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In BCR-ABL-positive cells, STAT-5 tyrosine-phosphorylation integrates signals induced by imatinib mesylate and Ara-C

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SPOTLIGHT

In BCR-ABL-positive cells, the transcription factor STAT-5 is constitutively activated by tyrosine phosphorylation. STAT-5 activation results in upregulation of bcl-X_L and increased resistance to induction of apoptosis. Here, we investigated the effects of imatinib mesylate and cytosine arabinoside (Ara-C) on STAT-5 tyrosine-phosphorylation, cellular proliferation and induction of apoptosis in cell lines and primary hematopoietic cells. Imatinib mesylate treatment strongly suppressed STAT-5 tyrosine-phosphorylation in K562 and primary CML blasts. In contrast to JAK-2 and PI-3-kinase inhibition, exposure of K562 cells to imatinib mesylate resulted in obvious suppression of proliferation. Reduced cell growth was due to specific induction of caspase activation followed by apoptotic cell death. In addition, we investigated the effects of Ara-C on STAT-5 tyrosine-phosphorylation. Exposure to Ara-C resulted in significant downregulation of STAT-5 tyrosine-phosphorylation and inhibition of DNA binding. Treatment of K562 cells with Ara-C in combination with imatinib mesylate revealed synergistic effects at the level of STAT-5 tyrosine-phosphorylation and DNA binding, Hck tyrosine-phosphorylation, cell growth and induction of apoptosis. Overall, in this report we demonstrate that STAT-5 tyrosine-phosphorylation is a specific target of imatinib mesylate and Ara-C. Our results suggest that, in combination therapy, inhibition of STAT-5 tyrosine-phosphorylation may be responsible for synergistic or additive effects on BCR-ABL-positive cells.
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Introduction

The BCR-ABL chimeric oncogene is caused by a reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome),[1] which places the c-ABL gene under the transcriptional control of the BCR gene.[2] Transcription of this hybrid gene results in the constitutively active p210 or p190 Bcr-Abl tyrosine kinases,[3,4] which can be detected in 95% of chronic myeloid leukemia (CML) and 20–30% of adult acute lymphoblastic leukemia (ALL), respectively.[4,5] Expression of the Bcr-Abl protein in hematopoietic cells induces growth factor independence,[6] alteration in cell–cell and cell–matrix interaction[7] and resistance to apoptosis after exposure to DNA damage.[8,9] This results in malignant transformation and leukemogenesis, as demonstrated in SCID mice transfected with the BCR-ABL oncogene.[10,11] Several signaling pathways are known to be activated by adaptor molecule recruitment or by direct phosphorylation, including the ras-raf, MAPK, PI-3-kinase, c-jun and c-myc pathways.[12–15] In addition, recent publications demonstrated the constitutive phosphorylation of STAT-5 in BCR-ABL-positive cells and its essential role in malignant transformation.[16–18] STAT-5 is a latent transcription factor, which is activated in response to several cytokines (Il-3, Epo, GM-CSF).[19,20] Upon tyrosine phosphorylation, STAT molecules dimerize, translocate to the nucleus and bind to specific response elements in the promoter of target genes,[21] including β-casein, CIS-1 and bcl-X_L.[22–24] The latter is strongly upregulated in BCR-ABL-positive cells, exhibiting antiapoptotic effects and resistance to chemotherapeutic drugs.[25]

As constitutive tyrosine kinase activity is the crucial event driving all known functions of Bcr-Abl, specific targeting of the chimeric oncogene is an essential approach for therapeutic strategies. Imatinib mesylate, a newly developed tyrosine kinase inhibitor with specificity for Abl, Bcr-Abl, c-Kit and PDGF receptor, binds to the ATP-binding site of Bcr-Abl and specifically inhibits cellular proliferation and tumor formation in BCR-ABL-positive cells.[26,27] In recent clinical trials, significant antileukemic activity with minimal toxicity has been demonstrated.[28,29] Although good clinical and molecular responses could be achieved in chronic as well as in advanced stages of disease, duration of remission in CML blast crisis is limited and molecular resistance is frequently observed.[30] Combination therapy with other antileukemic agents currently used in the treatment of CML may exhibit synergistic effects and prevent the development of resistance. To develop effective therapeutic strategies, understanding of intracellular and molecular mechanisms of therapeutic agents is essential. In this report, we investigated the effects of imatinib mesylate and cytosine arabinoside (Ara-C) on STAT-5 signaling using a number of hematopoietic cell lines and primary hematopoietic cells. Imatinib mesylate as well as Ara-C clearly downregulated STAT-5 tyrosine-phosphorylation and inhibited cellular proliferation. In combination therapy, this inhibition of STAT-5 tyrosine-phosphorylation may provide synergistic or additive effects on BCR-ABL-positive cells.

Materials and methods

Reagents

Ara-C (100 mg/ml) was obtained from cell pharm, Hannover, Germany. Ara-C was diluted in sterile water and used within 24 h of reconstitution. Imatinib mesylate was kindly provided by Dr Buchdunger (Novartis, Basel, Switzerland). A 10 mM stock solution in dimethyl sulfoxide (DMSO) was prepared and stored at −20°C. Stock solution was diluted in cell culture medium and added to cells directly. AG490, a JAK-2- inhibitor, and the PI-3-kinase inhibitor wortmannin were obtained from Calbiochem (San Diego, USA). Stock solutions of AG490 (1.7 mM in DMSO) and wortmannin (2.3 M in DMSO) were stored at +4°C, the final dilution (AG490: 0.25 μM, wortmannin: 0.1–0.25 μM) was prepared immediately before use.

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Z-DEVD-FMK, a specific caspase-3 inhibitor, was obtained from Calbiochem (San Diego, USA). A 1.5 mM stock solution in DMSO was prepared and stored at −20°C. Fresh stock solution was added to cells to a final concentration of 100 μM.

Antibodies used in these studies include anti-STAT-5A, anti-c-abl, anti-poly-(ADP-ribose)-polymerase (PARP), anti-Hck, anti-Lck and antiphospho-Hck (Santa Cruz, Heidelberg, Germany), antiphospho-tyrosine (Santa Cruz, Upstate and Sigma), anti-Lyn (Cell Signaling Technology) and anti-phosphotyrosine-STAT-5 (Upstate). Polyclonal anti-RFCp140 was prepared as described previously.[31]

Cell lines and cell culture

K562 and U937 cells were obtained from DSMZ (Braunschweig, Germany). Both cell lines were maintained in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, pH 7.3, 50 μM β-mercaptoethanol, 2 mM L-glutamine, penicillin G (100 U/ml) and streptomycin (100 μg/ml).

The murine myeloid precursor cell line 32D and 32D p210$^{bcr-abl}$-transfected cells were kindly provided by T Skorski (Philadelphia, USA). 3-dependent 32D cells were maintained in IMDM-CM (Iscoves modified Dulbecco medium), supplemented with 10% FBS, 20 mM HEPES, pH 7.3, 50 μM β-mercaptoethanol, 2 mM L-glutamine, penicillin G (100 U/ml) and streptomycin (100 μg/ml), and 10% WEHI-3B conditioned medium (WEHI-3B-CM) as a source of Il-3. The p210$^{bcr-abl}$-transformed 32D cells are Il-3 independent and were maintained in culture without Il-3. All cells were grown at 37°C, in a 5% CO_2-humidified incubator.

Primary human CML samples

Heparinized peripheral blood (50 ml) was obtained from patients in CML myeloid blast crisis after informed consent was obtained according to the institutional guidelines. Mononuclear cells were prepared by Ficoll–Hypaque (Seromed, Berlin, Germany) density-gradient centrifugation and cultured in RPMI 1640 medium supplemented as described above. All patients were 100% Philadelphia chromosome positive as revealed by cytogenetic analysis (data not shown).

Preparation of cellular extracts

Cells were washed twice in cold PBS and pelleted by centrifugation. For the preparation of cellular lysates, cells were resuspended in 1 ml lysis buffer (20 mM Tris-HCl; pH 7.0, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.2 mM sodium O-thovanadate, 50 mM NaF, 1 μM okadaic acid) per 100×10^6 cells.

Whole-cell extracts (WCEs) enriched in nuclear proteins were prepared as described previously.[31] Briefly, cells were resuspended in 1 ml per 100×10^6 cells of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and incubated for 10 min on ice. Cells were harvested by centrifugation and the pellet was resuspended in 100 μl of buffer C (20 mM HEPES, pH 7.9 (v/v) 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA; each buffer contained the above-mentioned phosphatase and protease inhibitors), incubated for 30 min on ice and finally centrifuged for 20 min at 4°C to remove nuclear debris. The supernatants were shock freezed and stored at −80°C. Protein concentration was measured using the Bradford assay.

Western blot analysis and immunoprecipitation

As previously described,[32] protein lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose membrane (ECL membrane, Amersham) using an SDS electroblotting system (BioRad, Munich, Germany) for 4 h at a constant voltage of 50 V. The filter was treated with blocking solution (Boehringer Mannheim, Germany) for 1 h, incubated with primary antibody (1:1000) overnight in blocking solution and washed twice in TPBS. Filters were incubated for 1 h with appropriate secondary antibody (1:2000 horseradish peroxidase-linked anti-rabbit, anti-mouse or anti-goat immunglobulin; Santa Cruz) and specific proteins were visualized using the ECL detection system (Amersham, Germany). To verify application of equal amounts of protein, blots were stripped and reprobed.

Electrophoresis mobility shift analysis (EMSA)

WCEs (5 μg of total protein) were incubated with 3 μg poly(dI-dC) and 6 μl of binding buffer (60 mM HEPES pH 7.9, 1.8 mM EDTA, 30% (v/v) glycerol, 3 mM DTT, 15 mM $MgCl_2$ and 12 mM Tris-HCl, pH 7.9) for 5 min at room temperature, followed by 20 min incubation with 2 ng of end-labeled DNA harboring the N5-GAS-site (5′-TCGAGCCTGATTTCCCCGAAATGACGGC-3′) as described.[31] Samples were separated on a 6% polyacrylamide gel in 5 × TBE, gels were dried and visualized by autoradiography.

Proliferation assay

Cells were treated with different inhibitors for the indicated times. Relative number of viable cells was analyzed by trypan blue exclusion assay.

Cell-cycle analysis

Cell-cycle analysis was performed as described previously.[32] Briefly, K562 cells treated with imatinib mesylate were harvested at different times, washed twice with sample buffer (0.1% glucose in PBS) and fixed with 1 ml ice-cold ethanol (70% v/v in water) for at least 1 h at 4°C. After centrifugation, cells were resuspended in a sample buffer containing 50 μg/ml propidium iodide solution and 100 U/ml RNase A (Boehringer, Mannheim, Germany), incubated in the dark for 15 min and finally analyzed by flow cytometry (FACScan, Becton Dickinson, Germany). Cell-cycle distribution was analyzed using the Multi-Cycle program (Phoenix Flow Systems).

TUNEL assay

The presence of DNA nicking in apoptotic cells was assayed, with minor modifications, according to the protocol of the MEBSTAIN Apoptosis kit Direct (Immunotech, France). Briefly, $1–3 \times 10^6$ cells were collected, washed twice with washing solution (0.2% BSA in PBS) and fixed in 4% formaldehyde in PBS at 4°C for 30 min. Cells were washed twice, resuspended in

cold ethanol 70% and stored at −20°C for 30 min. After rehydration, cells were resuspended in TdT buffer and FITC–dUTP in the presence or absence of TdT enzyme and incubated at 37°C for 1 h. Cells were washed twice in 0.2% BSA in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, Germany).

Statistical analysis

For statistical analysis, the number of viable cells upon treatment with imatinib mesylate +/− wortmannin or Ara-C +/− imatinib mesylate were compared using the alternate (Welch), unpaired *t*-test (1993 GraphPad Software Inc.).

Results

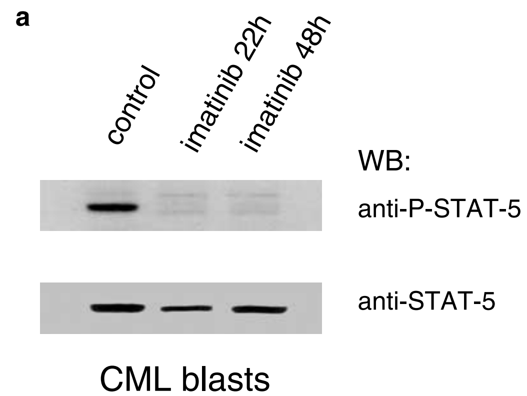
In primary CML blasts, imatinib mesylate inhibits tyrosine phosphorylation and DNA-binding activity of STAT-5 and induces apoptosis

The effects of imatinib mesylate on Bcr-Abl downstream pathways are well documented in BCR-ABL-positive cell lines.[33] However, so far only a few of these effects have been investigated in primary hematopoietic cells from CML patients. Therefore, we analyzed the effects of imatinib mesylate on STAT-5 and on induction of apoptosis in primary CML blasts. Primary CML blasts were treated with imatinib mesylate at a final concentration of 1 μM. At this concentration, selective suppression of growth of CFU-GM and burst-forming unit-erythroid (BFU-E) has been demonstrated.[34] As revealed by Western blot analysis, complete down-regulation of STAT-5 tyrosine-phosphorylation was already observed upon treatment with imatinib mesylate for 22 h (Figure 1a).

Upon tyrosine phosphorylation, STAT-5 proteins usually form homodimers, translocate to the nucleus and bind to specific DNA motifs.[21] As imatinib mesylate downregulates tyrosine phosphorylation of STAT-5, we also expected a reduced STAT-5-DNA-binding capacity. As a positive control STAT-5 DNA binding was investigated in GM-CSF-treated U937 cells (Figure 1b). In untreated primary CML blast, EMSA analysis showed constitutive STAT-5-DNA binding (Figure 1b). Upon imatinib mesylate treatment for 24 h, binding to the N5-GAS-site was strongly downregulated and completely suppressed after incubation for 63 h. These results strongly suggest reduced transcriptional activity of STAT-5 in imatinib mesylate-treated primary CML cells. As noted above, in primary hematopoietic cells from CML patients, little is known concerning the growth-inhibitory effects of imatinib mesylate and induction of apoptotic cell death. To investigate induction of apoptosis, primary myeloid CML blasts were treated with imatinib mesylate (1 μM) for different time periods, and TUNEL assays were performed. Upon exposure for 63 h, apoptotic cell death was observed in 17% of primary CML blasts (Figure 2). In untreated control cells, the basal rate of apoptosis was 7% (Figure 2). No difference in percentage of apoptotic cells was seen upon incubation for 24 and 48 h with and without imatinib (data not shown).

Imatinib mesylate treatment selectively inhibits proliferation due to induction of apoptosis

To compare the growth kinetics of bcr-abl-positive cells upon treatment with various kinase inhibitors, cell proliferation assays



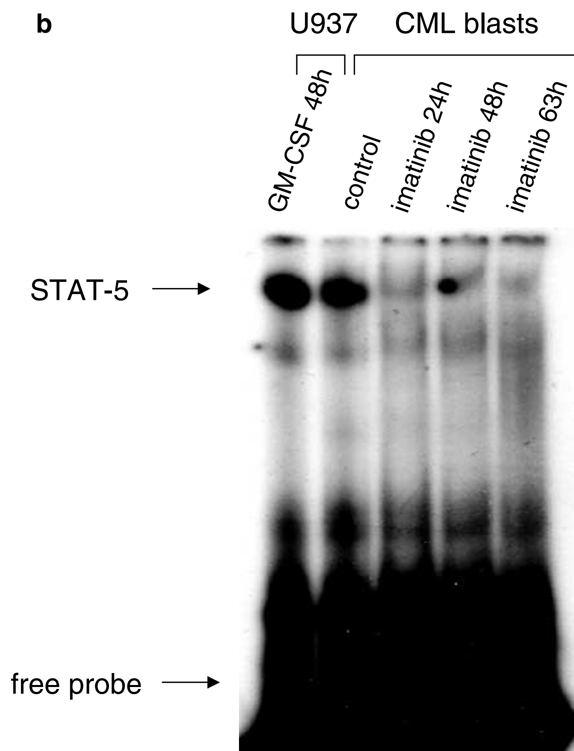


Figure 1 (a) Analysis of STAT-5 tyrosine-phosphorylation in primary hematopoietic cells. Primary CML blasts were maintained in RPMI 1640 +10% FCS and left untreated or treated with 1 μM imatinib mesylate for various time periods. Protein lysates were prepared and subjected to SDS-PAGE. Tyrosine phosphorylation of STAT-5 was analyzed by Western blot with a specific antiphosphotyrosine-STAT-5 antibody. The levels of STAT-5 were analyzed to assure equal loading. (b) Reduced STAT-5-DNA-binding activity upon imatinib mesylate treatment. Primary CML blasts were treated with imatinib mesylate for the indicated times. Nuclear extracts were prepared and EMSA was performed using an end-labeled GAS probe as described in Material and methods. U937 cells, incubated with GM-CSF for 48 h, were used as positive control. Arrows indicate STAT-5-specific DNA-binding complexes and free probe, respectively.

were performed using cells exposed to AG490, a JAK-2 inhibitor, and wortmannin, an irreversible PI-3-kinase inhibitor and imatinib mesylate. The JAK-2 and PI-3-kinase pathways have been described to be activated in BCR-ABL-positive cells.[35,13] In K562 cells, growth inhibition induced by imatinib mesylate occurred within 48 h (data not shown). Therefore, we evaluated the number of viable cells after 24 and 48 h. Upon

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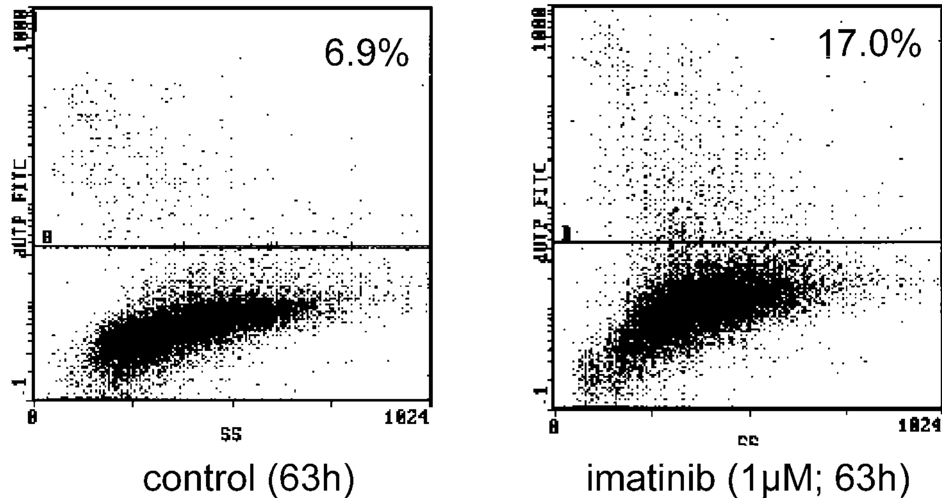

Figure 2 Induction of apoptosis in primary CML blasts. Primary cells were kept in RPMI 1640 +10% FCS with and without imatinib mesylate (1 μM) treatment for 63 h. Cells (2×10^6) were harvested and induction of apoptosis was investigated by TUNEL assay analysis. Numbers represent the percentage of cells undergoing apoptosis.

exposure to AG490 or wortmannin (0.25 μM), no apparent inhibition of proliferation was seen (Figure 3). Combination of imatinib mesylate (1.0 μM) plus wortmannin at concentrations ranging from 0.1 to 0.25 μM did not result in a statistically significant increase in inhibition of proliferation (Figure 3 and data not shown).

TUNEL assay was performed to confirm that the mechanism of imatinib mesylate in the inhibition of proliferation of K562 cells is induction of apoptosis. As shown in Figure 4a, significant induction of apoptotic cell death (51%) was seen as compared to untreated control cells. Specificity of imatinib in the induction of apoptosis was controlled in 32D cells transfected with and without BCR-ABL. Upon imatinib mesylate treatment for 12 h, significant induction of apoptosis was observed in 32D BCR-ABL p210-positive cells (Figure 4b, right panel), whereas no apoptosis was demonstrated in parental cells (Figure 4b, left panel). This result clearly indicates that the presence of the BCR-ABL oncogene is a prerequisite for induction of apoptosis by imatinib mesylate.

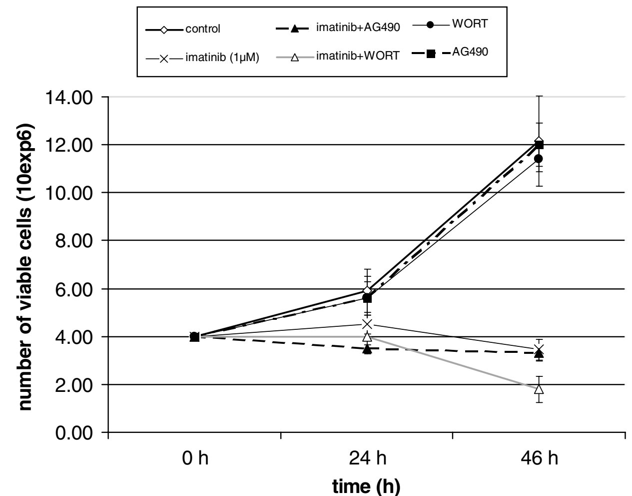

Figure 3 K562 cells were maintained in RPMI 1640 + FCS 10% and were treated with imatinib mesylate (1 μM), AG 490 (0.25 μM), wortmannin (0.25 μM) and wortmannin (0.25 μM) plus imatinib mesylate (1 μM) for the indicated times. Viable cells were counted after staining with trypan blue. As a control, cells were treated with 5 and 12 μM AG490, respectively. No effect on cell viability was noted (data not shown). The difference in the number of viable cells upon treatment with imatinib and imatinib plus wortmannin is not statistically significant (unpaired alternate *t*-test (Welch), $P = 0.11$).

Imatinib mesylate-induced activation of caspases

To further investigate the mechanism of apoptotic cell death, intracellular apoptotic pathways resulting in the activation of caspases were investigated. The DNA-binding subunit of replication factor C (RFCp140) and PARP are both specific targets of caspase-3.[36,37] Upon imatinib mesylate exposure of K562 cells, a time-dependent decrease in RFCp140 and PARP (116 kDa) protein levels could be demonstrated. In addition, the PARP cleavage product (89 kDa) was detected after 24 h (Figure 5a). No cleavage products were observed in BCR-ABL-negative U937 control cells (Figure 5a). To determine whether caspase-3 is involved in the downregulation of RFCp140 protein levels, we next analyzed RFCp140 protein expression in the presence of the specific caspase-3 inhibitor Z-DEVD-FMK. Pretreatment for 30 min followed by imatinib mesylate incubation resulted in a partial rescue of the imatinib mesylate-induced decrease in RFCp140 levels as demonstrated in Figure 5b. Thus, caspase-3-induced proteolytic cleavage of RFCp140 is involved in the downregulation of RFCp140 by imatinib mesylate.

Downregulation of constitutive STAT-5 tyrosine-phosphorylation upon Ara-C treatment

Development of clinical and molecular resistance in patients with CML blast crisis is an important issue in imatinib mesylate therapy. Combination with chemotherapy may have an advantage because of rapid elimination of most of the tumor cells. Recent publications demonstrated synergistic effects of imatinib mesylate in combination with Ara-C,[38–40] but the molecular mechanism is unclear. Inhibition of STAT activation through antileukemic drugs has been described previously.[41] Therefore,

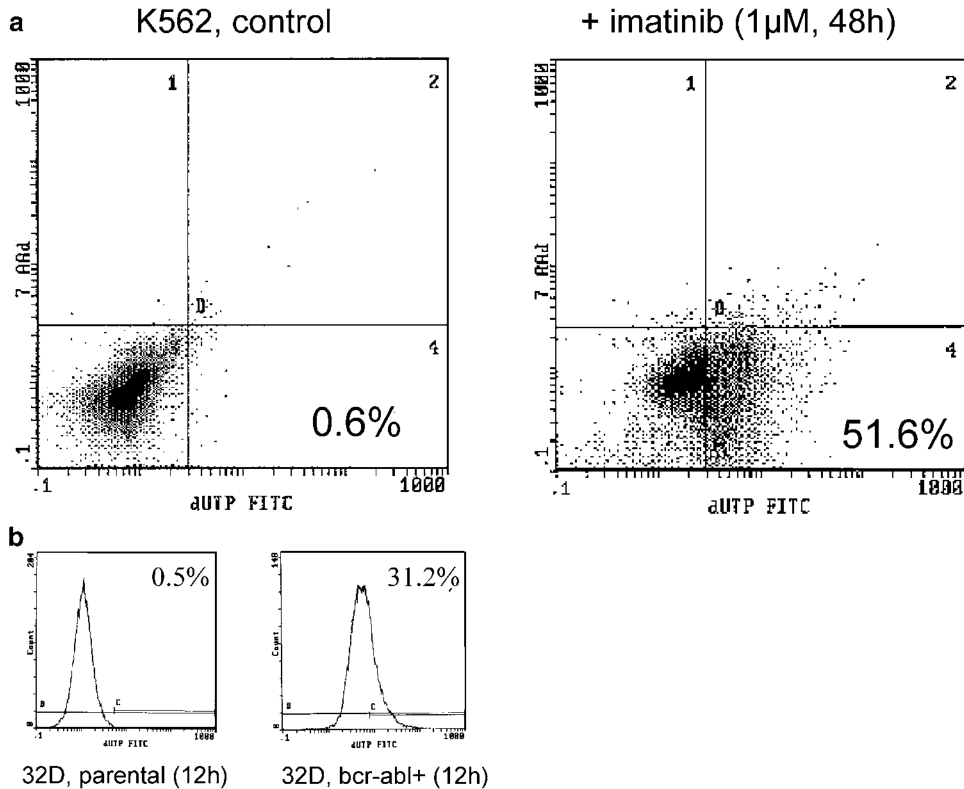

Figure 4 Induction of apoptosis in K562, parental 32D and 32D^{p210} cell lines. K562 cells (a) were maintained in RPMI 1640+10% FCS, parental and p210 32D cells (b) in IMDM-CM+10% FCS with and without Il-3, respectively. Cells were treated with 1 μM imatinib mesylate for the indicated times and induction of apoptosis was quantified by TUNEL assay. Numbers represent the percentage of cells in apoptosis.

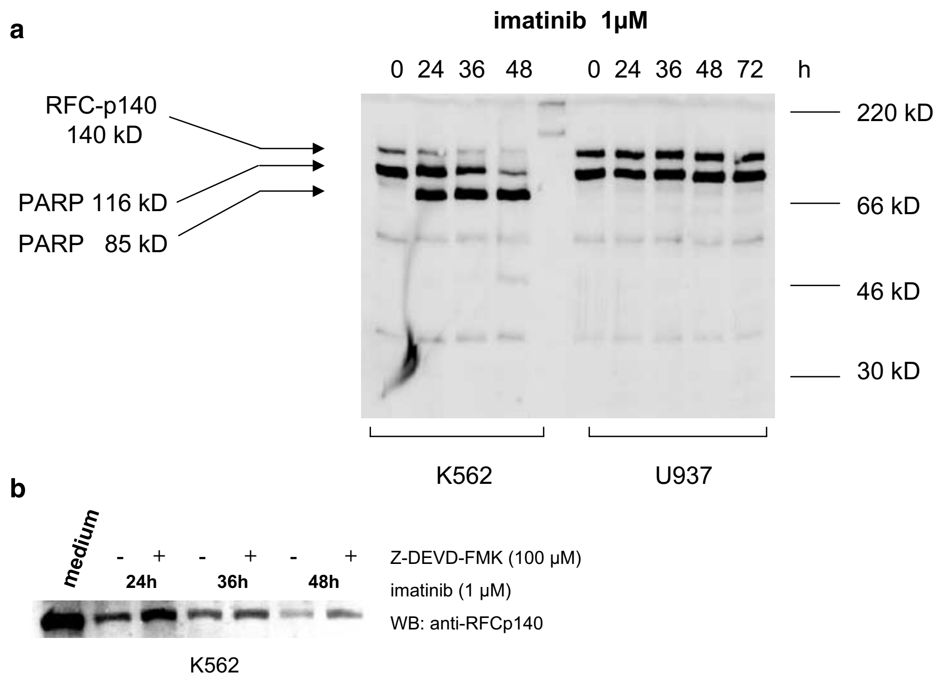

Figure 5 Activation of intracellular apoptotic pathways. K562 and U937 cells (a) were treated with imatinib mesylate (1 μM) for the indicated times. Whole-cell lysates were prepared and equal amounts of protein were subjected to SDS-PAGE. Western blot analysis was performed using antibodies specific to PARP and RFCp140. (b) K562 cells were pretreated with 100 μM Z-DEVD-FMK for 30 min followed by treatment with imatinib mesylate (1 μM) for different times. Western blot analysis using a specific anti-RFCp140 antibody was performed.

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we investigated the state of STAT-5 tyrosine-phosphorylation upon Ara-C treatment. K562 cells and primary CML blasts were treated with Ara-C (5 μM) for various time periods. As demonstrated in Figure 6a, STAT-5 tyrosine-phosphorylation was downregulated by Ara-C in a time-dependent manner in K562 cells. Almost complete inhibition of STAT-5 tyrosine-phosphorylation was seen in primary CML blasts upon incubation for 12 h (Figure 6b). In contrast, in K562 cells, tyrosine-phosphorylated STAT-5 protein levels could still be detected upon treatment for 48 h. To demonstrate that the observed decrease in tyrosine phosphorylation of STAT-5 does not represent a global effect on tyrosine phosphoproteins, Western blot analysis using an antiphosphotyrosine antibody was performed. No change in overall tyrosine phosphorylation of protein lysates from Ara-C-treated K562 cells could be demonstrated (Figure 6c).

Synergistic effects of Ara-C and imatinib mesylate on the level of STAT-5 tyrosine-phosphorylation

To investigate a possible synergistic effect on the level of STAT-5 tyrosine-phosphorylation, K562 cells were treated with Ara-C, imatinib mesylate or in combination. As 1 μM of imatinib mesylate almost completely suppressed STAT-5 tyrosine-phosphorylation (Figure 1a), K562 cells were treated with 0.1 μM of imatinib mesylate for 24 h. Incubation with imatinib mesylate (0.1 μM) and with Ara-C, respectively resulted in significant, but not complete, downregulation of STAT-5 tyrosine-phosphorylation (Figure 7). Upon treatment with both compounds almost complete inhibition of STAT-5 tyrosine-phosphorylation could be demonstrated, consistent with a synergistic effect of imatinib mesylate and Ara-C (Figure 7). Further, STAT-5 DNA-binding

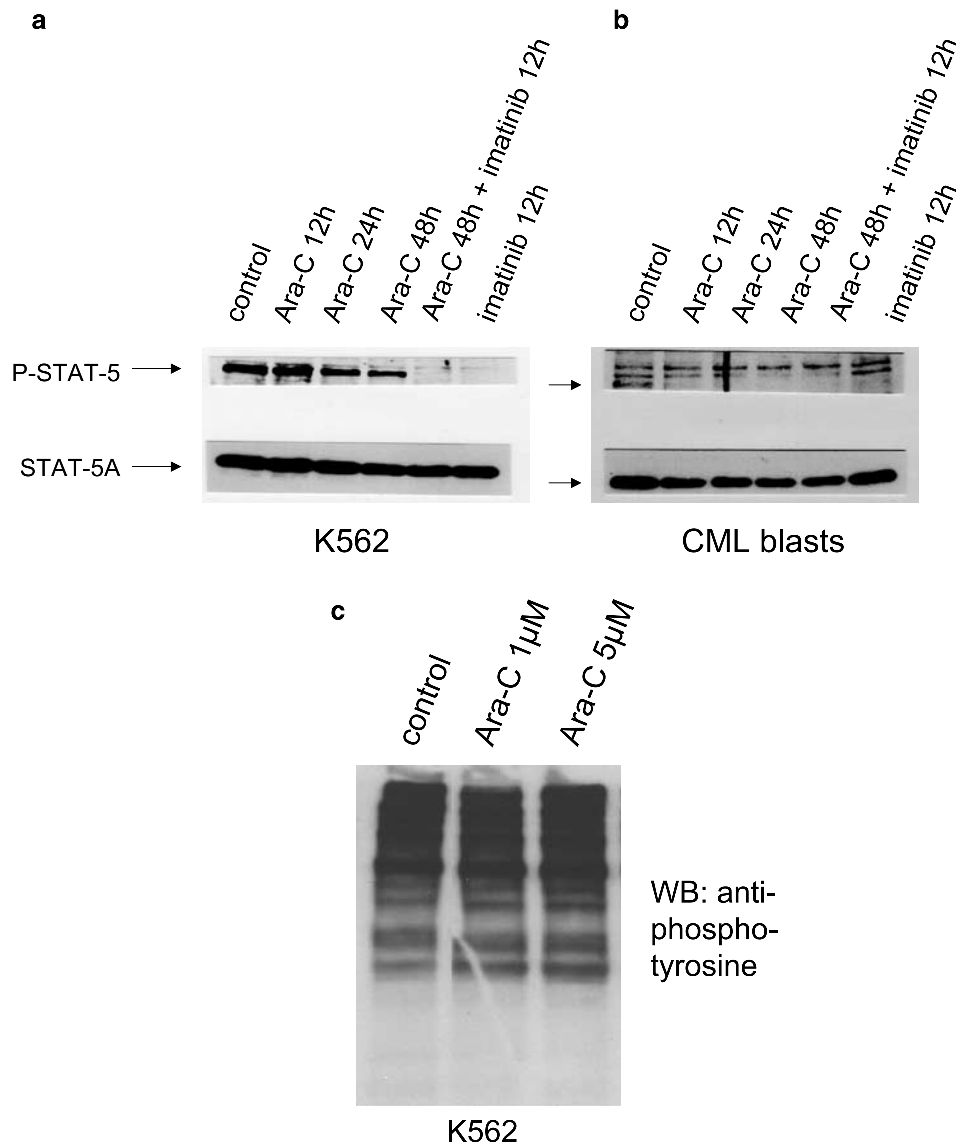

Figure 6 Downregulation of STAT-5 tyrosine-phosphorylation upon Ara-C treatment. K562 (a) and primary CML cells (b) were treated with Ara-C (5 μM) and imatinib mesylate (1 μM) for the indicated time period. Whole-cell lysates were prepared and equal amounts of protein were subjected to SDS-PAGE. Western blot analysis was performed using a specific antiphosphotyrosine-STAT-5 antibody. To control equal loading, the blot was stripped and reprobed with anti-STAT-5A. Arrows indicate specific P-STAT-5 and STAT-5A bands, respectively; the upper bands in Figure 6b are nonspecific. One representative experiment out of a total of five (a) and two (b), respectively, is shown. (c) K562 cells were treated with 1 or 5 μM Ara-C for 24 h. Protein lysates were prepared and subjected to SDS-PAGE. Overall tyrosine phosphorylation was analyzed by Western blot with a specific antiphosphotyrosine antibody. One representative experiment out of a total of two is shown.

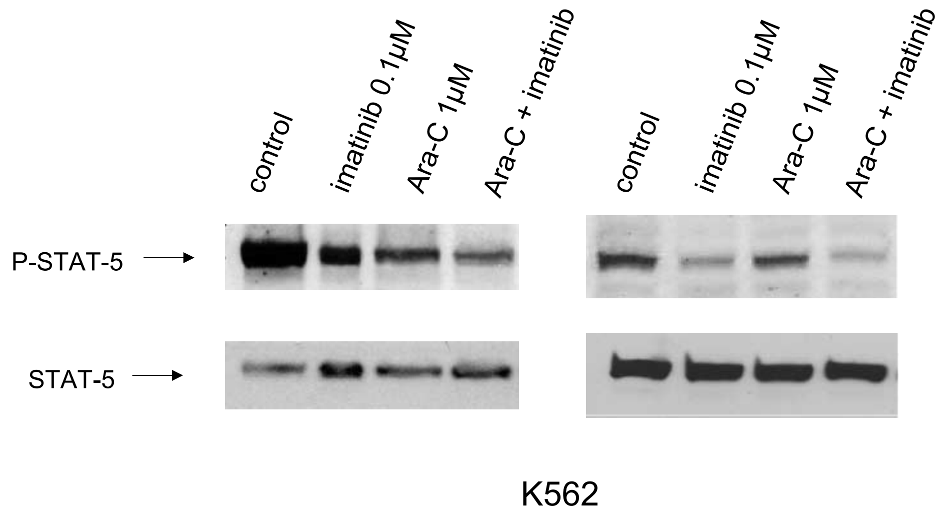

Figure 7 Synergistic effects of imatinib mesylate in conjunction with Ara-C. K562 cells were treated with imatinib mesylate (0.1 μM), Ara-C (1 μM) or in combination as indicated. Whole-cell lysates were prepared and equal amounts of protein were subjected to SDS-PAGE. Western blot analysis was performed using a specific antiphosphotyrosine-STAT-5 antibody. To control equal loading, the blot was stripped and reprobed with anti-STAT-5. Figure 7 shows two out of five representative experiments.

capacity was investigated upon treatment with imatinib mesylate, Ara-C and a combination of both drugs, respectively. As expected, K562 cells treated either with Ara-C or imatinib mesylate (0.1 μM) showed impaired STAT-5 DNA-binding activity (Figure 8). Again, treatment with Ara-C plus imatinib mesylate at low concentration (0.1 μM) resulted in synergistic effects on the level of STAT-5 DNA binding (Figure 8).

Combination treatment results in decreased cell proliferation and increased apoptotic cell death

To evaluate functional consequences of the above-described synergistic effects on the level of STAT-5 tyrosine-phosphorylation, cell proliferation assays and cell-cycle analysis were performed. K562 cells were treated with Ara-C, imatinib mesylate (0.1 μM) and with a combination of both drugs, respectively. Imatinib mesylate at a concentration of 0.1 μM only marginally impaired cellular proliferation within 48 h (Figure 9a). In contrast, Ara-C treatment resulted in significant inhibition of proliferation. Combination treatment further enhanced this effect (Figure 9a). To investigate the mechanism of inhibition of proliferation, cell-cycle analysis was performed. Minimal induction of cell death was seen upon incubation with imatinib mesylate (0.1 μM) for 48 h. Ara-C treatment resulted in a G2 arrest and minimal cell death (Figure 9b). In contrast, combination treatment was followed by a significant increase of the sub-G1 fraction, consistent with a synergistic effect of imatinib mesylate and Ara-C on induction of apoptosis (Figure 9b).

Inhibition of Hck tyrosine-phosphorylation upon Ara-C treatment

Interaction of Hck, a member of the src tyrosine kinase family, with Bcr-Abl appears to be essential for transformation by Bcr-Abl.[42] Recently, Hck has been demonstrated to couple Bcr-Abl to STAT-5 activation in myeloid leukemia cells.[43] To determine whether Hck plays a role in the observed inhibition of STAT-5

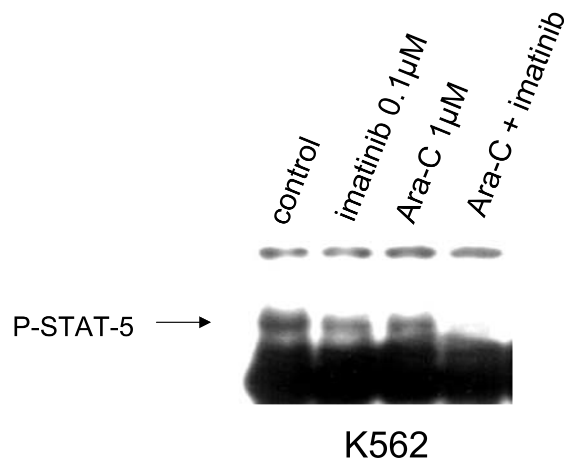

Figure 8 Reduced STAT-5-DNA-binding activity upon Ara-C treatment. K562 cells were treated with imatinib mesylate (0.1 μM), Ara-C (1 μM) or in combination for 24 h. Whole-cell lysates were prepared and EMSA was performed using an end-labeled GAS probe.

tyrosine-phosphorylation upon Ara-C treatment, Hck protein expression and tyrosine phosphorylation were examined. As shown in Figure 10, imatinib mesylate induced strong inhibition of Hck tyrosine-phosphorylation. Further, Ara-C treatment (1 μM) resulted in downregulation of Hck tyrosine-phosphorylation. Again, combination of the two agents caused a synergistic effect, similar to Ara-C- and imatinib mesylate-induced inhibition of STAT-5 tyrosine-phosphorylation (Figure 7).

Discussion

The effects of Bcr-Abl on signal transduction pathways have been demonstrated in several studies.[12–18] In BCR-ABL-negative cells, a number of these signaling pathways are shared by cytokines like GM-CSF or Il-3, resulting in proliferation and increased viability.[44,45] The JAK-STAT pathways play a major

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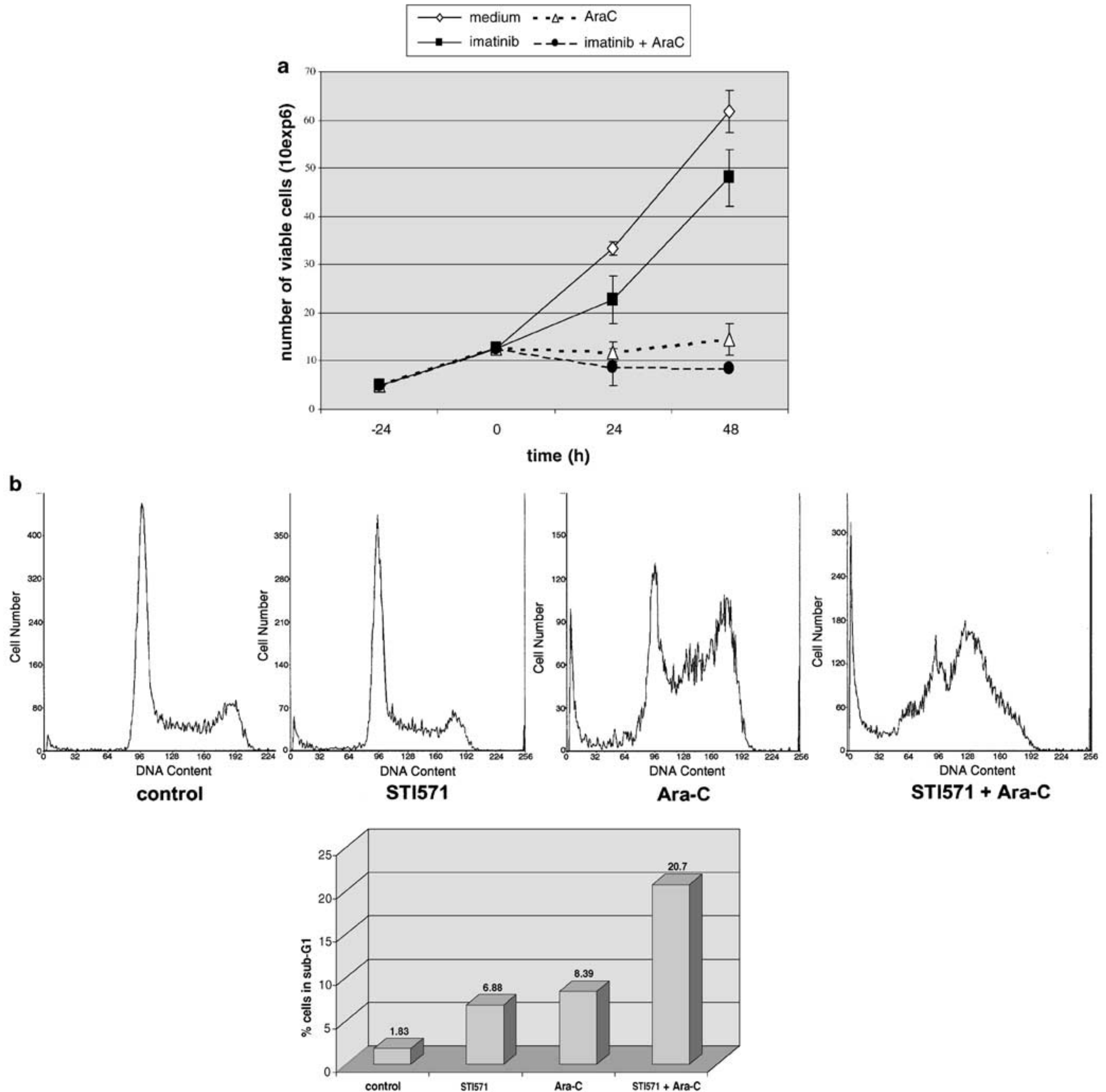

Figure 9 (a) K562 cells were maintained in RPMI 1640 +FCS 10% and were treated with imatinib mesylate (0.1 μM), Ara-C (1 μM) and Ara-C (1 μM) plus imatinib mesylate (0.1 μM) for the indicated times. Viable cells were counted after staining with trypan blue. One representative experiment out of a total of three is shown. The difference in the number of viable cells upon treatment with Ara-C and Ara-C plus imatinib is not statistically significant (unpaired alternate *t*-test (Welch), $P=0.087$). (b) Cell-cycle analysis of K562 cells upon imatinib mesylate and Ara-C treatment. K562 cells were treated with 0.1 μM imatinib mesylate, 1 μM Ara-C or imatinib mesylate+Ara-C. After 48 h, propidium iodide-stained cells were analyzed for their DNA content by flow cytometry. The percentage of hypoploid cells is indicated.

role in normal hematopoiesis, and recent reports demonstrate an essential function of STAT-5 conferring growth factor independence and resistance to apoptosis in BCR-ABL-positive cells.[18,46,47]

In this report, we investigated the effects of imatinib mesylate and of Ara-C on STAT-5 tyrosine-phosphorylation in primary CML blasts and in BCR-ABL-positive cell lines. Upon treatment with imatinib mesylate, STAT-5 tyrosine-phosphorylation is almost completely abrogated, resulting in abolished STAT-5-DNA-binding capacity and induction of apoptosis. In addition, we investigated the mechanisms of induction of apoptosis in BCR-ABL-positive cell lines upon exposure to imatinib mesylate. Proliferation and TUNEL assays clearly demonstrated the specificity of imatinib mesylate for Bcr-Abl, as no inhibitory effect could be demonstrated neither in U937 cells (data not shown) nor in parental 32D cells. Recently, JAK-kinase-

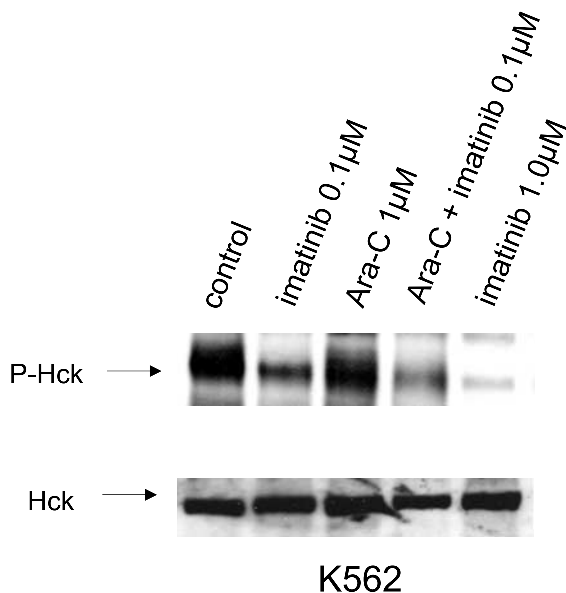

Figure 10 Downregulation of Hck tyrosine-phosphorylation upon Ara-C treatment. K562 cells were treated with Ara-C (1 μM), imatinib mesylate (0.1 μM) or in combination for the indicated time period. Whole-cell lysates were prepared and equal amounts of protein were subjected to SDS-PAGE. Western blot analysis was performed using an antiphosphotyrosine-Hck antibody. To control equal loading, the blot was stripped and reprobed with anti-Hck. Arrows indicate specific phospho-Hck and Hck bands, respectively. One representative experiment out of a total of two is shown. Crossreactivity with phospho-Lyn and phospho-Lck was excluded by immunoblotting with α-Lyn and α-Lck (data not shown). This analysis showed that Hck, Lyn and Lck can be clearly distinguished by their apparent MW in this SDS-PAGE analysis.

dependent pathways have been shown to be involved in the regulation of bcl-X_L and apoptosis.[48] In addition, some reports described the involvement of PI-3-kinase and downstream proteins (Akt, Bad) in leukemogenesis and malignant proliferation.[49,50,13] In our investigation, we treated K562 cells with JAK-2- and PI-3-kinase inhibitors, but no effect on proliferation could be detected. Treatment with imatinib mesylate in combination with wortmannin resulted in a small increase of cellular sensitivity. These results suggest only minimal dependence of BCR-ABL positive K562 cells on these pathways. Activation of STAT-5 has been described to play a more significant role in BCR-ABL-positive cells.[16–18,25,47] This is in line with recent results detecting only minimal changes in MAPK- or PI-3-kinase activity in BCR-ABL-positive cells as compared to the change observed in STAT-5 phosphorylation.[51,52]

For this reason, targeting the STAT-5 molecule to exhibit synergistic or additive effects may be more useful as compared to targeting other downstream signaling pathways. In this report, we investigated the effect of Ara-C on STAT-5 phosphorylation. Ara-C is a commonly used and effective drug in CML therapy. Treatment with Ara-C caused a time-dependent downregulation of STAT-5 tyrosine-phosphorylation resulting in reduced DNA-binding activity in K562 cells and primary CML blasts. Further, combination treatment suppressed malignant proliferation because of synergistic induction of apoptotic cell death. Recent studies investigated the effects of imatinib mesylate in combination with Ara-C on viability.[38–40] These studies also demonstrated a synergistic or additive effect. Our results suggest that the molecular mechanism involved is inhibition of STAT-5 tyrosine-phosphorylation. In contrast to fludarabine, which caused specific depletion of STAT-1 protein and mRNA levels,[41] no decrease of STAT-5 protein levels was detected. The mechanism of Bcr-Abl-induced activation of STAT-5 is poorly understood. Immunoprecipitation experiments revealed only a small portion of STAT-5 in complex with Bcr-Abl[17,18] and also STAT-5 tyrosine-phosphorylation does not depend on Jak-2 activation.[17] Recent studies[42,43] provide evidence that downregulation of STAT-5 tyrosine-phosphorylation may be effected by inhibition of the tyrosine kinase Hck, a member of the src kinase family. In BCR-ABL-positive cells, Hck is strongly tyrosine phosphorylated and it appears to be essential for malignant transformation.[42] Further, Klejman *et al.*[43] identified Hck as the intermediate protein kinase responsible for Bcr-Abl-mediated activation of STAT-5 in myeloid cells. In our studies, Ara-C treatment of K562 cells resulted in inhibition of Hck tyrosine-phosphorylation, similar to that seen in STAT-5 tyrosine-phosphorylation. Thus, in view of the data mentioned above this suggests that inhibition of STAT-5 tyrosine-phosphorylation is mediated by the effects of Ara-C on Hck tyrosine-phosphorylation. Downregulation of Hck tyrosine-phosphorylation may be due to activation of phosphatases. As an example, it has recently been shown that Ara-C treatment results in the phosphorylation of SHPTP1 and in increased phosphatase activity.[53]

Overall, the data presented here demonstrate specific effects of imatinib mesylate on inhibition of proliferation and induction of apoptosis in BCR-ABL-positive cells. Targeting Bcr-Abl results in decreased STAT-5 tyrosine-phosphorylation followed by suppression of STAT-5 DNA binding and induction of apoptosis in cell lines as well as in primary CML cells. Furthermore, the data suggest that Ara-C exhibits its antiproliferative effect by targeting STAT-5 tyrosine-phosphorylation. This may result in the observed synergistic or additive effects on proliferation upon combination with imatinib mesylate.

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