Dual inhibition of Bcr-Abl and Hsp90 by C086 potently inhibits the proliferation of imatinib-resistant CML cells

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TRANSLATIONAL RELEVANCE

Although the discovery of tyrosine kinase inhibitors targeting the Bcr-Abl kinase revolutionized chronic myeloid 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 (LSCs) 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 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.
Abstract

Purpose: Although such tyrosine kinase inhibitors (TKIs) as imatinib provide an effective treatment against Bcr-Abl kinase activity in the mature cells of CML patients, 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 (CFU), long-term culture-initiating cells (LTC-ICs), and flow cytometry were used to test CML progenitor and stem cells.

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, 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 decreased 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.
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 chronic-phase chronic myeloid leukemia (CML) patients (1). The use of imatinib in CML patients 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 \textit{bcr-abl} gene, and the insensitivity of leukemia stem cells to imatinib (2-5).

Clinically observed mutations have been identified within several regions of the Bcr-Abl kinase domain. In this study, we examined 3 kinase domain variants, Q252H, Y253F, and T315I, and \textit{bcr-abl} gene amplification. These variants contain several functionally distinct kinase domain regions, including the nucleotide-binding P-loop (Q252H and Y253F) and 2 imatinib mesylate contact residues (Y253F and T315I).

There is considerable interest in developing alternative Abl kinase inhibitors capable of inhibiting the Bcr-Abl kinase domain mutants observed in relapsed patients. The second-generation tyrosine kinase inhibitors (TKIs), such as nilotinib and dasatinib, are able to override the majority of the mutations conferring resistance to imatinib, with the exception of the T315I mutation (6-11). GNF-2, a selective allosteric Bcr-Abl inhibitor, binds to the myristate-binding site of Abl, leading to changes in the structural dynamics of the ATP-binding site and rendering Abl unaffected by kinase mutations (12, 13). Another new
agent, ponatinib (AP24534), is a potent oral TKI that blocks native and mutated Bcr-Abl, including the gatekeeper mutant T315I, which is uniformly resistant to TKIs (14).

Although the discovery of TKIs targeting the Bcr-Abl kinase dramatically decreased disease burden, these drugs probably can not eliminate quiescent LSCs, allowing disease relapse. Therefore, it is essential that alternative strategies are developed to target the LSC population.

Based on its chaperone function in regulating many tumor-related client proteins, Hsp90 is a promising target for chemotherapy (15-18). Gorre et al. reported that Bcr-Abl point mutants isolated from patients with imatinib-resistant CML remain sensitive to the Hsp90 inhibitors geldanamycin (GA) and 17-allylaminogeldanamycin (17-AAG) (19). Moreover, Peng et al. reported that IPI-504, an Hsp90 inhibitor, had a dramatic inhibitory effect on these LSCs. This result indicates that the inhibition of Hsp90 can effectively reduce the survival and proliferation of LSCs (20). Unfortunately, the use of Hsp90 inhibitors has been limited due their significant hepatotoxicity in clinical trials (21). In an effort to identify new inhibitors that are safe for humans and to overcome the resistance to TKIs caused by Bcr-Abl mutations and LSCs, we used structure-based drug design and focused on synthetic libraries of curcumin analogues. Using a docking model, we identified C086 ((1E,6E)-4-(4-hydroxy-3-methoxybenzyl)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione) as a potent novel inhibitor binding to both Abl kinases and Hsp90. It is a new structural analog with the molecular weight 504 Daltons that was synthesized by our laboratory. We incorporated an active group (4-hydroxy-3-methoxy-phenyl-methyl) chain into the β-diketone structure at position 4 (Figure 1A), while retaining the β-diketone structure and
natural aryl pattern of curcumin. Our previous work showed that C086 exhibited a significant antiproliferative effect in a mouse xenograft model of SW480 cells while maintaining low toxicity (22). Here, we demonstrate for the first time that C086 can overcome imatinib resistance resulting from either Abl kinase mutation or LSC accumulation through dual inhibition of Bcr-Abl kinase activity and Hsp90 chaperone function.

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. (MA, USA). StemSpan® CC100, StemSpan® Serum-Free Expansion Medium (SFEM), EasySep® Human Whole Blood CD34-Positive Selection Kit, MethoCult® H4434, and MyeloCult® H5100 were purchased from STEMCELL Technologies Inc. (BC, Canada). Wild type and mutant ABL kinases were purchased from Millipore Corporation (MA, USA). The Abl kinase substrate was purchased from Enzo Life Science, Inc. (NY, USA).

Cell culture

The 32D-WT, 32D-T315I, 32D-Q252H, and 32D-Y253F cell lines used in this study were constructed as described previously (23). The cell lines have been tested and authenticated by real-time quantitative PCR, DNA sequencing, mycoplasma detection, and cell vitality detection. Human leukemic K562 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) 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 fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM 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 imatinib.

**Tyrosine kinase assay**

The ABL kinase activity was tested using the Kinase-Glo Luminescent Kinase Assay Platform (Cat# V6072, Promega Corporation, WI, USA), 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.

**Colony-forming unit (CFU) assays (24)**

Bone marrow samples were obtained with informed consent from patients with CML and from patients with non-myeloid hematologic disorders. All samples were handled accordance with requirements of Institutional Ethical Review Board of FMU. CD34+ cells were enriched using the EasySep® Human Whole Blood CD34 Positive Selection Kit. The CD34+ cells, cultured for 24 h alone or in the presence of C086, were plated in MethoCult® H4434 medium for 10-14 days, and the CFUs were counted.
Assessment of CAFC and LTC-IC (25)

M2-10B4 cells and SI/SI fibroblasts were established as feeder layers and then irradiated at 60 Gy. The mononuclear cells (MNCs) from normal or CML bone marrow were treated with or without C086 or imatinib for 24 h and then seeded by limiting dilutions (2700, 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 μM. 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 non-adherent and trypsinized adherent cell content was harvested and cultured in MethoCult® H4434 medium for 10-14 days. The presence of hematopoietic colonies was scored and those wells that contained one or more CFU were considered to be positive. The frequencies of CAFC and LTC-IC were computed using the L-calc software, version 1.0 (Stem Cell Technologies, Vancouver, BC, Canada).

Analysis of CD34+ leukemia progenitor/stem cell apoptosis

MNC cells from CML bone marrow were treated with or without C086 for 24 h or 48 h. 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 FMU. 1.5×10^7 MNCs from CML bone marrow were harvested and
transplanted via tail vein injection into 6-week-old, sub-lethally irradiated (2 Gy) NOD-SCID mice (Shanghai Experimental Animal Center Chinese Academy of Sciences, Shanghai, China). Mice were euthanized after 7 weeks, and the marrow 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, San Diego, CA).

**Cloning, expression, and purification of NHsp90 (26)**

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

**Fluorescence measurements (27)**

Fluorescence measurements were performed at 293 K, 303 K, and 310 K using a Cary Eclipse (Varian) and an excitation wavelength of 280 nm. 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 μM NHsp90 solution and successive additions of C086 solution from 0.5 μM to 50.0 μM. All tests were performed in triplicate.

**Hsp90 ATPase assay (28)**

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). 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 (29)**

C086 was conjugated with cyanogen bromide (CNBr)-activated Sepharose 4B (GE Healthcare). Briefly, C086 dissolved in coupling buffer (0.1 M NaHCO3 and 0.5 M 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 h 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 h. 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 small interfering RNA (siRNA)**

To down-regulate the expression of Hsp90 and Bcr-Abl with siRNA, 1×10^5 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, Genechem, Inc., Shanghai, China), or Bcr-Abl (X-GWDD38609) siRNA (final concentration 50 nM) along with Lipo 2000 transfection reagent (Dharmacon), according to the manufacturer’s protocol. After 24 h incubation, the transfection medium was removed and replaced with cell culture medium. The cells were allowed to grow for an additional 96 h 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 in ABL kinase were more resistant to imatinib when compared to the 32D cells transfected with WT Bcr-Abl (Figure 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 (Figure 1C).
To explore the relationship between the effect of C086 on the ABL kinase activity and leukemic 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 µM (Table 1) when ATP was present at 0.2 µM. 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 µM. Moreover, C086 exhibited strong inhibition on WT or mutant ABL kinase activity, as evidenced by the inhibition of ABL autophosphorylation (Figure 1D) directly in vitro.

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 (Figure 1E), and amplification of the bcr-abl gene was observed by FISH staining (Supplementary Figure S1A) (30). When K562 or K562/G01 cells were exposed to C086 at various concentrations for 48 h, cell growth was inhibited and apoptosis was induced in a concentration-dependent manner (Figure 1F and Supplementary Figure S1B and C). 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 µM and 1.44 µM, 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) monoclonal antibodies revealed a concentration-dependent reduction in the
phosphorylation of these Bcr-Abl targets (Figure 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 (Figure 2A). We then obtained the $K_D$ values at different temperatures: 8.77±0.383 μM (293 K), 7.97±0.523 μM (303 K), and 6.67±0.185 μM (310 K) (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 μM C086 (Figure 2B).

Third, we assessed the direct physical interaction between Hsp90 and C086 using C086-conjugated Sepharose 4B beads. A C086-conjugated Sepharose pull-down assay confirmed that Hsp90 interacts physically with C086 (Figure 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 multichaperone 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 p60\(^{\text{Hop}}\) (Figure 2D). Because heat shock transcription factor (Hsf1) is inactivated by Hsp90, most of the Hsp90 inhibitors, such as geldanamycin (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 Figure 2E, the immunoprecipitation of Hsp90 pulled down Hsp70, p23, and p60\(^{\text{Hop}}\). After C086 or GA treatment, the amount of p60\(^{\text{Hop}}\) protein in the immunoprecipitated Hsp90 complex decreased significantly. These data suggest that C086 acts in accordance with GA by disrupting the Bcr-Abl/Hsp90/p60\(^{\text{Hop}}\) complex and increasing the Hsp90/Hsp70 complex in Bcr-Abl-positive K562 cells. The multichaperone complex also changed in parallel to these results after ABL immunoprecipitation (Figure 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 (Figure 2G).

Taken together, these results indicate that the C086-induced inhibition of CML cell proliferation was associated with the blockage 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 blockage 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 Figure S1 B and C).

The mitochondrial pathway of apoptosis can be examined by JC-1. These experiments demonstrated that C086 treatment led to a significant reduction in MMP, which resulted in an increase in JC-1 green fluorescence and decrease in JC-1 red fluorescence (Supplementary Figure S2 A), with a dramatic decrease in the red/green ratio (Supplementary Figure S2 B). 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 Figure S2 C), which then cleave PARP nuclear proteins and induce apoptosis.

In addition, C086 arrested the cell cycle at the G2/M phase in both K562 and K562/G01 cells (Supplementary Figure 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 CML patients 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 μM C086 resulted in marked suppression of leukemia progenitor cells from CML patients by 41.3%, 58.3%, and 73.9%, respectively, in CFUs of CD34+ CML cells (Figure 2A) and moderate suppression of progenitor cells from healthy donors by 12.8%, 31.7%, and 42.4%, respectively (Figure 2A). Imatinib can also significantly inhibit leukemia progenitor cell...
growth (Figure 2B). Trypan blue exclusion assays produced parallel results (Figure 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 imatinib-resistant CML patients (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 (Figure 2E). As expected, imatinib had much more moderate effects on the progenitor cells from resistant patients (Figure 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 CML patient characteristics were described in Table S2 in supplemental data.

Another important mechanism of imatinib resistance is leukemia stem cell 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 Figure 3G and H. Exposure to C086 for 24 h 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 IM.

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 SFM alone. C086 induced apoptosis in both the CD34+CD38+ and CD34+CD38- fractions (Figure 4 A-C), highlighting the activity of this drug in both quiescent and cycling cell populations, which was confirmed by CFSE (carboxyfluorescein diacetate succinimidyl ester) staining (Figure 4 D and E). In addition, C086 is somewhat selective between normal and CML stem cells (Figure 4 C and E).

**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 to 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 (BM), spleen, and peripheral blood (PB) from CML MNCs engrafted mice at 7 weeks after transplantation (Figure 4 F). Furthermore, human CD45+CD33+, CD45+CD34+CD38+, and CD45+CD34+CD38- cells in the BM, spleen, and PB were decreased after C086 treatment (Figure 4 G-I). In addition, human CD45+, CD45+CD33+, CD45+CD34+CD38+, and CD45+CD34+CD38- cells in the BM, spleen, and PB from normal MNCs engrafted mice were not affected by C086 (Supplementary Figure 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 confirm 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+ cells. The expression of each transgenes was confirmed by western blotting (Figure 5A). Downregulation of either Hsp90 or Bcr-Abl expression significantly inhibited the growth of CML cells (Figure 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 multi-targeted 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/AP-24534, 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 leukemia stem cells, (ii) leukemia-committed progenitors that can dedifferentiate to become leukemia stem cells, and (iii) LSCs that can also result from differentiated mature leukemia cells that reacquire the stem cell capability of self-renewal (Supplementary Figure S5) (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 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 to the controls, the addition of 10 μM C086 significantly reduced the number of LTC-ICs compared to the no drug control.
Moreover, C086 induced apoptosis in both CD34+CD38+ and CD34+CD38- cells from CML patients, 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. Although no 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+ ALL (acute lymphoblastic leukemia), and as
other cancers resistant to treatment with tyrosine kinase inhibitors.

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AUTHORSHIP CONTRIBUTIONS

J Xu, L Wu, and Y Chen designed experiments, analyzed results, and wrote the manuscript; Y Liu designed and synthesized the compound; J Yu, R Chen, L Lou, Y Wu, and L Huang performed experiments and statistical analysis; M Huang and Y Wu obtained human bone marrow samples according to the ethics requirements of FMU and analyzed results.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).
<table>
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<th></th>
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<th>ATP 2 μM</th>
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<td>0.0798±0.0232</td>
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REFERENCES


2007;131:257-70.


Figure Legends

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, C) Proliferation of 32D-WT, 32D-T315I, 32D-Q252H, and 32D-Y253F cell lines in the presence of increasing concentrations of (B) imatinib mesylate (0-10 μM) or (C) C086 (0-3 μM). 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, F) The proliferation of K562 or K562/G01 cells in the presence of increasing concentrations of (E) imatinib (0-12.5 μM) or (F) C086 (0-10 μM). 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 blotting 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).

Figure 2. C086 physically binds to Hsp90 and disrupts its Hsp90 chaperone function. (A) The fluorescence quenching spectra of NHsp90 with C086 at different temperatures. Upper panel: The concentration of NHsp90 was fixed at 5 μM, and the ratio of C086 vs NHsp90 was from 1:1 to 10:1. Lower panel: The variation tendency of ΔF at different temperatures. (B) The ATPase activity of NHsp90 was suppressed by C086. (C) C086 binds to Hsp90. Whole-cell lysates were incubated with C086-Sepharose 4B. The levels of bound HSP90 in
the precipitates were monitored by western blotting analysis. (D) Effects of C086 on Hsp protein expressions in K562 cells. C086 treatment significantly up-regulated the expression of Hsp70. (E) Effects of C086 on the Hsp90/Hsp70/p23/p60Hop complex in K562 cells. K562 cells were treated with C086 or GA for 24 h and then harvested to extract the total proteins. The cell lysates were immunoprecipitated with anti-Hsp90 antibodies and then detected by western blotting with antibodies against Bcr-Abl, Hsp90, Hsp70, p60Hop, or p23. (F) Treatment with C086 for 24 h altered the composition of the multimolecular chaperone complex associated with Bcr-Abl in K562 cells. K562 cell lysates were immunoprecipitated with anti-c-Abl antibodies. The co-precipitation of members of the Hsp90 multimolecular complex was detected by western blotting with antibodies against Hsp90, Hsp70, p60Hop, or p23. (G) The effects of C086 on the degradation of Hsp90 client proteins in K562 cells. K562 cells were exposed to increasing concentrations of C086 for 24 h, and the level of client proteins, Bcr-Abl, Raf, p-Erk, Akt, p-Akt, and p-Src, was detected by western blotting.

**Figure 3. C086 potently inhibits the growth of human leukemia progenitor cells.** (A, 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 IL-3, rh IL-6) either without inhibitors (control) or in the presence of graded concentrations of C086 or imatinib (IM) for 24 h and then plated in methylcellulose progenitor culture (MethoCult® H4434 contains Methylcellulose in Iscove's MDM, Fetal Bovine Serum, Bovine Serum Albumin, 2-Mercaptoethanol, rh Stem Cell Factor, rh GM-CSF, rh IL-3, rh Erythropoietin) for 14 days. The data are presented as the percentage of CFU suppression of CML or normal CD34+ cells compared to untreated controls. Significant differences between
the CML and normal CD34+ cells (unpaired t tests; ***P<0.001) are indicated. (C, D) Dead CML CD34+ cells using trypan blue exclusion assay. CD34+ cells were treated with C086 or IM for 24 h 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 to untreated controls. Significant differences between the CML and normal CD34+ cells (unpaired t tests; ***P<0.001) are indicated. (E, F) Colony formation of imatinib-resistant CML CD34+ cells. The data are presented as the percentage of CFU suppression compared to untreated controls. Significant differences between the CML and normal CD34+ cells (unpaired t tests; ***P<0.001, *P>0.05) are indicated. The results represent the mean±SEM of triplicate experiments (n = 3). The error bars represent the SEM. (G, H) Frequency of CAFC and LTC-IC in normal or CML MNCs from human bone marrow. MNCs from CML or normal bone marrow were exposed to IM (0.4 μM) or C086 (2.5 or 5 μM) for 24 h. The cells were then seeded onto pre-formed, irradiated normal bone marrow feeder layers in 96-well plates at 5 different dilutions, with 24 replicates per dilution. After culture for 5 weeks as described in Materials and Methods, individual wells were scored as positive or negative for the presence of a CAFC consisting of 5 or more tightly packed phase-dark stromal embedded cells. After calculation of the percentage of negative wells at each dilution, limiting dilution analysis was used to estimate the frequency of CAFC/10^5 MNCs (G). To estimate the frequency of functional LTC-IC, the entire contents of the culture wells were placed in individual enriched methylcellulose for the CFU assay. After 14 days of incubation, the wells were scored as positive or negative for the presence of CFC, and the frequency of LTC-IC was estimated by limiting dilution analysis (H). The indicated mean±SEM graphed
for CML or normal cells is based on replicate experiments (CML, n=5; normal, n=3). The error bars represent the SEM. Significant differences between the control and C086 treatment groups (unpaired t tests; ***P<0.001, *P>0.05) are indicated.

**Figure 4. C086 induces apoptosis in CML primitive and committed progenitors in vitro and suppresses engraftment of CML cells in NOD-SCID mice.** (A-C) Apoptosis of CML (A-B) or normal (C) CD34^+CD38^- primitive and CML CD34^+CD38^+ committed progenitor induced by C086. CML CD34^+CD38^- primitive and CD34^+CD38^+ committed progenitors were exposed to C086 at the indicated concentrations for 24 (A) or 48 h (B). Apoptosis was analyzed by FACS as the percentage of cells positively labeled with Annexin V-APC. (D, E) Apoptosis of divided CD34^+CFSE^{low} and undivided CD34^+CFSE^{high}. Significant differences between the control and C086 treatment groups (unpaired t tests; ***P<0.001) are indicated. The results represent the mean±SEM of triplicate experiments (CML, n = 3). (F-I) C086 suppresses engraftment of CML MNCs in NOD-SCID mice. MNCs (1.5×10^7 cells per mouse) were transplanted into NOD-SCID mice. Mice were euthanized after 7 weeks, and the marrow content of the femurs was obtained, together with spleen cells and blood cells. The graphs show human cell engraftment in peripheral blood (PB), BM, and spleen at 7 weeks post-transplantation. Significant differences between the control and C086 treatment groups (n = 3, unpaired t tests; ***P<0.001) are indicated.

**Figure 5. RNA interference in CML cells.** (A) siRNA reduced the expression of Hsp90 and bcr-abl proteins in K562, K562/G01, and CML CD34+ cells. (B-D) Effects of siRNA on cell proliferation. Viable cells were counted by trypan blue exclusion assays. The inhibition rate was calculated by comparing the cell number of the siRNA treated group to that of the control.
The results represent the mean±SEM of triplicate experiments. The error bars represent the SEM.
Fig. 1
Fig. 3
Fig. 4
Clinical Cancer Research

Dual inhibition of Bcr-Abl and Hsp90 by C086 potently inhibits the proliferation of imatinib-resistant CML cells

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