Suppression of Survivin Induced by a BCR-ABL/JAK2/STAT3 Pathway Sensitizes Imatinib-Resistant CML Cells to Different Cytotoxic Drugs

Stefania Stella, Elena Tirro, Enrico Conte, Fabio Stagno, Francesco Di Raimondo, Livia Manzella, and Paolo Vigneri

Abstract

The BCR-ABL oncoprotein of chronic myelogenous leukemia (CML) displays exclusive cytoplasmic localization and constitutive tyrosine kinase activity leading to the activation of different pathways that favor cell proliferation and survival. BCR-ABL induces survivin expression at both the mRNA and protein level, thus inhibiting the apoptotic machinery of CML cells and contributing to the expansion of the leukemic clone. We report that, in human CML cell lines, BCR-ABL–mediated upregulation of survivin involves the JAK2/STAT3 pathway since silencing of either protein caused a consistent reduction in survivin expression. Cell lines unresponsive to imatinib mesylate (IM) because of BCR-ABL gene amplification were not resensitized to the drug after survivin downregulation. However, cells insensitive to IM because of point mutations in the BCR-ABL kinase domain were highly responsive to hydroxyurea (HU) after survivin silencing. To address the possible clinical applications of our results, we used shepherdin, a cell-permeable peptidomimetic compound that downregulates survivin expression by preventing its interaction with Hsp90. Incubation with shepherdin of immortalized cell lines both sensitive and resistant to IM enhanced cell death induced by HU and doxorubicin. Similarly, the combination of shepherdin with first- and second-generation tyrosine kinase inhibitors reduced the colony-forming potential of human progenitors derived from both patients with IM-sensitive and IM-resistant CML. These results suggest that strategies aimed at reducing survivin levels may represent a potential therapeutic option for patients with CML unresponsive to IM.

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Introduction

A reciprocal translocation between chromosomes 9 and 22 generates the abnormal Philadelphia (Ph) chromosome that, in turn, encodes for the chimaeric BCR-ABL oncogene (1). The ensuing BCR-ABL oncoprotein displays constitutive tyrosine kinase activity thereby activating multiple signaling pathways that lead to the development of chronic myeloid leukemia (CML; ref. 2). BCR-ABL–dependent activation of the phosphoinositide 3-kinase and the upregulation of Bcl-xL (3, 4) contribute to block cytochrome C release from the mitochondria and inhibit caspase activity, thus hampering the apoptotic machinery of CML cells and favoring the survival of leukemic myeloid progenitors. Treatment of CML cells with the ABL inhibitor imatinib mesylate (IM) suppresses BCR-ABL kinase activity reverting the ant apoptotic phenotype of the Ph-positive cells and resulting in the induction of cell death (5).

Survivin is a member of the inhibitor of apoptosis proteins (IAP) that is undetectable in normal tissues but highly expressed in most forms of human cancer and functions as both an inhibitor of cell death and a mitotic regulator (6–9). It has been reported that survivin overexpression reduces the sensitivity of neoplastic cells to death stimuli induced by gamma radiation (10) or chemotherapeutic agents (11, 12). The design of shepherdin, a cell-permeable peptidomimetic molecule that reduces survivin half-life by disrupting its binding with the molecular chaperone Hsp90, represents an attempt to increase the killing of cancer cells while preserving the viability of their normal counterpart (13).

We previously reported a significant correlation between BCR-ABL levels and the expression of survivin in a series of 44 patients with CML (14), suggesting that BCR-ABL could directly regulate survivin expression. Indeed, subsequent evidence indicated that BCR-ABL upregulates survivin at the mRNA and protein levels (15). A further study by Carter and colleagues suggested that the RAS/RAF/MAPK cascade was involved in
BCR-ABL–dependent induction of survivin in a human CML cell line and that suppression of survivin expression increased responsiveness to IM therapy (16).

The aims of this study were 3-fold. We initially wanted to ascertain the signaling pathway(s) triggered by the BCR-ABL kinase to induce survivin expression. We then wanted to establish whether survivin overexpression contributed to the failure of IM treatment in a representative panel of CML cell lines with well-established mechanisms of IM resistance. Finally, we wished to determine whether reduced survivin levels increased the antiproliferative activity of different cytotoxic compounds.

We found that JAK2/STAT3 signaling modulates BCR-ABL–dependent induction of survivin and that survivin downregulation enhances the apoptotic effect of IM on CML cells that are sensitive to this compound. Lowering survivin expression increased the cytotoxic effect of hydroxyurea (HU) and doxorubicin (Doxo) on multiple cell lines unresponsive to IM. Likewise, exposure of IM-resistant cells to shepherdin reduced survivin expression and induced higher cell killing by both cytotoxic drugs and other ABL tyrosine kinase inhibitors (TKI).

Materials and Methods

Cell lines, primary cells, drugs, and shepherdin treatments

Human CML cell lines K562, KCL22, KYO-1, LAMA84, K562 S, K562 R, KCL22 S, KCL22 R, LAMA84 S, LAMA84 R, and murine Ba/F3 cells expressing either wild-type BCR-ABL (Ba/F3p210) or different BCR-ABL mutants (Ba/F3p210Y253F and Ba/F3p210T315I) were grown in RPMI-1640 (Sigma-Aldrich Corp) supplemented with (Symansis), 1 mmol/L HU (Sigma), 1 mmol/L IM (a gift from Novartis), 12.5 nmol/L nilotinib (a gift in informed consent. Total leukocytes were separated by blood of patients with CML after receiving written specified BCR-ABL constructs.

After viral transduction of the Ba/F3 pro-B cell line (purified), IM-resistant clone. Ba/F3BCR-ABLp210, Ba/F3p210Y253F, and Ba/F3p210T315I were grown in RPMI-1640 (Sigma-Aldrich Corp) supplemented with 10% heat-inactivated FBS (Lonza), 2 mmol/L L-glutamine RPMI-1640 (Sigma-Aldrich Corp), and penicillin/streptomycin (100 U/mL). Cells were maintained in parallel cultures without IM for the time required to obtain the IM-resistant clone. Ba/F3BCR-ABLp210, Ba/F3p210Y253F, and Ba/F3p210T315I were generated in our lab after viral transduction of the Ba/F3 pro-B cell line (purified) with lentiviral vectors encoding for the specified BCR-ABL constructs.

Primary CML cells were obtained from the peripheral blood of patients with CML after receiving written informed consent. Total leukocytes were separated by red cell lysis.

The following drug treatments were used: 1 µmol/L IM (a gift from Novartis), 12.5 nmol/L nilotinib (a gift from Novartis), 50 µmol/L PD98059 (Calbiochem), 20 µmol/L LY294002 (Sigma), 7.5 nmol/L TG101209 (Symansis), 1 mmol/L HU (Sigma), 1 µmol/L doxorubicin (Sigma), 5 nmol/L dasatinib (a gift from Bristol-Myers Squibb), 17-Allylamino-17-demethoxygeldanamycin (17-AAG, InvivoGen; Fig. 1).

In further experiments, we used 30 µmol/L of cell-permeable shepherdin (biotin-X-KKWKMRNNQFVWK-VQRLFACGSSHK-CONH2; a gift from Dario Altiere, Wistar Cancer Institute, Philadelphia, PA) or an equimolar concentration of the corresponding scrambled peptide (biotin-X-KKWKMRNNQFVWKVQRGHSFCALKS-CONH2). The Antennapedia homeodomain sequence is underlined. X = EAHX, hexanoic acid spacer.

Immunoprecipitation and immunoblotting

Cell pellets were resuspended in isotonic buffer [25 mmol/l Trizma base (pH 8.5), 100 mmol/l NaCl, 7 mmol/l β-mercaptoethanol, 1× protease inhibitor cocktail (Roche)], sonicated and harvested by centrifugation at 14,000 rpm for 10 minutes at 4°C.

For immunoprecipitation experiments, 2 µg of antiphosphotyrosine antibody (clone 4G10, Millipore) were incubated with protein G-Sepharose (Amersham Biosciences) were incubated with protein G-Sepharose (Amersham Biosciences) were incubated with protein G-Sepharose (Amersham Biosciences) were incubated with protein G-Sepharose (Amersham Biosciences). Appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) were added and proteins were detected using the enhanced chemiluminescence reagent Lite Ablot (Euroclone).

Chemiluminescence images were captured and densitometric quantification of protein bands was carried out using the ImageJ 1.36b software (NIH, Bethesda, MD).

Cell proliferation and cell death assays

To evaluate cell viability, 2×10⁶ cells were incubated, for 24 hours in 96-well plates with different drugs, siRNAs, or peptides and cell proliferation was subsequently determined using the luminescence ATP detection assay system ATPlite 1 step (Perkin-Elmer), following the manufacturer’s instructions. To assess cell death, 5×10⁶ cells were treated with different drugs or peptides, alone or in combination, for 24 hours. Cells were harvested and triplicate samples were selected to determine apoptosis using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) following the manufacturer’s instructions. Apoptotic events were recorded as a combination of Annexin V+/PI– (early apoptosis) and
Figure 1. Chemical structures of the specified drugs.

Imatinib Mesylate

Nilotinib

PD98059

LY294002

TG101209

Hydroxyurea

Doxorubicin

Dasatinib

17-AAG
Annexin V+/PI+ (late apoptosis) events and results were expressed as percentage of Annexin V+ cells.

Transfection and lentiviral transduction

For the production of the lentiviral supernatant, transfected vectors pGIPZ nonsilencing shRNA (Cat no. RHS4346) or pGIPZ shRNA anti-JAK2 (NM_004972, Cat no. RHS4430) were cotransfected with the packaging plasmids in the HEK293T cell line, using the calcium phosphate transfection method (Trans-Lentiviral shRNA Packaging Kit, Open Biosystems). Viral supernatants were harvested 48 hours after transfection. CML cells were then transfected by a double round of spin-infection. Cells were centrifuged at 1,200 g for 90 minutes at 32°C in the presence of 1 mL of viral supernatant and 8 μg/mL polybrene (Sigma-Aldrich). Transduced cells were finally selected with 6 μg/mL puromycin (Sigma-Aldrich) for 72 hours.

Lentiviruses containing the pLEX empty vector or pLEX \textit{STAT3}C were produced using the calcium phosphate transfection method (Trans-Lentiviral ORF Packaging Kit, Open Biosystems) as previously described.

RNA silencing experiments

For antisense oligonucleotides, 1 × 10^6 cells were resuspended in 0.8 mL of culture media with 500 nmol/L antisense oligonucleotides against \textit{STAT3} (AS \textit{STAT3}) or with a control oligonucleotide (AS Scr) and the electroporated in a 0.4 cm cuvette using the Gene Pulser Electroporation apparatus (Bio-Rad) with a double pulse protocol (25 μF, 500 V). Both the \textit{STAT3} antisense (5'-GCTCCAGACATCTGCTGCTT-3') and the control mismatch oligonucleotide (5'-GCTCCAAATACCGTTGCTT-C') were synthesized with a phosphorothioate backbone and with a 2'-O-methoxymethyl modification of the first and last 5 nucleotides (Eurofins MWG Operon).

For siRNA electroporation of Ba/F3 cells, cells were resuspended as previously described and mixed with 100 nmol/L of si Surv or with the corresponding scrambled control (si Scr; Dharmacon). si RNA electroporation was carried out as described above using different electrical settings (960 μF, 250 V).

\textbf{STAT3 construct and mutagenesis}

\textit{STAT3} was amplified by PCR using the indicated forward (FLAG-tag sequence underlined) 5'-ATAAGAATTCGCCCCCATCATGATTACAAAGGATGGCAGCATGATGCGCCAATGGAATAGATGATGATGGAGGAGGAGGAGGAGGACGCGAGTAAGATGGCGCGCGCGCGCATATTATCAGCTAC-3' and reverse 5'-CCGCGTCTGATGACATGCGGCGAGGAGGAGGACGCGAGTAAGATGGCGCGCGCGCATATTATCAGCTAC-3' primers and cloned in the \textit{NotI} and \textit{XhoI} unique sites of the pLEX lentiviral vector (Open Biosystems).

\textbf{Colony-forming assays}

To evaluate the effect of different treatments on the colony-forming potential of CML myeloid progenitors, 5 × 10^4 primary cells were treated for 24 hours with sheepdin alone or in combination with IM, nilotinib, or dasatinib. Drug-treated cells were then seeded in Methocult H4435 methylcellulose medium (StemCell Technologies). Formation of colonies was assessed 14 days after plating.

\textbf{Statistical analysis}

Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software Inc). Unpaired, single-tail \textit{t} tests with 95% confidence intervals were used to compare cell viability in different experimental conditions. The one-way ANOVAs according to Bonferroni’s posttest were used to compare the effect of the combination of sheepdin with different tyrosine kinase inhibitors.

\textbf{Results}

\textbf{Survivin induction by BCR-ABL tyrosine kinase activity involves JAK2}

We have previously reported that, in primary hematopoietic cells isolated from patients diagnosed with CML, the amount of BCR-ABL transcript significantly correlates with the expression levels of survivin (14). Our findings suggested that BCR-ABL might directly regulate survivin expression in CML cells and indeed, subsequent manuscripts have shown that BCR-ABL upregulates survivin at both the transcriptional and protein levels (15–17). However, these reports used single immortalized cell lines and attributed BCR-ABL–dependent induction of survivin to different signaling pathways.

To clarify these contrasting observations, we conducted an antisurvivin immunoblot on a wide panel of human CML cells before and after IM or nilotinib treatment. BCR-ABL inactivation produced a striking decrease in survivin levels in CML cells, confirming that BCR-ABL constitutive tyrosine kinase activity induces survivin expression (Fig. 2A and B). We next wanted to establish the BCR-ABL–induced pathway(s) responsible for this effect. Carter and colleagues had previously suggested that the RAS/RAF/MAPK cascade was involved in BCR-ABL–dependent upregulation of survivin (16). In a different cell line, Fang and colleagues identified JAK2/P38K/c-myc signaling as the main regulator of BCR-ABL–dependent induction of survivin (17). However, when we suppressed mitogen-activated protein kinase (MAPK) activity with PD98059 in a set of 4 human CML cells, we detected a reduction in survivin levels only in the K562 line (Fig. 2C). Likewise, suppression of phosphoinositide 3-kinase (P38K) activity by LY294002 induced a decrease in survivin expression only in LAMA84 cells (Fig. 2D). We therefore turned our attention to JAK2, as previous evidence...
Figure 2. JAK2 inhibition reduces survivin expression in CML cells. The indicated cell lines were treated for 24 hours with 1 μmol/L IM (A), 12.5 nmol/L nilotinib (B), 50 μmol/L PD98059 (C), 20 μmol/L LY294002 (D), 7.5 nmol/L TG101209 (E), and subsequently assessed for their proliferation rate (left). Lysates were then assayed for the expression and phosphorylation of the specified proteins (middle; a blot representative of 3 separate experiments is shown). Histograms (right) depict survivin expression in each cell line normalized to actin, MAPK, AKT, or JAK2 as quantified by densitometric analysis, with survivin levels in untreated cells arbitrarily set at 1. Columns represent average ± SD of 3 independent experiments.
A

K562

Survivin

sh Jak2  
sh NS

Actin

KYO -1

Survivin

sh Jak2  
sh NS

Actin

B

K562

Survivin

AS STAT3  
AS Scr

Actin

KCL22

Survivin

AS STAT3  
AS Scr

Actin

C

K562

Survivin

STAT3C

ev

Actin

KCL22

Survivin

FLAG

Actin

KYO -1

Survivin

FLAG

Actin

LAMA84

Survivin

FLAG

Actin

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Figure 3. JAK2/STAT3 signaling contributes to BCR-ABL–mediated induction of survivin. A, protein extracts from the specified cell lines lentivirally transduced with either control (sh NS) or JAK2-specific (sh JAK2) shRNAs were blotted for survivin or JAK2 to verify the efficacy of the silencing procedure. B, to study the contribution of STAT3, the same cells were electroporated with antisense oligonucleotides against STAT3 (AS STAT3) or with a scrambled control (AS Scr). After 48 hours, lysates were blotted for survivin or STAT3 to confirm the decrease of the latter protein. Histograms on the right represent the decrease of the latter protein. Columns show average ± SD of 3 separate experiments. C, to further confirm the role of STAT3 in the regulation of survivin, cell lysates from CML cells lentivirally transduced with pLEX STAT3C or pLEX empty vector were blotted using antisurvivin (top), anti-FLAG (middle), or antiaactin (bottom) antibodies. Panels show a blot representative of 3 independent experiments. *", P < 0.01; **", P < 0.001; ns, not significant.

had shown that BCR-ABL binds to JAK2 and phosphorylates it on tyrosine 1007, thereby activating its kinase activity (18). In turn, catalytically active JAK2 phosphorylates different STAT monomers inducing their dimerization, nuclear translocation, and transcriptional activity (18, 19). After blocking JAK2 signaling with the selective inhibitor TG101209, we observed a reduction in survivin expression in all CML lines tested (Fig 2E). Prior evidence had established that JAK2 inhibition reduced survivin levels in AML cells (20). Furthermore, Fang and colleagues proposed that survivin upregulation was largely controlled at the transcriptional level through a JAK2–dependent mechanism (17). Our findings suggested a role for JAK2 in modulating survivin levels in CML cells.

**JAK2/STAT3 signaling contributes to BCR-ABL–dependent induction of survivin**

To confirm that the BCR-ABL–dependent induction of survivin involves JAK2, we stably transduced CML cell lines with lentiviral vectors expressing a JAK2-specific shRNA or a nonsilencing control (Fig. 3A) and detected a reduction in survivin in all cell lines. Interestingly, a constitutive activation of the JAK2/STAT3 pathway by the constitutive BCR-ABL tyrosine kinase has been previously described in CML progenitors (21) and several reports have shown that STAT3 induces survivin expression in both solid and hematologic malignancies (22–24). Moreover, Bewry and colleagues have suggested that STAT3 might contribute to the development of IM resistance (25).

To determine whether STAT3 was the pivotal downstream mediator of the BCR-ABL/JAK2 pathway, we electroporated STAT3-targeted antisense oligonucleotides or their scrambled counterpart in 4 CML lines. Immunoblot analyses showed that STAT3 downregulation reduced survivin levels in each leukemic cell line, whereas this was not the case with the scrambled control (Fig. 3B). To further confirm the role of STAT3 in the regulation of survivin, we generated an activated STAT3 (STAT3C) as previously reported by Bromberg and colleagues (Fig. 3C; ref. 26). We found that STAT3C promoted an increase in survivin levels in all 4 CML cells tested, implying that the JAK2/STAT3 cascade is a major regulator of survivin expression in CML cells.

**Survivin silencing sensitizes IM-resistant cells expressing BCR-ABL mutants to hydroxyurea**

Resistance to IM has emerged as a major caveat in the long-term treatment of CML (27). BCR-ABL amplification or point mutations in the oncprotein kinase domain represent 2 major mechanisms underlying IM failure. We wanted to establish whether survivin downregulation by specifically targeted siRNAs would increase the apoptotic effect of HU on CML cell lines displaying different mechanisms of IM resistance. To this end, we initially used LAMA84 R cells that are unresponsive to IM because of BCR-ABL point mutations. These results support the notion that lowering survivin expression can increase HU efficacy on CML cells unresponsive to IM (Fig. 4D and E, middle). Likewise, suppression of survivin in Ba/F3p210 cells expressing the Y253F and T315I mutants significantly increased the response to HU (Fig. 4D and E, middle). As anticipated, a reduction in survivin levels failed to restore responsiveness to IM (Fig. 4D and E, right), as point mutations induce conformational changes of the BCR-ABL kinase domain that prevent IM binding. These findings support the notion that lowering survivin expression can increase HU efficacy on CML cells unresponsive to IM because of BCR-ABL point mutations. These results also confirm that survivin does not affect the structural modifications of the BCR-ABL kinase domain induced by different amino acidic substitutions. Hence, reducing survivin levels fail to resensitize BCR-ABL mutants to the effect of IM.

The Hsp90 peptidomimetic shepherdin sensitizes IM-resistant cells to different cytotoxic drugs

The design of shepherdin, a compound that reduces survivin expression by disrupting its interaction with Hsp90, represents an attractive opportunity to determine the possible therapeutic potential of survivin downregulation in CML cells. We therefore incubated IM-resistant cell lines K562 R, KCL22 R, LAMA84 R, and their IM-sensitive counterpart with shepherdin alone or in combination with HU or doxorubicin. As expected, addition of shepherdin, but not of the scramble control peptide, reduced survivin expression in all cell lines (Fig. 5A, D, and G). When we assayed cell proliferation and survival in the presence of shepherdin and HU or doxorubicin, we...
found significant reductions in the growth and viability of K562 and KCL22, regardless of their IM sensitivity (Fig. 5B, C, E, and F). Likewise, LAMA84 S cells were highly sensitive to the combination of shepherdin and different chemotherapeutic drugs (Fig. 5H). However, in agreement with previous silencing data (Fig. 4B), shepherdin failed to sensitize LAMA84 R to either HU or doxorubicin (Fig. 5I), confirming that resistance caused by BCR-ABL amplification is independent from survivin. These results were further validated by using the Ba/F3 cell model...
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Figure 5. Treatment with the Hep92 inhibitor shepherdin sensitizes IM-resistant cells to different cytotoxic drugs. IM-sensitive and IM-resistant cell lines were incubated for 24 hours with 30 μmol/L shepherdin (Shep) or with an equal concentration of a scrambled peptide (Scr). Alternatively, cells were exposed to 2 μmol/L doxorubicin (Doxo) in association with either Shep or Scr. Cell lysates were then examined by immunoblot to confirm survivin downregulation in the presence of shepherdin (A, D, and G). The effect of each combination was then assessed using the ATPlite luminescence assay (B, C, E, F, H, and I, left) or Annexin V-FITC/PI double staining (B, C, E, F, H, and I, right). Columns represent average ± SD of 3 independent experiments conducted in triplicate (for the ATPlite assay untreated cells were arbitrarily set at 100). *, P < 0.05; **, P < 0.01; ###, P < 0.001; ns, not significant.
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transformed by either wild-type (Ba/F3p210) or mutant BCR-ABL (Ba/F3p210Y253F, Ba/F3p210T315I). In the context of these cell lines, shepherdin efficiently suppressed survivin expression (Fig. 6A) and its association with either HU or doxorubicin significantly reduced cell proliferation and cell viability (Fig. 6B, C, and D).

Shepherdin increases the cytotoxic effect of nilotinib and dasatinib on human CML myeloid progenitors

To further ascertain the efficacy of shepherdin on BCR-ABL-expressing clones, we isolated primary cells from the peripheral blood of 3 different patients with CML: 2 individuals with newly diagnosed disease and a subject that had failed treatment with IM, nilotinib, and dasatinib. We then assessed the colony-forming potential of these cells after treatment with shepherdin alone or in combination with one of the 3 TKIs. Interestingly, we observed a significant increase in overall colony number when shepherdin was associated with nilotinib and dasatinib, regardless of the TKI sensitivity of the primary cells (Fig. 7A, B, and C). These findings further reinforced the conclusion that downregulation of survivin expression.

Figure 6. Treatment with shepherdin sensitizes IM-resistant cells expressing BCR-ABL mutants to HU and doxorubicin (Doxo). Ba/F3 cells expressing either wild-type BCR-ABL or the indicated IM-resistant mutants were incubated for 24 hours with 30 μmol/L shepherdin (Shep) or with an equal concentration of a scrambled peptide (Scr). Alternatively, cells were exposed to 1 mmol/L HU or 1 μmol/L Doxo in association with either shepherdin or Scr. Cell lysates were then examined by immunoblot to confirm survivin downregulation in the presence of shepherdin (A). The effect of each combination was then assessed using the ATPLite luminescence assay (B-D, left) or Annexin V-FITC/PI double staining (B-D, right). Columns represent average ± SD of 3 independent experiments conducted in triplicate (for the ATPLite assay untreated cells were arbitrarily set at 100). *P < 0.05; **P < 0.01; ***P < 0.001.
TKI-dependent inhibition of BCR-ABL kinase activity. It may represent a potential therapeutic strategy to eliminate CML cells both responsive and unresponsive to the TKI-dependent inhibition of BCR-ABL kinase activity.

Discussion

Although IM has changed the natural course of CML achieving 89% overall survival rates after 8 years of follow-up (31), approximately 50% of patients with CML fail to obtain an optimal response as defined by the current European Leukemia Net (ELN) recommendations (32). To address these critical issues, second-generation TKIs (i.e., dasatinib and nilotinib) have been generated, tested, and approved for the treatment of patients with CML that fail IM (33). Despite the early success of these compounds, 50% of patients receiving either dasatinib or nilotinib will not obtain long-term benefit from these drugs and will progress to the advanced phases of the disease (34). Thus, the need for additional therapeutic strategies that will benefit this selected patient population (34, 35).

Carter and colleagues have previously shown that BCR-ABL induces the expression of the IAP survivin through the MAPK and PI3K pathways, and that reduction of survivin expression increases both spontaneous and IM-dependent death of a CML cell line (16). However, it is unclear whether these results could: (i) be extended to a more representative panel of CML cells both sensitive and resistant to IM; (ii) be used to potentiate the antiproliferative activity of other pharmacologic compounds; (iii) be exploited to devise a possible therapeutic strategy aimed at reducing survivin levels in patients with CML.

We report here that BCR-ABL kinase activity induced survivin expression in CML cell models via the JAK2/STAT3 pathway. Silencing of survivin reduced the viability of CML cells and enhanced the efficacy of IM. Moreover, survivin downregulation significantly increased the cytotoxic activity of HU, doxorubicin, nilotinib, and dasatinib on cells unresponsive to IM because of either point mutations (including T315I) in the BCR-ABL catalytic domain or other yet uncharacterized mechanisms. These results were obtained with the use of survivin-targeted siRNAs or by using shepherdin, a peptidomimetic compound that reduces survivin half-life by abrogating its interaction with the Hsp90 chaperone (13). These findings were further confirmed by experiments on human myeloid progenitors derived from both IM-sensitive and IM-resistant patients with CML.

Our observations lead to several considerations. First, and at variance with the data reported by Carter and colleagues, we found that only MAPK and PI3K signaling cascades modestly contribute to the regulation of survivin expression in CML. Conversely, we identified the JAK2/STAT3 pathway as a major contributor of BCR-ABL-mediated induction of survivin. This discrepancy could be due to the different cells used in the 2 studies. It is also possible that, in certain cell types, both JAK2 and MAPK contribute to survivin overexpression, as published evidence suggests that JAK2 and MAPK stimulate phosphorylation of STAT3, thus enhancing its biologic activity (36).

We have also found that suppression of survivin is unable to restore IM activity regardless of the mechanisms responsible for drug failure. These results are not unexpected as IM resistance attributable to BCR-ABL gene amplification is due to the enhanced signaling of a higher absolute number of BCR-ABL molecules (37). Intuitively, this phenomenon cannot be constrained by lowering survivin expression. Likewise, point mutations associated with IM failure induce alterations in the 3-dimensional
Figure 8. The combination of 17-AAG and HU or doxorubicin (Doxo) does not increase death rates of Ba/F3 cells expressing BCR-ABL mutants. Ba/F3 cells expressing either wild-type BCR-ABL or the indicated IM-resistant mutants were incubated for 24 hours with 0.5 µmol/L or 0.75 µmol/L 17-AAG. Cell lysates were then examined by immunoblot to ascertain changes in BCR-ABL expression (A). Alternatively, the same cells were exposed to 1 mmol/L HU or 1 µmol/L Doxo in association with 0.5 µmol/L 17-AAG. The effect of each combination on cell viability was then assessed using Annexin V-FITC/PI double staining (B-D). Columns represent average ± SD of 3 independent experiments conducted in triplicate. ns, not significant.

structure of the BCR-ABL catalytic domain that either mimic an active conformation or affect specific amino-acidic residues required for the physical interaction with IM (38, 39). Thus, it is evident that suppression of survivin per se fails to restore sensitivity to IM, as survivin does not associate with BCR-ABL and is therefore incapable of modulating the conformation of its kinase domain.

However, suppression of survivin levels significantly increased the cytotoxic activity of different compounds on CML cells failing IM because of yet uncharacterized mechanisms or point mutations in the BCR-ABL kinase domain. In these contexts, suppression of survivin may represent a potential therapeutic strategy to reduce the viability of these leukemic clones. To this end, we have conducted several experiments using the peptidomimetic shepherdin to lower survivin expression in IM-sensitive and IM-resistant CML cells. Previous studies investigating the use of shepherdin have described strong antitumor activity in human models of both solid and hematologic malignancies (13, 40). Our findings indicate that shepherdin-dependent reduction of survivin levels significantly increased the cytotoxic activity of HU and doxorubicin on all cell lines expressing BCR-ABL mutants. In addition, as further compounds (ISIS 23722, SPC3042, and YM155) are currently being developed as inhibitors of survivin expression (41), any pharmacologic strategy aimed at reducing survivin levels could be of significant value in patients with IM-resistant clones carrying point mutations in the BCR-ABL kinase domain.

Finally, the reduction in cell viability that we observed after shepherdin treatment was superior to that detected after siRNA-mediated silencing, suggesting that shepherdin may increase CML cell death by both survivin-dependent and survivin-independent mechanisms. Kang and colleagues have previously shown that shepherdin disables a mitochondrial chaperone network that cancer cells use to protect themselves from multiple apoptotic stimuli (42). Hence, it is possible that part of shepherdin’s efficacy on CML cells depends on this mechanism. Indeed, when we treated Ba/F3 cells expressing different BCR-ABL mutants with HU or doxorubicin in association with 17-AAG (a cytosolic inhibitor of Hsp90), we found no significant increases in cell death (Fig. 8). These preliminary findings support the hypothesis that shepherdin kills CML cells through a double mechanism: (i) a reduction in survivin expression and (ii) the mitochondrial inhibition of Hsp90 chaperones.

In summary, our results suggest that pharmacologic reduction of survivin expression may provide a new therapeutic approach for the molecular targeting of IM-insensitive CML cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Stella, E. Tirro, E. Conte, P. Vigneri Development of methodology: S. Stella, E. Tirro Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Stella, E. Conte, F. Stagno, F.D. Raimondo Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Stella, E. Tirro, F. Stagno, F.D. Raimondo, P. Vigneri Writing, review, and/or revision of the manuscript: S. Stella, E. Tirro, E. Conte, F. Stagno, F.D. Raimondo, L. Manzella, P. Vigneri
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