukemia stem cells remaining in the culture. FACS analysis showed that compared with the untreated group, imatinib treatment did not lower the percentage and the number of leukemia stem cells, whereas IPI-504 treatment had a dramatic inhibitory effect on the stem cells (Figure 5A, P < .001). We next tested whether IPI-504 inhibits leukemia stem cells in CML mice. Mice with BCR-ABL-T315I-induced CML were treated with a placebo, imatinib, or IPI-504 for 6 days, and bone marrow cells were analyzed by FACS for GFP+Lin−c-Kit+c-KitSca-1+ cells. Consistent with the in vitro results, imatinib treatment did not lower the percentage and number of leukemia stem cells, compared with the untreated group, whereas IPI-504 treatment had a dramatic inhibitory effect on the stem cells (Figure 5B). To determine whether IPI-504 had an effect on normal HSCs in mice, WT mice were treated with IPI-504 or placebo for 2 weeks. Analysis of bone marrow from these mice showed that there was no change in levels of Lin−c-Kit−Sca-1+ cells from any treatment group (Figure 5C), indicating that IPI-504 treatment did not inhibit survival of normal HSCs.

**Inhibition of Hsp90 prevents emergence of the T315I-expressing clones over the WT clones**

The effectiveness of IPI-504 in prolonging survival of mice with CML and B-ALL induced by the T315I mutant (Figures 2-3) suggests that inhibition of Hsp90 would preferentially prevent emergence of the T315I-expressing clones over the WT clones. To test this hypothesis, studies were performed in mice bearing both populations of leukemic cells. In the first study, bone marrow cells (BMCs) from Ly 5.1 and Ly5.2 C57BL/6 mice were transduced with BCR-ABL-T315I and BCR-ABL-WT, respectively. Equal numbers of donor BMCs were mixed and transplanted into recipient mice. Mice were treated with a placebo, imatinib, or IPI-504. During the treatment, FACS analysis was performed to assess the percentages of GFP+Gr-1+Ly5.1+ (representing T315I-expressing cells) and GFP+Gr-1−Ly5.1− (representing WT-expressing cells) cells in peripheral blood of the CML mice (Figure 6A). In placebo-treated mice, the ratio between T315I- and WT-expressing cells remained unchanged, and in imatinib-treated mice, T315I-expressing cells became dominant. In contrast, with continuous treatment of IPI-504, T315I-expressing cells gradually decreased to a low level (Figure 6A). Mice treated with IPI-504 lived significantly longer than those treated with imatinib (Figure 6A). Consistent with previous data, these results indicate that inhibition of Hsp90 preferentially suppresses T315I-expressing leukemic clones over the WT-expressing clones. In the second study, BMCs from BALB/c mice were transduced with BCR-ABL-T315I and BCR-ABL-WT, respectively, and equal numbers of the transduced cells were mixed and transplanted into recipient mice. Mice were treated with a placebo, imatinib, IPI-504, or both agents (Figure 6B). Mice treated with the combination of IPI-504 and imatinib survived significantly longer than those treated with IPI-504 or imatinib alone. Results from these studies suggest that the combined use of IPI-504 and imatinib would be a viable strategy for preventing emergence of imatinib-resistant clones in the clinic.

**Other imatinib-resistant BCR-ABL mutants are also sensitive to Hsp90 inhibition**

Other resistance-conferring BCR-ABL kinase domain mutations have been observed in imatinib refractory CML patients, including E225K, M351T, and Y253F.5,9 Consistent with the increased
cells were 1:1 mixed, and 0.5 cells from C57BL/6-Ly5.2 mice were transduced by BCR-ABL-WT, and bone marrow
BCR-ABL-T315I mutation prior to therapy had no objective
trials with dasatinib revealed that patients known to have the
B-ALL harboring the BCR-ABL-T315I mutation. Recent clinical
no drugs have been effective in treating patients with CML and
secondary resistance accounting for up to 90% of cases. Currently,
mechanisms of secondary resistance have been very well character-
demonstrate that direct inhibition of Hsp90 with IPI-504
represents an alternative treatment strategy that results in
degradation of the offending BCR-ABL protein regardless of its
mutational status. Herein, we
dependency of BCR-ABL-T315I on Hsp90, IPI-504 also pro-
depends on BCR-ABL-T315I–expressing myeloid cells. Gr-1
A plausible explanation is that BCR-ABL cooperates with a
loss of Hsp90 may serve as an effective
target for treating imatinib- and dasatinib-resistant CML patients,
as well as patients with blast crisis or with Ph+ ALL. The
simultaneous use of IPI-504 and imatinib in chronic-phase CML
patients might prevent the development of imatinib-resistant clones and inhibit growth of highly proliferative leukemic cells through inhibition of BCR-ABL kinase activity, thereby providing a rationale for combination strategy. Likewise, early use of IPI-504
to suppress initial B-ALL clones may help prevent the transition of

Figure 6. Inhibition of Hsp90 by IPI-504 preferentially reduces growth of
myeloid leukemic cells harboring the BCR-ABL-T315I mutant. (A) Bone marrow
from C57BL/6-Ly5.2 mice were transduced by BCR-ABL-WT, and bone marrow
from C57BL/6-Ly5.1 mice were transduced by BCR-ABL-T315I. The transduced
cells were 1:1 mixed, and 0.5 × 10⁶ mixed cells were injected into each recipient
mouse (C57BL/6-Ly5.2). The mice were treated with a placebo (n = 10), imatinib
(100 mg/kg, twice a day) (n = 10), and IPI-504 (50 mg/kg, once every 2 days)
(n = 10), respectively, beginning at 8 days after BMT. At days 12 and 15 after BMT,
GFP+ cells viable cells in peripheral blood of the mice were analyzed for Gr-1 “Ly5.1”
cells that represented BCR-ABL-WT–expressing myeloid cells. Percentages of BCR-ABL-
T315I–expressing myeloid cells in peripheral blood of IPI-504–treated CML mice
were further analyzed at days 21 and 28 after BMT. The FACS results for one
representative mouse from each treatment group were shown. IPI-504 but not
imatinib significantly prolonged survival of the CML mice. (B) Simultaneous inhibition
of Hsp90 and BCR-ABL kinase activity with IPI-504 and imatinib significantly prolongs
survival of CML mice carrying both T315I-expressing and WT-BCR-ABL leukemia
cells. BALB/c mice were used to induce CML, and each treatment group had 10 mice.

Discussion
While the mechanism of primary resistance to imatinib and
dasatinib therapy in CML patients is poorly understood, the
mechanisms of secondary resistance have been very well charac-
terized. Kinase domain mutations represent the predominant form of
secondary resistance accounting for up to 90% of cases. Currently,
no drugs have been effective in treating patients with CML and
B-ALL harboring the BCR-ABL-T315I mutation. Recent clinical
trials with dasatinib revealed that patients known to have the
BCR-ABL-T315I mutation prior to therapy had no objective
response to treatment.16 Thus, as newer tyrosine kinase inhibitors
(TKIs) that effectively block other resistant mutations become
clinically available, the T315I mutation may become the predomi-
nant acquired resistance mutation. The challenge for development
of an effective Ph+ leukemia therapy is therefore to develop an
alternative treatment strategy that does not rely solely on kinase
domain inhibition but rather results in degradation of the offending
BCR-ABL protein regardless of its mutational status. Herein, we

Figure 7. Other imatinib-resistant BCR-ABL mutants are also sensitive to
Hsp90 inhibition. IPI-504 treatment prolonged survival of mice with CML induced by
imatinib-resistant BCR-ABL-E225K (n = 10), -M351T (n = 10), or -Y253F (n = 10).
CML to advanced B-ALL caused by the BCR-ABL-T315I mutation. While IPI-504 was active in BCR-ABL–induced B-ALL, the activity was not as pronounced as in CML. Studies to evaluate the mechanism for this difference showed that Hsp70 was more strongly induced in myeloid cells compared with lymphoid cells. Hsp70 is reported to exert antiapoptotic effects in a variety of settings and cell types, including leukemia cells that are exposed to Hsp90 inhibitors.\(^7\) In separate studies, inhibition of Hsp90 was shown to result in increased binding of BCR-ABL to Hsp70, thereby favoring proteasome-mediated degradation of BCR-ABL.\(^9,13\) Thus, on one hand, Hsp70 inhibition could counter the effects of Hsp90 inhibition, while other studies suggest that Hsp70 could have a positive influence on the ability of Hsp90 inhibition to result in degradation of BCR-ABL. Elucidation of the mechanism of differential sensitivity to Hsp90 inhibition between myeloid and lymphoid leukemia will require more extensive studies, as variation in Hsp70 induction is not likely the cause.

In summary, IPI-504 represents a novel therapeutic approach whereby inhibition of Hsp90 in CML patients and Ph\(^+\) ALL may significantly advance efforts to develop a cure for these diseases. The rationale underlying the use of IPI-504 for kinase inhibitor–resistant CML has implications for other cancers that display oncogene addiction to kinases that are Hsp90 client proteins. While resistant conferring kinase-domain mutations were originally described in CML, analogous mutations have been observed in lung cancer, gastrointestinal stromal tumor, and the hypereosinophilic syndrome with resistance to kinase inhibitor therapy.\(^7\) IPI-504 is currently in clinical trials to evaluate its potential for treating cancer that has become resistant to therapy with tyrosine kinase inhibitors such as imatinib.

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**Authorship**

Contribution: C.P. performed experiments and analyzed the data; J.B. provided reagents and helped with the paper; Y.H., L.K., and A.G. helped with the experiments; D.G. provided reagents and helped with the paper; M.R. provided reagents and helped with the paper; R.P. provided reagents; S.L. designed and performed experiments, analyzed the data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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**References**


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