A systems biology understanding of the synergistic effects of arsenic sulfide and Imatinib in BCR/ABL-associated leukemia

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In this study, we show that combined use of Imatinib (IM) and arsenic sulfide [As2S4 (AS)] exerts more profound therapeutic effects in a BCR/ABL-positive mouse model of chronic myeloid leukemia (CML) than either drug as a single agent. A systematic analysis of dynamic changes of the proteome, phosphoproteome, and transcriptome in K562 cells after AS and/or IM treatment was performed to address the mechanisms underlying this synergy. Our data indicate that AS promotes the activities of the unfolded protein reaction (UPR) and ubiquitination pathway, which could form the biochemical basis for the pharmacological effects of this compound. In this CML model, AS targets BCR/ABL through the ubiquitination of key lysine residues, leading to its proteasomal degradation, whereas IM inhibits the PI3K/AKT/mTOR pathway. Combination of the 2 agents synergistically arrests the cell cycle, decreases activity of BCR/ABL, and leads to activation of intrinsic and extrinsic apoptosis pathways through complex modifications to both transcription and protein levels. Thus, these results suggest potential clinical benefits of IM/AS combination therapy for human CML.

Up to 95% of chronic myeloid leukemia (CML) patients harbor the (9;22) chromosomal translocation that gives rise to expression of the 210-kDa BCR/ABL fusion protein and its associated aberrant protein tyrosine kinase (PTK) activity. BCR/ABL aberrantly phosphorylates a large number of substrates, leading to activation of many downstream effectors including those that confer antiapoptotic and growth advantages to CML cells. Research evidence suggests that abnormalities in protein translation, modification (mainly phosphorylation), and degradation play critical roles in initiation, development, blast crisis (BC), and induction of drug-resistance in CML (1). Thus, these abnormalities should be targeted when designing novel strategies for the treatment of CML. Imatinib (IM) (STI571; Gleevec) was designed to selectively inhibit the abnormal PTK activity of BCR/ABL and is now a standard treatment for CML for inducing cytogenetic and molecular remission. However, resistance to IM is commonly observed in patients at accelerated phase (AP) and BC and can also occur during the chronic phase after long-term exposure to this drug (2). Thus, the development of novel targeted therapeutic agents or use of IM in combination with other drugs to improve response rates and overcome drug-resistance is required (3). Arsenic compounds such as arsenic trioxide (As2O3) and arsenic sulfide (As2S4, AS) have been used in the treatment of CML before the era of modern chemotherapy and more recently have been shown to be effective, particularly in combination with all-trans retinoic acid, in the treatment of acute promyelocytic leukemia (APL) (4). Indeed, some previous studies have reported the potentiating effects of As2O3 when used in combination with IM in CML cells (5,6). A combination of AS and IM also induced apoptosis in CML cells (7) and the fact that AS can be used orally in humans as a relatively safe agent (8) makes it potentially a more appropriate partner for IM in anti-CML therapy.

In this study, we tested the therapeutic efficacy of AS/IM treatment in a mouse model bearing the BCR/ABL oncogene. Using 2D electrophoresis (2DE), MALDI-TOF-TOF mass spectrometry (MS), and cDNA microarray analysis, we systematically analyzed the regulatory networks modulated in human CML cell lines at the levels of the proteome, phosphoproteome, and transcriptome and explored some of the key molecular mechanisms underlying the therapeutic effects observed in the mouse model. Importantly, we found that AS activated an array of factors involved in protein ubiquitination and proteasomal degradation, which correlated with the catabolism of BCR/ABL and may form the basis for AS synergy with IM in CML treatment.

Results

AS Potentiates the Efficacy of IM in a CML Mouse Model. We compared the efficacy of combined use of IM (25 mg/kg/d) and AS (6 mg/kg/d) with each monotherapy in the P210 BCR/ABL mouse model. Within 5 weeks of transplantation, 100% of PBS-treated control mice died from a CML-like illness characterized by granulocytosis with an average white blood cell (WBC) count >200 × 106 cells per milliliter, splenomegaly, and infiltration of bone marrow (BM), liver, and spleen by leukemic cells. In contrast, all drug-treated mice showed a reduction in the leukemic burden with a diminished degree of leukemia cell infiltration in major hematopoietic organs [Fig. 1A and supporting information (SI) Fig. S1A and B]. However, when compared with the PBS control group, only the IM/AS combination-treated group achieved statistically significant differences when the criteria of liver- or spleen-to-body-weight ratio, percentage of GFP cells (%GFP) reflecting the BCR/ABL-expressing leukemia population in peripheral blood (PB) and WBC counts were used. Of note, when WBC counts and %GFP cells were compared, the combination group also displayed statistically better result than the groups treated with a single agent (WBC, P = 0.01; P = 0.001; and P = 0.005 versus 50 mg/kg/d IM, 25 mg/kg/d IM, and AS, respectively; %GFP, P = 0.007; P < 0.001; and P = 0.005 versus 50 mg/kg/d IM, 25 mg/kg/d IM and AS,

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those in K562 cells treated with As2O3 (5) with a less pronounced concentrations, AS induced transcriptome patterns resembled posttranscriptional modification of proteins (Fig. 2).

SOM analysis of transcriptomic data showed that differences in imposed expression patterns of AS and IM (Fig. 2). The SOM outputs of proteome (A), phosphoproteome (B Right), and transcriptome (D) expression data of all treatment series were visually shown. Each presentation illustrates a sample-specific, proteome-wide or transcriptome-wide proteins/genes regulation map. Color-coding index stands for log-transformed (base 2) ratios.

Dynamic Changes in Molecular Profiling of K562 Cells Treated with AS/IM. To gain insights into the mechanisms of synergistic effect of AS/IM in treating CML, transcriptomic and proteomic analysis was performed in human CML cell line K562 treated with AS and/or IM. Expression data from 1,278 protein spots with good reproducibility were obtained (Fig. 2A). SOM analysis of these proteomic data showed the protein modulation intensity and range of each series appeared as time-dependent patterns. Moreover, the modulation intensity and scope of proteins regulated by AS was greater than that of IM, probably because of the lower specificity of AS. In general, protein expression was more noticeably down-regulated rather than up-regulated in the IM, AS, or cotreatment series, indicating a functional importance in triggering growth arrest and potentially apoptosis (Fig. 2C). SOM analysis of phosphoproteome expression data from 426 phosphoprotein spots with good reproducibility indicated that the IM/AS combination exerted significantly differential effects on protein phosphorylation. Interestingly, the expression pattern of the cotreatment series appeared similar to the super-imposed expression patterns of AS and IM (Fig. 2B). Meanwhile, SOM analysis of transcriptomic data showed that differences in the expression profiles of genes were less significant than those of proteins, suggesting that the 2 agents might act primarily on posttranscriptional modification of proteins (Fig. 2D). At similar concentrations, AS induced transcriptome patterns resembled those in K562 cells treated with As2O3 (5) with a less pronounced effect, pointing to a more moderate mode of action of AS. Furthermore, the changes in the transcriptome pattern observed in the cotreatment series, albeit more pronounced, were very similar to those in IM-treated ones, suggested that IM plays a more important role in transcriptional regulation than AS. Hence, the complementatory effects of the 2 agents on both the proteome and transcriptome may be responsible in large part for the molecular basis for their synergy. Additionally, most of the significantly modulated genes/proteins (228, 72, and 3,350 from the 2DE, phosphoproteome and cDNA arrays, respectively) were related to cytoskeleton, ubiquitin–proteasome pathway, unfolding protein reaction (UPR), glucose metabolism, cell cycle, and apoptosis (Fig. 3A–C; see also Table S1, Table S2, and Table S3). PCA analysis of these data indicated that in the AS-treated and cotreatment series many proteins in UPR and ubiquitin–proteasome pathways were significantly up-regulated. IM, however, modulated components of the PI3K/AKT/mTOR pathway (PI3K, AKT1, EIF4E, EIF4B, S6K1, and PP2A), which plays a crucial role in the pathogenesis of CML. Notably, down-regulation of proteins that play key roles in the G1/S and G2/M phases (CCND3, CDK4; CDC2, CCNA2, etc.) and glycolysis metabolism (HK4, ALDOC, PDHB, ALDH2, etc.) was observed in both IM-treated and cotreatment series, but not in the AS treatment group.

Identification of Key Pathways Underlying the Synergistic Effects of AS/IM in CML Cells. The PI3K/AKT/mTOR Pathway. We analyzed the expression and phosphorylation status of the main components of the PI3K/AKT/mTOR pathway by Western blot. Fig. 3D shows that AS considerably down-regulated EIF4E and 2 phosphorylation forms of 4EBP (4EBP-Thr-37/46 and 4EBP-Thr-70) but had no obvious effect on other signaling proteins in this pathway. On the other hand, although significantly down-regulating the expression of the main elements in this pathway
such as mTOR, PI3K, PS6K, 4EBP-Thr-37/46/H11032/4EBP-Thr-70, and EIF4E, IM significantly induced the expression of PP2A, resulting in the inhibition of the activity of PI3K/AKT/mTOR pathway. Cotreatment with AS/IM induced greater changes of some key elements of PI3K/AKT/mTOR pathway (e.g., mTOR and 4EBP) compared with IM monotherapy, suggesting that the activity of IM underlies these effects, whereas AS may have a potentiating role.

**AS promotes the ubiquitin–proteasome pathway and UPR.** Analysis of the transcriptome and proteome revealed that many mRNA transcripts and proteins related to the ubiquitin–proteasome pathway, especially the E3 ubiquitin ligase (CUL1, CBL, FBXO16, and FBXW3, etc.), were significantly up-regulated in the AS-treated and cotreatment series (Fig. 3A and B). These changes were much less pronounced in the IM-treated series, suggesting that AS provides a major contribution. Moreover, AS up-regulated many important components of UPR such as PERK, ATF6, ATF4, XBP1, and HSPAB. Meanwhile, induction of mRNA and proteins required for correct protein folding (P4HB, PDLA3, and PDLA4) was observed (Fig. 3A and B), whereas the chaperone HSP90 (Fig. 3E), which is important for the stability of some key regulatory protein was down-regulated. We examined several key factors involved in these pathways (USP5, ATF6, and PDIA3) in the BM of the mice treated with IM/AS and obtained results consistent with in vitro cell culture experiments (Fig. 3F).

**Synergistic/additive effects of IM and AS on apoptosis.** Proteome and transcriptome analysis showed that both AS and IM regulated the intrinsic and extrinsic apoptotic pathways (Fig. 3A and B). IM induced apoptosis primarily through the intrinsic pathway, whereas AS induced the UPR leading to apoptosis via the endoplasmic reticulum (ER) stress-mediated pathway. In the cotreatment series, the intrinsic apoptotic pathway was significantly promoted at an early stage (12 h), with a similar pattern of enhancement observed in UPR and ER stress-mediated apoptosis. The modulation of proteins involved in UPR and ER stress was mainly directed by AS, whereas the modulation of apoptotic factors appeared to be potentiated by IM. In the cotreatment but not mono-treatment series, proapoptotic factors (CASP8, FADD, and DEDD2) were up-regulated whereas negatively acting regulators (CFLAR, TNFRSF1R, and BIRC2) were down-regulated (Fig. 3). By using different bioinformatics methods, a model for how IM and AS synergistically target the proliferation/survival advantage in CML was summarized (Fig. 4).

**Combined Effects of AS and IM on BCR/ABL Oncoprotein.** In K562 cells, AS but not IM decreased the level of BCR/ABL, and cotreatment did not enhance AS-mediated change (Fig. S2B). BCR/ABL kinase activity was inhibited in IM, and cotreatment significantly enhanced this effect (Fig. 5A). Real-time PCR analysis showed that the expression of BCR/ABL mRNA was not significantly changed in the AS and/or IM treatment series compared with controls (Fig. S2A). Interestingly, AS caused an overall increase in ubiquitination levels in K562 cells (Fig. 5B Upper), suggesting that arsenic could also induce ubiquitination of BCR/ABL. In fact, neither the lysosome inhibitor chloroquine nor the broad-spectrum caspase inhibitor Z-VAD-FMK could inhibit AS-mediated degradation of BCR/ABL (Fig. S2C). An increased BCR/ABL polyubiquitination was seen after treatment with AS, and pretreatment with the proteasome inhibitor MG132 greatly enhanced this effect (Fig. 5B Lower).

**MS Characterization of Ubiquitinated BCR/ABL.** GFP-tagged BCR/ABL was immunoprecipitated from 293T cells by using anti-GFP
antibody, trypsinized, and subjected to LC-MALDI-MS/MS analysis. BCR/ABL/GFP fusion protein was identified with the highest confidence. Specific signatures corresponding to the C-terminal BCR and N-terminal ABL regions not contained in the fusion protein were not detected, indicating that the majority of immunoprecipitated material was purified BCR/ABL/GFP. Moreover, distinct 1460.8-Da fragments produced by digestion of K48-linked polyubiquitin were detected with 60% relative intensity. Further MS/MS analysis demonstrated that this fragment originated from polyubiquitin (Fig. S3A), suggesting the presence of a large amount of K48 polyubiquitin conjugated to BCR/ABL. Isopeptide-linked ubiquitin was cleaved by trypsin at the junction between Arg 74 and Gly 75, producing a –GG signature peptide (Fig. S3B), and tandem MS analysis of BCR/ABL showed existence of these distinct –GG signature peptides. Peptide segments 1427.8 (amino acids 1986–1996, K1990) (Fig. 5C), 1677.7 (amino acids 27–39, K39), and 1301.3 (amino acids 791–800, K795) were identified. Additionally, the distinct –GG signatures were also detected on a peptide fragment 1695.7 (amino acids 204–213, K213) of BCR/ABL immunoprecipitated from K562 cells (Fig. S3C–E). To address why the lysine residues at amino acid positions 39, 213, 795, and 1990 are preferentially polyubiquitinated, we analyzed the available crystal structure of BCR/ABL. However, there were only crystallographic data for the protein domains containing K39 and K1990 of BCR/ABL (9, 10). Interestingly, both K39 and K1990 were exposed residues (Fig. 5D and E), facilitating their K48-linked polyubiquitination.

The fact that ubiquitinated BCR/ABL could be detected in the absence of any drugs and the fusion protein accumulated in the presence of proteasome inhibitor (Fig. 5B Lower) strongly suggests that BCR/ABL polyubiquitination is an intrinsic cellular self-regulatory process. Importantly, this process was enhanced by AS, leading to accelerated degradation of BCR/ABL.

Discussion

In this article, we examined the effect of IM in combination with AS in treating P210 BCR/ABL BM transplant mice. Although AS as a single agent yielded an efficacy close to the low-dose IM, it dramatically enhanced the efficacy of IM, leading to improved reduction of tumor burden and longer survival without obvious cardiac toxicity. Our data thus suggest that IM/AS cotreatment for CML has clinical value. This approach may represent an extension of the well-established concept of cancer polychemotherapy (11) and point to new lines of investigation using drug combinations targeting multiple pathways.

An investigation of the molecular mechanisms underlying the in vivo and in vitro effects of IM and AS led to the identification of focal points in regulatory networks targeted by both agents. Although IM is an inhibitor that specifically targets PTK activity, multiple downstream pathways are affected. The PI3K/AKT/mTOR pathway is, however, of particular interest because it is activated excessively by the aberrant PTK activity of BCR/ABL and, in turn, activation of this pathway promotes the translation initiation of BCR/ABL, forming a positive-feedback cycle (12, 13). We show in this study that IM inhibited the PI3K/AKT/mTOR pathway, targeting the cell proliferation/survival advantage conferred by CML. With regard to AS, the mechanisms of action are even more complex. Arsenic is known to affect the modification of many proteins (14), and As2O3, for example, has been shown to induce the hyperacetylation of histones H3 and H4 and to modulate the phosphorylation of multiple proteins involved in signaling (15, 16). Because the mode of action of AS in CML was poorly understood, an “omics” platform was used.

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Western blot analysis using an anti-ubiquitin antibody. (Upper) The product ion spectrum of the precursor ion 1427.8 Da from the digestion product of immunoprecipitation. (Lower) K562 cells were treated as described in Materials and Methods. Then, cell lysates were immunoprecipitated against BCR and Western blot against polyubiquitin (Upper) and c-ABL (Lower). (C) The product ion spectrum of the precursor ion 1427.8 Da from the digestion product of immunoprecipitation. (D and E) The protein crystallographic structures of BCR/ABL from 1 to 67 and 925 to 2,031 aa indicate the K39 and K1990 of BCR/ABL exposed outside.

Fig. 5. Effects of IM and/or AS on BCR/ABL. (A) PTK activity was analyzed in K562 cells after treatment with AS and/or IM for 4, 12, 24, and 48 h (n = 3). One-sided paired t test was used for statistical analysis (∗, P < 0.05 versus control; ∗∗, P < 0.01 versus control; ▲, P < 0.05 versus IM, and AS groups.). (B) Upper Western blot analysis using an anti-ubiquitin antibody. (Lower) K562 cells were treated as described in Materials and Methods. Then, cell lysates were immunoprecipitated against BCR and Western blot against polyubiquitin (Upper) and c-ABL (Lower). (C) The product ion spectrum of the precursor ion 1427.8 Da from the digestion product of immunoprecipitation. (D and E) The protein crystallographic structures of BCR/ABL from 1 to 67 and 925 to 2,031 aa indicate the K39 and K1990 of BCR/ABL exposed outside.

Materials and Methods

Cell Culture and Sample Treatments. AS and IM were kindly provided by Novartis and the BCR/ABL-positive K562 cell line was cultured as previously described (7). After treatment with 0.25 μM IM or/and 2 μM AS for 4, 8, 12, 24, 48, and 72 h, 1.5 × 10^5 K562 cells were collected and used for subsequent experiments. K562 cells treated with PBS were used as a control.

BCR/ABL Mouse Model and AS/IM Treatment Regimen. The mouse BM transplantation model of CML coexpressing P210 BCR/ABL and GFP was established as previously reported (29, 30). Animals were randomized and treated with PBS, AS (6 mg/kg/d; vena caudalis injection, which gave better result than intragastric administration in mouse experiments), IM (50 or 25 mg/kg/d; intragastric administration), and AS plus IM (25 mg/kg/d, respectively).

Antibodies and Western Blot Analysis. Antibodies used in this study include anti-Phospho-P56K, anti-Phospho-Thr37/46-4EBP, anti-Phospho-Thr70-4EBP, anti-Phospho-eIF4E, anti-Phospho-PDK1 (Cell Signaling Technology), anti-HSP90, anti-BCR, anti-PP2A, anti-PI3K, anti-mTOR, anti-c-ABL (Abcam) and anti-ubiquitin (FK2) (Affinity Research Products). Immunoblot analysis of BCR/ABL. First, arsenic inhibits the initiation of translation of BCR/ABL via suppression of the PI3K/AKT/mTOR pathway (12). Consistent with this finding, we show here that AS significantly down-regulated the E1E4E protein, which has been previously demonstrated to undergo enhanced ubiquitination and proteolysis in response to sodium arsenite treatment, in turn contributing to the inhibition of BCR/ABL translation. AS can also directly promote the degradation of BCR/ABL via the ubiquitin–proteasome pathway, which is consistent with studies showing that arsenic facilitates the ubiquitination-dependent degradation of several oncoproteins in leukemia, including the PML-RARα in APL and AME/EVII/CDC25C in several myeloid disorders (22, 23). Our study also revealed that AS down-regulated HSP90 (Fig. 3E), an important chaperone for BCR/ABL, the decreased level of which has been shown to promote the degradation of BCR/ABL by the ubiquitin–proteasome pathway (24). Arsenic can also induce oxidative stress and reactive oxygen species (ROS) generation in K562 cells, which may alter the structure of or induce modification of BCR/ABL, thus rendering it more susceptible to ubiquitination-dependent degradation (15, 25). In this study, we show that BCR/ABL may be conjugated to a K48-linked polyubiquitin chain at key lysine residues (K39, K213, K795, and K1990) of the fusion protein. Thus, AS targets BCR/ABL for ubiquitin-dependent proteasomal degradation. It remains to be established which specific E3 ligase is responsible for the polyubiquitination of BCR/ABL among the multiple E3 ligases up-regulated upon AS treatment. In this regard, the CBL family of E3 ligases could be of particular interest because they have been shown to target activated tyrosine kinases for ubiquitination and subsequent degradation (26).

Finally, our results support the notion that targeting a complex disease such as CML, which contains multiple deregulated pathways, with a single agent will eventually induce drug resistance (27). Here, IM is able to cause synergism in inducing apoptosis when combined with AS (Fig. 4). Both the intrinsic and extrinsic apoptotic pathways were activated, and this effect was enhanced by activation of the UPR/ER stress pathway. In the context of CML, it is significant that both agents targeted BCR/ABL through the PI3K/AKT/mTOR pathway, with IM inhibiting substrate phosphorylation and AS up-regulating phosphatases such as PP2A. The reduction of PTK activity by IM was also strengthened by the induction of BCR/ABL catabolism by AS. Furthermore, as recently reported, AS can modulate PML, a protein involved in the maintenance of quiescent leukaemia-initiating cell status, and its targeting plays a critical role for possibly eradicating the CML stem cells (28). These data suggest that a randomized clinical trial is warranted to test the efficacy of IM/AS combination for CML.

to obtain an overview, and we then focused on key pathways underlying the observed effects. Through this approach, we found that AS up-regulated the expression of many phosphatases in K562 cells, which is consistent with the observed wide-ranging decrease in protein phosphorylation (Fig. 2B). It is well known that arsenic binds to a variety of proteins containing dithiols formed by vicinal cysteine structures, such as the binding sites of numerous binding domains of signaling factors or key enzymatic activity sites (17). Almost all known phosphotyrosine phosphatases have the –SH radical and are sensitive to thiol reagents. However, the biological consequence of the binding of phosphatase by arsenic may depend on cell type and environment, special protein structures, and category and dose of arsenicals. For example, although Cavigelli et al. (18) reported that arsenite could inhibit JNK phosphatase, Luo et al. (19) and our data indicate that AS up-regulates PP2A, 1 of the 4 major Ser/Thr phosphatases.

How arsenic acts on the ubiquitin–proteasome pathway remains controversial. Most studies suggest arsenic compounds increase the activity of the ubiquitin–proteasome pathway and promote the proteasome-dependent degradation of many proteins (20), whereas others indicate that arsenicals can inhibit the system, and this inhibitory effect could be related to the oncogenicity of arsenic compounds (21). These discrepancies may also arise because of different experimental conditions among cell/organism/disease models. This study supports the notion of an activation of the ubiquitin–proteasome pathway by evidenced by increased expression of a number of E3 ubiquitin ligases and multiple elements of the UPR. Most importantly, we demonstrated that BCR/ABL is the target of this activated pathway. Thus far, studies have demonstrated at least 2 mechanisms underlying the activities of arsenic compounds in down-regulation...
was performed by using standard procedures on 5 $\times$ 10^6 K562 cells or cells harvested from BM of CML mice.

PTK Activity. The PTK activity was assayed as described in the manufacturer’s manuals (Chemicon International). Assays were performed in triplicate, and the 2-sided paired t test was used for statistical analysis.

Real-Time RT-PCR. Real-time RT-PCR analysis of BCR/ABL transcripts was performed as previously described (5, 7). All experiments were performed 4 times, and the 2-sided paired t test was used for statistical analysis.

Two-Dimensional Electrophoresis, cDNA Array, and Data Processing. Two-dimensional gel electrophoresis and cDNA arrays were performed as previously described (31). For phosphoproteome detection, after 4 h, gels were first stained with Pro-Q Diamond (Molecular Probes), followed by staining with Deep purple. A conservative 2-fold change threshold (i.e., treated sample versus untreated reference) was used to determine regulated genes/proteins. All experiments were performed at least in duplicate. The 2DE and transcriptome expression data were preprocessed and analyzed as described previously by using self-organization map (SOM) and principle component (PCA) analysis toolboxes for Matlab 7.1 (Mathworks). By literature reading and with the help of some bioinformatics tools (Panther, iHOP, and KEGG), the ideogram illustrating dynamic changes of pathways impacted was built up manually.

Transfection and Immunoprecipitation. The 293T cells were transfected with Migr1-BCR/ABL/GFP plasmid (kindly provided by Rui-bao Ren, Brandeis University, Waltham, MA) by using a Superfect kit (Invitrogen), then exposed to MG132 or AS for 4 h. Immunoprecipitation was performed by using anti-GFP antibodies for BCR/ABL-GFP-expressing 293T cells and anti-BCR ones for K562 cells, followed by further purification with ultratitration membrane (Millipore). Purified immunoprecipitates from K562 cells cotreated with AS and MG132 were immunoblotted with FK2 (anti-ubiquitin) or c-ABL antibody. Immunoprecipitates from MG132 treated 293T and K562 cells were used for trypsin digestion and separation by 2D nano HPLC, followed by analysis with MALDI-TOF-TOF mass spectrometry.

Tandem MS Analysis. Significant protein spots revealed by PCA were digested by trypsin. Digestion products from spots and separated immunoprecipitates were identified by 4700 MALDI-TOF-TOF MS (ABI) with 0.15-Da and 0.25-Da mass tolerance for PMF and MS/MS, respectively (31). Identiﬁcations with a GPS conﬁdence interval of >99.9% were accepted.

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Fig. S1. The phenotypes of the mouse model of CML at 2 weeks after cotreatment with AS and IM. (A) PB, BM, and spleen cell smears from treatment groups. (B) Liver/ or spleen/body weight ratios of mice treated with PBS control, AS and/or IM for 2 weeks were calculated. (P values were 0.018 and 0.014 for liver/ and spleen/body weight ratios, respectively, when cotreatment of AS and 25 mg/kg/d IM groups was compared with PBS control group). (C) Heart's ejection fraction, fractional shortening, and cardiac output of mice treated with AS and/or IM were calculated. No treatment groups exhibited significant cardiomyocyte damage (P > 0.05).
Fig. S2. Effects of AS and/or IM on BCR/ABL. (A) Real-time PCR assay of relative transcript expression level of BCR/ABL-b3a2 in K562 cells treated by AS and/or IM for 0, 4, 12, 24, and 48 h. One-side paired t test was used for statistical analysis; no treatment groups exhibited significant changes ($P > 0.05$). (B) Western blot analysis of BCR/ABL of K562 cells treated by AS and/or IM for 0, 4, 12, and 24 h. (C) Inhibition assay as AS-mediated BCR/ABL degradation in K562 cells treated by lysosome inhibitor chloroquine or the broad-spectrum caspase inhibitor Z-VAD-FMK for 4 h; neither of them exhibited significant inhibition.
Fig. S3. Mass spectrometry analysis of BCR/ABL ubiquitination. (A) The spectrum of the digested polyubiquitin fragment-1460.8778 from the digestion product of immunoprecipitation. (B) The model of the polyubiquitin conjugated to proteins. The peptide backbone in the tandem mass spectrometer would produce the predicted fragment ion masses shown (b- and y-type ions) with the diglycine modification at Lys 48. (C–E) The spectra were the product ion spectra of the precursor ions 1301.3 Da, 1677.7 Da and 1695.7 Da from the digestion product of immunoprecipitation. The sequences of these precursor ions were denoted by single-letter code on the spectra.

Other Supporting Information Files

Table S1 (XLS)
Table S2 (XLS)
Table S3 (XLS)