Dual Inhibition of Bcr-Abl and Hsp90 by C086 Potently Inhibits the Proliferation of Imatinib-Resistant CML Cells

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Abstract

Purpose: Although tyrosine kinase inhibitors (TKI) such as imatinib provide an effective treatment against Bcr-Abl kinase activity in the mature cells of patients with chronic myelogenous leukemia (CML), TKIs probably cannot eradicate the leukemia stem cell (LSC) population. Therefore, alternative therapies are required to target both mature CML cells with wild-type (WT) or mutant Bcr-Abl and LSCs. To investigate the effect of C086, a derivative of curcumin, on imatinib-resistant cells, we explored its underlying mechanisms of Bcr-Abl kinase and heat shock protein 90 (Hsp90) function inhibition.

Experimental Design: Biochemical assays were used to test ABL kinase activity; fluorescence measurements using recombinant NHsp90, Hsp90 ATPase assay, immunoprecipitation, and immunoblotting were applied to examine Hsp90 function. Colony-forming unit, long-term culture-initiating cells (LTC-IC), and flow cytometry were used to test CML progenitor and stem cells.

Introduction

The Bcr-Abl kinase inhibitor imatinib is a standard treatment for Ph+ leukemia and has been shown to induce a complete hematologic and cytogenetic response in most patients with chronic-phase chronic myelogenous leukemia (CML; ref. 1). The use of imatinib in patients with CML has led to an enormous reduction in 5-year mortality. Despite outstanding clinical results, imatinib-resistant leukemia and clinical relapse eventually emerge. In fact, upon discontinuation of therapy, the disease aggressively relapses in the majority of patients. The mechanisms of resistance to imatinib include mutations of the Bcr-Abl kinase domain, the amplification of the bcr-abl gene, and the insensitivity of leukemia stem cells (LSC) to imatinib (2–5).

Clinically observed mutations have been identified within several regions of the Bcr-Abl kinase domain. In this study, we examined three kinase domain variants, Q252H, Y253F, and T315I, and bcr-abl gene amplification. These variants contain several functionally distinct kinase domain regions, including the nucleotide-binding P-loop (Q252H and Y253F) and two imatinib-resistant variants, Q252H and Y253F, and another new agent, ponatinib (AP24534), is a potent oral TKI that blocks native and

Results: Biochemical assays with purified recombinant Abl kinase confirmed that C086 can directly inhibit the kinase activity of Abl, including WT and the Q252H, Y253F, and T315I mutations. Furthermore, we identified C086 as a novel Hsp90 inhibitor with the capacity to disrupt the Hsp90 chaperone function in CML cells. Consequently, it inhibited the growth of both imatinib-sensitive and -resistant CML cells. Interestingly, C086 has the capacity to inhibit LTC-ICs and to induce apoptosis in both CD34+/CD38− and CD34+/CD38+ cells in vitro. Moreover, C086 could decrease the number of CD45+, CD45−CD34+CD38−, and CD45−CD34−CD38− cells in CML NOD-SCID mice.

Conclusions: Dual suppression of Abl kinase activity and Hsp90 chaperone function by C086 provides a new therapeutic strategy for treating Bcr-Abl–induced leukemia resistant to TKIs.

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Translational Relevance

Although the discovery of tyrosine kinase inhibitors targeting the Bcr-Abl kinase revolutionized chronic myelogenous leukemia therapy, resistant leukemia and clinical relapse eventually emerge. The mechanisms of resistance to imatinib include mutations of the Bcr-Abl kinase domain, the amplification of the bcr-abl gene, and the insensitivity of leukemia stem cells (LSC) to imatinib. There were reports indicating that inhibition of Hsp90 can effectively reduce the survival and proliferation of LSCs. Unfortunately, the use of Hsp90 inhibitors has been limited due to their significant hepatotoxicity in clinical trials. Here, we identified C086 as a dual inhibitor of Abl kinase activity and Hsp90 chaperone function. In addition to the inhibition of kinase activity of Bcr-Abl, the elimination of Bcr-Abl by C086 via Hsp90 inhibition provides a new therapeutic strategy for treating Bcr-Abl–induced leukemia and other cancers resistant to tyrosine kinase inhibitors.

Materials and Methods

Reagents

The PathScan Bcr/Abl Activity Assay Multiplex Western Detection and Apoptosis Antibody Sampler Kit were purchased from Cell Signaling Technology, Inc. StemSpan CC100, StemSpan Serum-Free Expansion Medium (SFEM), EasySep Human Whole Blood CD34-Positive Selection Kit, MethoCult H4434, and MyleoCult H5100 were purchased from StemCELL Technologies Inc. Wild-type (WT) and mutant ABL kinases were purchased from Millipore Corporation. The Abl kinase substrate was purchased from Enzo Life Science, Inc.

Cell culture

The 32D-WT, 32D-T315I, 32D-Q252H, and 32D-Y253F cell lines used in this study were constructed as described previously (23). These cell lines have been tested and authenticated by realtime quantitative PCR, DNA sequencing, mycoplasma detection, and cell vitality detection. Human leukemia K562 cells were obtained from the Cell Bank of the Chinese Academy of Sciences, where they were characterized by DNA fingerprinting, mycoplasma detection, and cell vitality detection. These cell lines were immediately expanded and frozen. K562 cells were cultured and passaged in RPMI 1640 containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM/L glutamine (medium A) in a 5% humidified CO2 atmosphere at 37°C. Imatinib-resistant K562/G01 cells were purchased from the Institute of Hematology, Chinese Academy of Medical Sciences, and Peking Union Medical College (Tianjin, China), where they were characterized by FISH analysis for the detection of Bcr-abl gene copies, mycoplasma detection, and cell vitality detection. These cell lines were immediately expanded and frozen such that they could be restarted every 3 to 4 months from a frozen vial of the same batch of cells. The K562/G01 cells were maintained in medium A either containing or lacking 4 μM/L imatinib.

Tyrosine kinase assay

The ABL kinase activity was tested using the Kinase-Glo Luminescence Kinase Assay Platform (Cat# V6072; Promega Corporation), which quantitated the amount of ATP remaining in the solution following a kinase reaction. The assays were performed by adding a volume of Kinase-Glo Reagent and measuring the resulting luminescence using the Thermo Scientific Varioskan Flash reader.

Colonoy-forming unit assays

Bone marrow samples were obtained with informed consent from patients with CML and from patients with non-myeloid hematologic disorders (24). All samples were handled in accordance with requirements of Institutional Ethical Review Board of FUMIL. CD34+ cells were enriched using the EasySep Human Whole Blood CD34 Positive Selection Kit. The CD34+ cells, cultured for 24 hours alone or in the presence of C086, were plated in MethoCult H4434 medium for 10 to 14 days, and the colony-forming units (CFU) were counted.

Assessment of cobblestone area-forming cell and long-term culture-initiating cell

M2–10B4 cells and SI/SI fibroblasts were established as feeder layers and then irradiated at 60 Gy (25). The mononuclear cells (MNC) from normal or CML bone marrow were treated with or
without C086 or imatinib for 24 hours and then seeded by limiting dilutions (2,700, 900, 300, 99, or 33 cells/well) on the irradiated feeder layers in MyeloCult H5100 medium supplemented with hydrocortisone at a final concentration of 1 μmol/L. The cultures were maintained at 33°C for 5 weeks, with weekly changes of half of the medium. At week 5, the percentage of wells with one or more cobblestone area-forming cells (CAFC), each containing at least five hematopoietic cells in the stromal layer, was determined using inverted phase-contrast microscopy. At the end of this culture period, the nonadherent and trypsinized adherent cell content was harvested and cultured in MethoCult H4434 medium for 10 to 14 days. The presence of hematopoietic colonies was scored and those wells that contained one or more CFUs were considered to be positive. The frequencies of CAFC and long-term culture-initiating cell (LTC-IC) were computed using the L-calc software, version 1.0 (Stem Cell Technologies).

Analysis of CD34+ leukemia progenitor/stem cell apoptosis
MNCs from CML bone marrow were treated with or without C086 for 24 or 48 hours. The cells were labeled with CD34-PE-Cy7 and CD38-PE and then analyzed by flow cytometry for apoptosis via Annexin V–APC staining.

Engraftment of human CML cells into NOD-SCID nude mice
NOD-SCID nude mice (6 weeks old) were used according to the animal protocol. All animals were handled in strict accordance with good animal practice, as defined by the Institutional Ethical Review Board of FUM. MNCs (1.5 x 10^6) from CML bone marrow were harvested and transplanted via tail vein injection into 6-week-old, sublethally irradiated (2 Gy) NOD-SCID mice (Shanghai Experimental Animal Center Chinese Academy of Sciences). Mice were euthanized after 7 weeks, and the marrow

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**Figure 1.**

C086 suppresses the kinase activity of both kinase domain mutants and WT ABL overexpression in vitro and in CML cells. A, the chemical structure of C086. The molecular weight of C086 is 504 Da. B and C, proliferation of 32D-WT, 32D-T315I, 32D-Q252H, and 32D-Y253F cell lines in the presence of increasing concentrations of (B) imatinib (IM) mesylate (0–10 μmol/L) or (C) C086 (0–3 μmol/L). Cell growth was assessed by MTT-based viability assays. The inhibition rate was calculated by comparing the cell number in the drug-treated group to that in the control group. D, C086 inhibits the autophosphorylation of WT and mutant ABL kinase activity. ABL served as a control for protein loading. E and F, the proliferation of K562 or K562/G01 cells in the presence of increasing concentrations of (E) imatinib (0–12.5 μmol/L) or (F) C086 (0–10 μmol/L). Cell growth was assessed by an MTT-based viability assay. The results represent the mean ± SEM of triplicate experiments (n = 3). The error bars represent the SEM. G, C086 inhibits tyrosine phosphorylation of Bcr-Abl and the downstream targets STAT-5 and CrkL. Western blot analysis of phospho-Bcr-Abl, phospho-STAT5, phospho-CrkL, and eIF4E (protein loading control) was performed using a Pathscan Bcr/Abl Multiplex Western Detection kit (Cell Signaling Technology).
contents of their femurs were obtained, together with their spleen cells and blood cells. Human cell engraftment was assessed by labeling with anti-human CD45-APC antibody and analyzed by flow cytometry. Mice exhibiting <1% human CD45+ cells in the bone marrow were considered nonengrafted and were excluded from the final analysis. Specific cell subsets were detected using antibodies against human CD34-PE, CD33-FITC, and CD38-PE-Cy7 (eBioscience, Inc.).

Cloning, expression, and purification of NHsp90

The recombinant vector NHsp90-pET28a was donated by a laboratory of the School of Life Sciences of Xiamen University, China (26). Transformed E. coli BL21 (DE3) were cultured in Luria-Bertani medium containing 50 mg/L kanamycin and then induced with 1 mM IPTG for 4 to 6 hours at 30°C. The collected cells were lysed, purified using an Ni2+–NTA agarose gel (GE company) column, and detected using SDS-PAGE. The expected molecular weight was 30 kDa.

Immunoprecipitation and immunoblotting analyses

Immunoprecipitation and immunoblotting analyses were performed exactly as the C086-coupled CNBr-activated Sepharose 4B beads that were mock-treated CNBr-activated Sepharose 4B beads that were incubated with anti-human CD45-APC antibody and analyzed by flow cytometry. Mice exhibiting <1% human CD45+ cells in the bone marrow were considered nonengrafted and were excluded from the final analysis. Specific cell subsets were detected using antibodies against human CD34-PE, CD33-FITC, and CD38-PE-Cy7 (eBioscience, Inc.).

Fluorescence measurements

Fluorescence measurements were performed at 293 K, 303 K, and 310 K using a Cary Eclipse (Varian) and an excitation wavelength of 280 nm (27). The excitation and emission bandwidths were 2.5 nm, and the emission spectra were recorded from 290 to 500 nm. The measurements were obtained with 2.0 mL of 5.0 μmol/L NHsp90 solution and successive additions of C086 solution from 0.5 to 50.0 μmol/L. All tests were performed in triplicate.

Hsp90 ATPase assay

The ATPase activity of in vitro–reconstituted Hsp90 was measured by the detection of free inorganic phosphate (Pi) using a PiPer Phosphate Assay kit (Molecular Probes; ref. 28). The assay measures an increase in the fluorescence absorption of an Amplex Red reagent that is proportional to the amount of Pi in the sample. All the assays were performed in triplicate.

C086-Sepharose 4B generation and in vitro C086 pulldown assay

C086 was conjugated with cyanogen bromide (CNBr)–activated Sepharose 4B (GE Healthcare; ref. 29). Briefly, C086 dissolved in coupling buffer (0.1 mol/L NaHCO3 and 0.5 mol/L NaCl) was added to 1 mL of resuspended CNBr-activated Sepharose 4B beads and incubated overnight at 4°C to facilitate the coupling of C086 to the resin. The control consisted of mock-treated CNBr-activated Sepharose 4B beads that were processed exactly as the C086-coupled CNBr-activated Sepharose 4B beads. K562 cell lysate was mixed with 250 μL of the mock-treated or C086-immobilized beads and incubated for 4 hours at 4°C with mild shaking. The bound proteins were then resolved by SDS-PAGE, followed by immunoblotting with antibodies against Hsp90.

Immunoprecipitation and immunoblotting analyses

Dynabeads Protein A/G beads were incubated with anti-Abl or anti-Hsp90 mAb at 4°C for 2 hours. After washing the Protein A/G bead–coupled antibodies, total cell lysates were added and incubated overnight at 4°C. The immunoprecipitates were washed, and the proteins were eluted with SDS sample-loading buffer and analyzed by immunoblotting. PVDF membranes were probed using anti-Abl, anti-Hsp90, anti-Hsp70, anti-p23, anti-p60Hop, anti-phospho-c-Abl, anti-phospho-Stat5, or anti–phospho-Crkl antibodies overnight at 4°C, followed by incubation with the secondary antibodies. The membranes were developed using a chemiluminescence (ECL) substrate and scanned using a Carestream Image Station System.

Downregulation of Hsp90 and Bcr-Abl with siRNA

To downregulate the expression of Hsp90 and Bcr-Abl with siRNA, 1 × 107 K562, K562/G01, or CML CD34+ cells were seeded into six-well plates. After overnight incubation, the medium was removed and replaced with transfection media containing control (non-targeting), Hsp90 (X-GWDD38609; Genchem, Inc.), or Bcr-Abl (X-GWDD38609) siRNA (final concentration, 50 nmol/L) along with Lipo 2000 transfection reagent (Dharmacon), according to the manufacturer’s protocol. After 24-hour incubation, the transfection medium was removed and replaced with cell culture medium. The cells were allowed to grow for an additional 96 hours to test cell proliferation, and the cell number of the siRNA-treated group was compared with that of the control group to calculate the inhibition rate. The knockdown of Hsp90 and Bcr-Abl was confirmed by Western blotting.

Statistical analysis

The group differences were examined using an unpaired Student t test. Differences were considered significant at P < 0.05. All of the analyses were performed using SPSS (Statistical Package for the Social Sciences) software.

Results

C086 inhibits both domain mutant and overexpressed WT ABL kinase activity in vitro and in CML cells

Mutations in Bcr-Abl or Bcr-Abl overexpression render Bcr-Abl tyrosine kinase insensitive to imatinib. As expected, the 32D cells harboring T315I, Q252H, and Y253F mutations were more resistant to imatinib when compared with the 32D cells transfected with WT Bcr-Abl (Fig. 1B). In contrast, the IC50 values for C086 in 32D cells expressing WT Bcr-Abl kinase and T315I, Q252H, and Y253F mutations were not significantly different (Fig. 1C).

To explore the relationship between the effect of C086 on the Abl kinase activity and leukemia cell growth inhibition directly, a biochemical mechanism in vitro kinase assay was performed. All the Abl kinase variants were efficiently inhibited within an IC50 range of 0.06 to 0.18 μmol/L (Table 1) when ATP was present at 0.2 μmol/L. In this assay, the catalytic activity of ABL-T315I was inhibited in the same concentration range as the other ABL mutants and WT ABL, with an IC50 of 0.067 μmol/L. Moreover, C086 exhibited strong inhibition on WT or mutant ABL kinase activity, as evidenced by the inhibition of ABL autophosphorylation (Fig. 1D) directly in vitro.

| Table 1. IC50 (μmol/L) value for C086 on ABL kinase activity in vitro |
|------------------|------------------|------------------|
|                  | ATP 0.2 μmol/L   | ATP 2 μmol/L     |
| ABL-WT           | 0.0853 ± 0.0132  | 0.1276 ± 0.0234  |
| ABL-T351         | 0.0673 ± 0.0211  | 0.1233 ± 0.0368  |
| ABL-Q252H        | 0.1852 ± 0.0534  | 0.3554 ± 0.0426  |
| ABL-Y253F        | 0.0798 ± 0.0232  | 0.0529 ± 0.0215  |

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The overexpression of WT ABL kinase is another factor in imatinib resistance. The resistant cell line K562/G01 was generated by culturing K562 cells with gradually increasing concentrations of imatinib over a period of several months. The resultant cell line was over 30-fold more resistant to imatinib than the sensitive parental cell line (Fig. 1E), and amplification of the bcr-abl gene was observed by FISH staining (Supplementary Fig. S1A; ref. 30). When K562 or K562/G01 cells were exposed to C086 at various concentrations for 48 hours, cell growth was inhibited and apoptosis was induced in a concentration-dependent manner (Fig. 1F and Supplementary Fig. S1B and S1C). In addition, there was no significant difference in the sensitivity to C086 between the imatinib-sensitive and imatinib-resistant cell lines. The IC50 values of the K562 and K562/G01 cell lines were 1.875 and 1.44 μmol/L, respectively.

Next, we examined the tyrosine phosphorylation status (Tyr245) of Bcr-Abl and the downstream substrates Stat5 and CrkL. Specifically, an immunoblot analysis of the lysates from the C086-treated cells using anti–phospho-Stat5 (Tyr694) and anti–phospho-CrkL (Tyr207) mAbs revealed a concentration-dependent reduction in the phosphorylation of these Bcr-Abl targets (Fig. 1G). Together, these data establish that the C086-mediated effects on the proliferation of CML cells correlate with a dramatic reduction in the tyrosine phosphorylation of Bcr-Abl and its downstream targets Stat5 and CrkL.

C086 physically binds to Hsp90 and influences its ATPase activity in vitro

Using structure-based drug design, C086 was synthesized and may bind to both Abl kinases and Hsp90. Thus, we examined the effects of C086 on Hsp90. First, we tested the binding of C086 to Hsp90 by the fluorescence quenching of Hsp90. His-tagged N-terminal Hsp90 (NHsp90) displayed maximal fluorescence at 322 nm upon excitation at 280 nm, and the intrinsic fluorescence of NHsp90 gradually decreased when NHsp90 was incubated with increasing concentrations of C086 (Fig. 2A). We then...
obtained the $K_D$ values at different temperatures: $8.77 \pm 0.383$ μmol/L (293 K), $7.97 \pm 0.523$ μmol/L (303 K), and $6.67 \pm 0.185$ μmol/L (310 K; Supplementary Table S1). These results suggest that C086 could interact with Hsp90.

Second, because the chaperone function of Hsp90 is dependent on ATP binding and ATP hydrolysis, we examined the influence of C086 on ATPase activity of Hsp90. We found that C086 suppressed the ATPase activity of Hsp90 in a concentration-dependent manner, with an inhibition of up to 60% at 6 μmol/L C086 (Fig. 2B).

Third, we assessed the direct physical interaction between Hsp90 and C086 using C086-conjugated Sepharose 4B beads. A C086-conjugated Sepharose pulldown assay confirmed that Hsp90 interacts physically with C086 (Fig. 2C).

C086 disrupts Hsp90 chaperone function in CML cells

To determine whether physical interaction between Hsp90 and C086 in vitro resulted in the disruption of Hsp90 chaperone function in vivo, we tested whether C086 affects the Hsp90 multi-chaperone complex in K562 cells. First, we examined the level of the Hsp90 co-chaperones in the cells after treatment with C086 and found that C086 increased Hsp70 protein expression but did not affect the expression of p23 or p60Hop (Fig. 2D). Because heat shock transcription factor (Hsf1) is inactivated by Hsp90, most of the Hsp90 inhibitors, such as GA, activate the expression of Hsf1 target genes, including Hsp70. Thus, our results indirectly suggest that C086 could inhibit Hsp90 function.

Further investigation tested the influence of C086 on the interaction of Hsp90 with other co-chaperones. As shown in Fig. 2E, the immunoprecipitation of Hsp90 pulled down Hsp70, p23, and p60Hop. After C086 or GA treatment, the amount of p60Hop protein in the immunoprecipitated Hsp90 complex decreased significantly. These data suggest that C086 acts in accordance with GA by disrupting the Bcr-Abl/Hsp90/p60Hop complex and increasing the Hsp90/Hsp70 complex in Bcr-Abl–positive K562 cells. The multi-chaperone complex also changed in parallel to these results after ABL immunoprecipitation (Fig. 2F).

Given that C086 demonstrated an inhibitory effect on Hsp90 chaperon function in CML cells, we assessed its influence on the client proteins of Hsp90 in K562 cells. Our results indicated that C086 significantly decreased the level of Bcr-Abl and Raf. Moreover, C086 inhibited the kinase activity of Erk, AKT, and Src (Fig. 2G).

Taken together, these results indicate that the C086-induced inhibition of CML cell proliferation was associated with the blockade of Hsp90 chaperon function, resulting in the down-regulation of Hsp90 client proteins and the consequent modification of signaling cascades in CML cells.

C086 induces apoptosis in imatinib-sensitive and -resistant CML cells

To clarify whether the blockade of Hsp90 function by C086 led to apoptosis in CML cells, we evaluated apoptosis induced by C086 in K562 and K562/G01 cells. In agreement with the results for the suppression of cellular Hsp90 function, apoptosis was induced in a concentration-dependent manner in both imatinib-sensitive and -resistant CML cells (Supplementary Fig. S1B and S1C).

The mitochondrial pathway of apoptosis can be examined by JC-1. These experiments demonstrated that C086 treatment led to a significant reduction in mitochondrial membrane potential (MMP), which resulted in an increase in JC-1 green fluorescence and decrease in JC-1 red fluorescence (Supplementary Fig. S2A), with a dramatic decrease in the red/green ratio (Supplementary Fig. S2B). The alteration of MMP promotes the release of cytochrome C, which, in turn, results in the cleavage of procaspases 9, 7, and 3 to generate activated caspases 9, 7, and 3 (Supplementary Fig. S2C), which then cleave PARP nuclear proteins and induce apoptosis.

In addition, C086 arrested the cell cycle at the $G_2$–$M$ phase in both K562 and K562/G01 cells (Supplementary Fig. S3).

C086 potently inhibits the growth of human leukemia progenitor/stem cells

Previous reports have suggested that Hsp90 inhibitors can eradicate CML stem and progenitor cells in murine disease models (20). To determine whether C086 has these properties and whether leukemia progenitor/stem cells from patients with CML are more sensitive to C086 than from healthy donors, CD34+ cells were isolated, and the growth inhibition effects were tested by CFU, CAFC, and LTC-IC assays. It is significant that treatment with 2.5, 5, and 10 μmol/L C086 resulted in marked suppression of leukemia progenitor cells from patients with CML by 41.3%, 58.3%, and 73.9%, respectively, in CFUs of CD34+ CML cells (Fig. 2A) and moderate suppression of progenitor cells from healthy donors by 12.8%, 31.7%, and 42.4%, respectively (Fig. 2A). Imatinib can also significantly inhibit leukemia progenitor cell growth (Fig. 2B). Trypan blue exclusion assays produced parallel results (Fig. 2C and D). These findings demonstrate that C086 exerted relatively little toxicity toward normal bone marrow progenitor cells. Given that C086 can potently inhibit imatinib-resistant K562/G01 cells, the compound might also kill progenitor cells isolated from the bone marrow of patients with imatinib-resistant CML [primary resistance, evaluated by National Comprehensive Cancer Network (NCCN) guidelines]. The results of the CFU assay demonstrated that C086 was able to efficiently suppress CFU growth of samples from imatinib-resistant patients (Fig. 2E). As expected, imatinib had much more moderate effects on the progenitor cells from resistant patients (Fig. 2F). Hence, primary CML progenitor cells from both imatinib-sensitive and imatinib-resistant patients, similar to other leukemia cells, were sensitive to C086. In addition, C086 is somewhat selective between normal and CML progenitor cells. The characteristics of patients with CML were described in Supplementary Table S2 in Supplementary Data.

Another important mechanism of imatinib resistance is LSC accumulation in patients undergoing imatinib therapy. Thus, we investigated the effects of C086 on CML stem cells and compared these effects with those of imatinib by testing week 5 CAFCs and LTC-ICs, which have been correlated with the long-term repopulating ability of hematopoietic cells. The effects of C086 and imatinib on CML LTC-ICs are depicted in Fig. 3G and H. Exposure to C086 for 24 hours resulted in a significant inhibition of the growth of CML primitive stem cells. The survival of CML and normal primitive stem cells was not affected by imatinib.

To illustrate the effect of C086 on both mature and primitive CML progenitor cells directly, CD34+CD38– and the more primitive CD34+CD38+ fractions were analyzed to determine their apoptosis ratio after culture in serum-free medium alone. C086 induced apoptosis in both the CD34+CD38– and CD34+CD38–
C086 potently inhibits the growth of human leukemia progenitor cells. A and B, colony formation of human CD34+ cells. CML (n = 3) or normal CD34+ cells were isolated and cultured with a low level of growth factors (rh Flt-3 Ligand, rh Stem Cell Factor, rh IL3, rh IL6) without inhibitors (control) or in the presence of graded concentrations of C086 or imatinib (IM) for 24 hours and then plated in methylcellulose progenitor culture (MethoCult H4434 contains Methylcellulose in Iscove’s MDM, FBS, BSA, 2-Mercaptoethanol, rh Stem Cell Factor, rh GM-CSF, rh IL3, rh Erythropoietin) for 14 days. The data are presented as the percentage of CFU suppression of CML or normal CD34+ cells compared with untreated controls. Significant differences between the CML and normal CD34+ cells (unpaired t tests; ***P < 0.001) are indicated. C and D, dead CML CD34+ cells using trypan blue exclusion assay. CD34+ cells were treated with C086 or imatinib for 24 hours and then incubated with trypan blue, and the number of dead cells taking up blue dye was counted. The results are presented as the percentage of dead cells compared with untreated controls. Significant differences between the CML and normal CD34+ cells (unpaired t tests; ***P < 0.001) are indicated.

Figure 3.

C086 treatment reduces CML stem and progenitor cell growth in vivo

Next, we evaluated the effect of C086 on CML LSCs in vivo. After CML MNCs engraftment for 2 weeks, mice were treated for 5 weeks with C086 (200 mg/kg/day intravenously). C086-treated mice did not lose weight (data not shown) compared with control mice, and no hepatotoxicity was observed (data not shown). Mice exhibiting <1% human CD45+ cells in the bone marrow were considered nonengrafted and were excluded from the final analysis.

C086 significantly reduced human CD45+ cells (P < 0.001) in the bone marrow, spleen, and peripheral blood from CML MNC-engrafted mice at 7 weeks after transplantation (Fig. 4F). Furthermore, human CD45+CD33+, CD45+CD34+CD38+, and CD45+CD34+CD38− cells in the bone marrow, spleen, and peripheral blood were decreased after C086 treatment (Fig. 4G–I). In
addition, human CD45<sup>+</sup>, CD45<sup>+</sup>CD33<sup>+</sup>, CD45<sup>+</sup>CD34<sup>+</sup>CD38<sup>+</sup>, and CD45<sup>+</sup>CD34<sup>+</sup>CD38<sup>−</sup>/C0<sup>−</sup> cells in the bone marrow, spleen, and peripheral blood from normal MNC-engrafted mice were not affected by C086 (Supplementary Fig. S5). These results indicate that C086 is, indeed, reducing the maintenance of CML LSCs and their repopulating capacity in vivo while sparing normal hematopoiesis.

Downregulation of Hsp90 and Bcr-Abl with siRNA confirms the essential role of both targets for CML cell survival

To further validate the essential role of Hsp90 and Bcr-Abl in CML cell survival, we transfected siRNA targeting Hsp90 and Bcr-Abl into K562, K562/G01, or CML CD34<sup>+</sup> cells. The expression of each transgene was confirmed by Western blotting (Fig. 5A). Downregulation of either Hsp90 or Bcr-Abl expression significantly inhibited the growth of CML cells (Fig. 5B–D). Moreover, combined knockdown of Hsp90 and Bcr-Abl reduced cell proliferation to a greater extent than either knockdown alone. These results, along with data from the C086 experiments, confirmed that dual inhibition of Hsp90 and Bcr-Abl is more efficient than inhibition of either gene alone.

Discussion

Here, we report for the first time that C086 can efficiently inhibit both Abl kinase activity and Hsp90 chaperone function. According to our findings, C086 is able to suppress LSCs, a property that renders this compound more effective than Abl kinase inhibition alone. Although the parent compound curcumin is a multitargeted anticancer agent interfering HER2, tumor necrosis factor, EGFR, and Bcr-abl, the poor bioavailability of
curcumin has limited its clinical application (31, 32). To improve the activity and solubility of curcumin, we designed and synthesized C086. However, further investigation is required to determine whether C086 inhibits multiple signaling pathways like its parent compound.

Although newly approved third-generation TKI, ponatinib/AP24534, was found to be highly active in heavily pretreated patients with resistance to TKIs, including patients with the Bcr-Abl T315I mutation (14), three new Bcr-Abl mutations were discovered in 2012: L248R, T315V, and F317R (33). These new mutations confer high resistance to imatinib, bosutinib, dasatinib, and nilotinib and intermediate resistance to ponatinib (33). Thus, although third-generation TKIs are encouraging for patients affected by Ph+ leukemias, clinicians need to be aware of the extreme heterogeneity in advanced cancers, in which millions of clones with different mutations can develop. Therefore, targeting more than a single pathogenic event in a highly heterogeneous cancer could produce better results. Furthermore, in addition to the Abl kinase mutations contributing to imatinib resistance, the accumulation of LSCs plays a critical role in CML relapse after imatinib therapy (4, 5). Importantly, TKIs can effectively target proliferating mature cells but do not eradicate quiescent LSCs, thereby allowing disease persistence despite treatment. Accordingly, it is essential to develop alternative strategies to target the LSCs population.

There are three potential origins of LSCs: (i) normal hematopoietic stem cells that can mutate to LSCs, (ii) leukemia-committed progenitors that can dedifferentiate to become LSCs, and (iii) LSCs that can also result from differentiated mature leukemia cells that reacquire the stem cell capability of self-renewal (Supplementary Fig. S5; refs. 34, 35). Thus, the only way to cure leukemia is to eradicate differentiated mature leukemia cells, leukemia progenitors, and LSCs simultaneously. In the present study, C086 was demonstrated to be capable of suppressing human leukemia in different mature states, as observed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), CFU, and LTC-IC assays.

The LTC-IC assay is recognized as the most stringent method to detect very primitive human hematopoietic stem cells in vitro. Compared with the controls, the addition of 10 μmol/L C086 significantly reduced the number of LTC-ICs compared with the no drug control. Moreover, C086 induced apoptosis in both CD34+CD38− and CD34+CD38+/C0 cells from patients with CML, and it effectively inhibited CML LSCs in vivo.

Recently, several new targets or drugs have been reported to inhibit LSCs in cultured human CD34+ CML cells or in mouse models of Bcr-Abl–induced CML, including Hsp90 inhibitors, an Alox5 pathway inhibitor, and BMS-214662 (36–39). Hsp90 is a chaperone of several oncoproteins and facilitates the folding of a variety of client proteins, such as Bcr-Abl, Her2, and Raf, affecting the stability of these proteins. Interestingly, when Bcr-Abl is mutated, it becomes even more dependent on Hsp90 in vitro (20). In 1994, the first Hsp90 inhibitor was identified, and Hsp90 was reported to be a target for anticancer therapeutics. In the last decade, there have been 17 distinct Hsp90 inhibitors entered into clinical trials.
Hsp90 inhibitor has achieved regulatory approval, recently, there has been significant progress in Hsp90 inhibitor clinical development (40).

Interestingly, we identified C086 as a novel Hsp90 inhibitor. C086 could physically bind to Hsp90 and influence its ATPase activity in vitro, consistently, C086 demonstrated the capacity to disrupt Hsp90 chaperone function in CML cells, causing the subsequent degradation of Hsp90 client proteins (Bcr-Abl and Raf) in K562 cells. Our findings support previous results indicating that, in addition to inhibiting CML cells that harbor mutations or Bcr-Abl overexpression, C086 is able to kill leukemia progenitor/stem cells.

In conclusion, we identified C086 as a dual inhibitor of Abl kinase activity and Hsp90 chaperone function. As opposed to merely inhibiting the kinase activity of Bcr-Abl, the elimination of Bcr-Abl by C086 via Hsp90 inhibition provides a new therapeutic strategy for treating Bcr-Abl–induced leukemia, such as Ph+ acute lymphoblastic leukemia and as other cancers resistant to treatment with TKIs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Wu, J. Yu, R. Chen, L. Huang, J. Xu
Writing, review, and/or revision of the manuscript: L. Wu, Y. Chen, J. Xu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Wu, J. Xu
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Other (conducted experiments): L. Lou

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