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Epigenetic down-regulation of *BIM* expression is associated with reduced optimal responses to imatinib treatment in chronic myeloid leukaemia

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ABSTRACT

Background: Expression of the pro-apoptotic BCL-2-interacting mediator (*BIM*) has recently been implicated in imatinib-induced apoptosis of *BCR-ABL1*$^+$ cells. However, the mechanisms involved in the regulation of *BIM* in CML and its role in the clinical setting have not been established.

Design and methods: We analysed the mRNA expression of *BIM* in 100 newly diagnosed patients with CML in chronic phase by Q-RT-PCR and the protein levels by Western blot analysis. Methylation status was analysed by bisulphite genomic sequencing and MSP. CML cell lines were treated with imatinib and 5-aza-2'-deoxycytidine, and were transfected with two different siRNAs against *BIM* and cell proliferation and apoptosis were analysed.

Results: We demonstrated that down-regulation of *BIM* expression was present in 36% of the patients and was significantly associated with a lack of optimal response to imatinib as indicated by the decrease in cytogenetic and molecular responses at 6, 12 and 18 months in comparison with patients with normal *BIM* expression ($p < 0.05$). Expression of *BIM* was mediated by promoter hypermethylation as demonstrated by restoration of *BIM* expression after treatment of CML cells with 5-aza-2′-deoxycytidine. Using CML cell lines with low and normal expression of *BIM* we further demonstrated that the expression of *BIM* is required for imatinib-induced CML apoptosis.

Conclusion: Our data indicate that down-regulation of *BIM* is epigenetically controlled by methylation in a percentage of CML patients and has an unfavourable prognostic impact, and that the combination of imatinib with a de-methylating agent may result in improved responses in patients with decreased expression of *BIM*.

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1. Introduction

The deregulated activity of the BCR-ABL1 tyrosine kinase encoded by the *BCR-ABL1* oncogene remains a therapeutic target in all phases of chronic myelogenous leukaemia (CML). Expression of BCR-ABL1 is associated with the deregulation of a number of pathways implicated in cell proliferation and survival leading to resistance to apoptosis.[1] CML cells express a number of antiapoptotic molecules that may contribute to enhanced survival of leukaemic cells[2] such as the members of the BCL-2 family including BCL-xL, MCL-1 and BCL-2.[1,3] However, the relative contribution of each of these molecules to inhibition of apoptosis in CML cells has not been clarified. A major inhibitor and antagonist of BCL-2 is the BCL-2-interacting mediator (BIM), a BH3-only protein of the BCL-2 family which is considered to have pro-apoptotic effects in various cell types.[4]

It has recently been described that BCR-ABL1$^+$ cells from patients with CML as well as CML cell lines established from patients in blast crisis express significantly lower amounts of *BIM* mRNA and BIM protein than normal cells, and that the BCR-ABL1 inhibitors, imatinib and nilotinib, promote expression of BIM in these cells.[5–8] Moreover, studies using siRNA and cells from gene-targeted mice revealed that BIM plays a major role in imatinib-induced apoptosis of BCR-ABL1 leukaemic cells and that the loss of BIM abrogates this killing.[8] These results suggest that BIM is an important downstream target that supports cell survival of BCR-ABL1-expressing haematopoietic cells and is a possible cause of imatinib resistance. However, its role in the clinical setting has not been established.

We studied the expression and regulation of *BIM* in a large group of patients with CML and demonstrated that down-regulation of *BIM* is associated with a poor prognosis in patients treated with imatinib, in part due to *BIM*-dependent resistance to imatinib. Furthermore, *BIM* expression is at least in part epigenetically controlled by methylation supporting a role for treatment with a de-methylating agent in patients with CML and hypermethylated *BIM*.

2. Material and methods

2.1. Cell lines and samples

Five human Ph$^+$ CML (K562, KU812, KYO-1, TCC-S and BV173) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in RPMI 1640 (BioWhitaker, Walkersvill, MD) supplemented with 20% foetal bovine serum (Gibco, Grand Island, NY), penicillin/streptomycin (BioWhitaker, Walkersvill, MD) and 20 mM Hepes buffer (BioWhitaker, Walkersvill, MD) until harvested for extraction of DNA, RNA and protein. Heparinised bone marrow cells were collected from patients in chronic phase (CP) CML at diagnosis and from healthy marrow donors. We studied 100 patients with Ph$^+$ CP-CML and treated them with imatinib as the front-line therapy. The study was approved by the Investigational Review Boards in accordance with the policies of the Department of Health and Human Services. All patients gave informed consent for the use of their samples. Risk categories according to the Sokal and Hasford score systems were determined as described.[9,10] Haematologic, cytogenetic and molecular responses and disease progression were defined as reported by an expert panel of the European LeukemiaNet.[11] All patients were evaluated for a response at 6, 12 and 18 months on imatinib therapy following the recommendations of the same panel of experts.[12]

2.2. Expression of BIM mRNA

Expression of *BIM* transcripts was analysed by the Quantitative real-time PCR technique. Total RNA was extracted from marrow samples with Ultraspec (Biotecx, Houston, TX) following the manufacturer's instructions. Reverse transcription was performed on 1-μg total RNA, after heating at 70 °C for 5 min, with random hexamers as reaction primer. The reaction was carried out at 42 °C for 45 min in the presence of 12 U Avian Myeloblastosis virus reverse transcriptase. Q-RT-PCR for *BIM* expression was performed with the LightCycler technology, using 1 μl of cDNA in 20-μl reaction volume with 0.4 μmol/l of each primer (forward: 5′- AGTGGGTATTTCTCTTTTGACACAG-3′; reverse: 5′- TCAATGCCTTCTCCATACCAGACG-3′), and 2 μl of 10× LightCycler FastStar DNA Master SYBR Green I. The final Mg^{2+} concentration in the reaction mixture was adjusted to 3.5 mmol/l. The following programme conditions were applied for Q-RT-PCR running: denaturation programme, consisting in one cycle at 95 °C for 8 min; amplification programme, consisting in 45 cycles at 95 °C for 5 s, 60 °C for 10 s and 72 °C for 15 s; melting programme, one cycle at 95 °C for 0 s, 40 °C for 60 s and 90 °C for 0 s; and cooling programme, one cycle at 40 °C for 60 s. The temperature transition rate was 20 °C/s, except in the melting programme, which was 0.4 °C/s between 40 °C and 90 °C. *Abelson* gene (*ABL1*) was employed as the reference gene, and it was amplified in the same run following the same procedure described above (forward: 5′-CCCAACCTTTTCGTTGCACTGT-3′; reverse: 5′-CGGCTCTCGGAGGAGACGTAGA-3′). In order to reduce the variation between different assays and samples, a procedure based on the relative quantification of target genes versus their controls/calibrators in relation to the reference gene was used. Calculations were automatically performed by LightCycler software (RealQuant, version 1.0). The normalised ratio (N_{Bim}), expressed as the percentage of the control/calibrator, was obtained by RealQuant software. The selected controls/calibrators were bone marrow specimens from healthy donors. They were considered as 100% expression.

2.3. Western blot analysis

Proteins extracted from the CML cell lines TCC-S, KYO, BV173 and KU812 were analysed by polyacrylamide gel electrophoresis, and the protein bands were electrophoretically transferred onto nitrocellulose membranes as previously described.[13] The membranes, after being blocked, were incubated first with primary antibody for BIM (diluted 1:5000 for 4 h; Stressgen, Ann Arbor, MI), PARP (1:1000 o/n; Promega Corp., Madison, WI) or β-Actin (1:4000 for 1 h; Sigma, St. Louis, MO) and then with alkaline phosphatase-conjugated secondary antibodies (1:10000 for 1 h; Sigma, St. Louis, MO). Bound antibodies were revealed by a chemiluminescent reagent (Tropix, Bedford, MA) and were detected using HyperfilmTM

ECL (Amershan Biosciences, Little Chalfont, Buckinghamshire, England). β-Actin was used as a loading control.

2.4. Methylation analysis

Methylation status of *BIM* promoter was analysed by bisulphite genomic sequencing and Methylation-Specific PCR (MSP) techniques. One microgram of genomic DNA was modified using the CpGenomic™ DNA Modification Kit (Intergen Company, Purchase, NY). Bone marrow DNA from healthy donors was used as the unmethylated control and human male genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY, USA) was used as the methylated control. After bisulphite modification, (a) for sequencing analysis: *BIM* promoter was amplified by nested PCR. First PCR was realised using 3 μl of modified DNA and BIM-SD (5′-GGATTT AGTTGTAGATTTTGTAGG-3′) and BIM-SB4 (5′- TAAAAAATA CCCCCAAACAAAATAC-3′) primers and the second PCR was realised using 1 μl of the first PCR product and BIM-SB3 (5′-GTTGGAGTTATAAATTTTATTGTGA-3′) and BIM-SR (5′- CTCTT TACCCAAAACAAACTTCTTC-3′) primers.[14] Both PCRs were carried out in a total volume of 25 μl, with 1 U high fidelity Platinum Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 50 pmol of each primer and were performed under the following conditions: 94 °C for 10 min, 30 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min, and a final elongation cycle at 72 °C for 10 min. The second 244-bp PCR products, containing 16 CpG dinucleotides, were subcloned into pCR® 4-TOPO® plasmid and candidate plasmid clones were sequenced as previously described by our group.[15] (b) For MSP: PCR amplifications were carried out in a total volume of 25 μl, using 3 μl of modified DNAs, 1 U AmpliTaq Gold DNA Polymerase, 1.5 mM MgCl2, 0.2 mM dNTPs and 50 pmol of each primer: BIM-MD (5′- AGTATTTTCGGTAAATAATGGGGTC-3′) and BIM-MR (5′-GAATAAATCAAAAACTCCCAACG-3′) for the methylated reaction and BIM-UD (5′-GTATTTTTGGTAAATAATGGGGTTG-3′) and BIM-UR (5′- CAAATAAATCAAAAACTCCCAACA-3′) for the unmethylated reaction, which amplified a 139-bp product. PCR conditions for all the primers were 94 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min. The final extension was at 72 °C for 10 min. MSP assay products were separated on a 1.8% agarose gel, stained with ethidium bromide and visualised under UV light.

2.5. In vitro treatment with 5-aza-2′-deoxycytidine and imatinib

CML-derived cell lines were grown at a cell density of 1×10^6 cells/ml and were treated with 0.5, 1 and 2 μM of 5-aza-2′-deoxycytidine for 24 h or with 4 μM for 4 d. Imatinib (generously provided by Dr. Elisabeth Buchdunger, Novartis, Basel, Swizterland) was used to treat CML cell lines for 24 h at 0.1, 2 and 10 μM. The CML cell line BV173 was treated with 2 μM of imatinib in combination with 1 μM of 5-aza-2'-deoxycytidine for 24 h.

2.6. Cell transfection studies

Cell transfections were done using 100 μl of Cell Line Nucleofector Solution V (Amaxa GmbH, Köln, Germany) and the program T-06 from the Nucleofector device (Amaxa GmbH, Köln, Germany). First, we optimised the *BIM* siRNA transfection protocol for TCC-S cells by assaying five different siRNA concentrations, by time intervals (24, 48, 72 and 96 h) and by the number of transfections (single transfection or three repetitive transfections each for 24 h). For this cell line, the concentration of 50 nM was identified as the best one (data not shown) so cells were nucleofected with two different *BIM* Silencer Select siRNAs (s195012 and s19474) and Silencer Select Negative Control-1 siRNA (Ambion, Austin, TX) at a final concentration of 50 nM. We used two different siRNAs against *BIM* target to demonstrate that the results obtained with *BIM* siRNA nucleofection are not due to a combination of inconsistent silencing and sequence specific off-target effects. Silencer Select Negative Control-1 siRNA was used to demonstrate that the nucleofection did not induce non-specific effects on gene expression. After transfections, viable cells were always isolated by density gradient centrifugation (Ficoll-Paque method). The *BIM* mRNA expression was analysed by Q-RT-PCR (*GUS* was employed as the reference gene) 24 h after a single transfection and BIM protein was analysed by Western blot analysis 24 h after three repetitive transfections. Transfection efficiency was determined by flow cytometry using the BLOCK IT Fluorescent Oligo (Invitrogen Life Technologies, Paisley, UK).

2.7. Cell proliferation assays

Cell proliferation was analysed by three different techniques: (1) *Cell cycle analysis*: 250,000 cells were cultured at a density of $1x10^6$ cells/ml, washed twice with PBS, resuspended in 0.2% Tween-20 in PBS and 0.5 mg/ml RNase A (Sigma, St. Louis, MO) and were incubated for 30 min at 37 °C. Subsequently, cells were stained with 25 μg/ml of propidium iodide (Sigma, St. Louis, MO) and were analysed by FACS using Becton Dickinson flow cytometer BD FACScan (Becton Dickinson, San Jose, CA). (2) *Neutral red staining assay*: cells were cultured at a density of 1×10^6 cells/ml in 96-well plates (200,000 cells/well). After treatment, 250 μl of neutral red solution (50 μl of neutral red at 0.5 mg/ml diluted in saline + 200 μl of growth medium) (Sigma, St. Louis, MO) was added to cells. After 2 h at 37 °C, plates were centrifuged at 800 g for 10 min and the medium was removed. The plates were rinsed thrice with PBS. Then 100 μl of 0.05 M sodium phosphate (monobasic) in 50% ethanol was added. The plates were vortexed in a plate shaker, incubated for 10 min at room temperature and read in a plate reader at 540 nm absorbance. Data were calculated as the percentage of total absorbance of treated cells / absorbance of non-treated cells and were expressed as means ± SEM. (3) *Cell viability assay*: the CellTiter-Glo Luminescent Cell Viability Assay (Promega Corp., Madison, WI) was used following the manufacturer's instructions. This method of determining the number of viable cells in culture is based on the quantisation of the ATP present, which signals the presence of metabolically active cells. Data were calculated as the percentage of total luminescence of treated cells/luminescence of non-treated cells and were expressed as means ± SEM.

2.8. Apoptosis assays

Apoptosis was analysed by two different techniques: (1) *Caspase-3 analysis by FACS*: the FITC-Conjugated Monoclonal Active Caspase-3 Antibody Apoptosis Kit (BD Pharmingen, San Diego, CA) was used following the manufacturer's instructions. The mean percentage of cells expressing active caspase-3 is shown. (2) *Detection of the 85-kDa fragment of PARP*: the detection by Western blot analysis of the 85-kDa fragment of PARP that results from caspase-3 cleavage was also used as a marker for apoptosis.

2.9. Statistical analysis

All calculations were performed with the SPSS statistical package (SPSS, Chicago, IL). The medians, standard deviations (SDs) and interquartile ranges for age and the most relevant clinical and laboratory findings at diagnosis were calculated for patients with and without *BIM* methylation and were tested for any significant differences with the Mann–Whitney *U* test (for continuous variables) or χ^2 analysis and Fisher exact test (for categorical variables). Disease progression was defined by any of the following events, whichever came first: (1) death from any disease-related cause during treatment, (2) the development of accelerated-phase CML (defined by the presence of at least 15% blasts in the blood or bone marrow, at least 30% blasts plus promyelocytes in the blood or bone marrow, >20% peripheral basophils, or thrombocytopaenia $<100\times10^9$/L unrelated to treatment), (3) blast-phase CML (defined by the presence of at least 30% blasts in the blood or bone marrow or extramedullary blastic involvement), (4) loss of complete haematologic response (defined by the appearance of any of the following findings in two blood samples obtained at least one month apart: a white cell count $>20\times10^9$/L, a platelet count $>600\times10^9$/L, the appearance of extramedullary disease, the appearance of at least 5% myelocytes and metamyelocytes in the peripheral blood, or the appearance of blasts or promyelocytes in the peripheral blood), (5) loss of major cytogenetic response (defined as >30% increase in the Ph^+ cells in two studies performed at least one month apart), or (6) an increasing white cell count (defined as a doubling of the count to more than 20×10^9/L in two occasions at least 1 month apart in a patient who had never strictly had a complete haematologic response despite the maximum tolerated doses of therapy).

Progression-free survival (PFS) was measured from CML diagnosis to the appearance of progression as defined above or death without disease progression, and it was censored only for those patients alive and without evidence of progression at the last follow-up. Distributions of PFS curve were estimated by the method of Kaplan and Meier, with the 95% confidence intervals being calculated by means of Greenwood's formula. Comparison of PFS between groups was based on the log-rank test.

3. Results

3.1. Expression of BIM in patients with CP-CML correlates with cytogenetic and molecular responses

As previous studies indicate that the expression of *BIM* is down-regulated in patients with CML[5] and in order to demonstrate if expression was associated with the response to treatment, we analysed BM cells from a group of 100 patients with CP-CML at diagnosis. Clinical and laboratory features of CML patients are shown in Table 1. Normalised ratios of *BIM* expression were determined using BM-MNC from 30 healthy controls. N_{bim} ratios fell between 82% and 100% (mean N_{bim}: 90 ± 7%) in healthy individuals so N_{bim} values equal to or below 69% (determined as the mean N_{bim} from normal individuals minus three SDs) were chosen to define reduced expression of *BIM* in CML samples. Using this cut-off value, down-regulation of *BIM* was found in 36% of CML patients (mean N_{bim}: 34 ± 12%) while the mean expression in the other 64 patients was 85 ± 9%. Interestingly, cytogenetic and molecular responses were significantly worse in patients with down-regulation of *BIM* expression than in patients with normal *BIM* levels ($p < 0.05$) with an increase in suboptimal responses and failures[11] in patients with low *BIM* expression (Fig. 1). In addition, time to achieve a complete cytogenetic response was significantly longer for patients with reduced *BIM* expression (9.3 ± 5.6 months) than for those expressing nor-

Table 1 – Clinical characteristics of patients with CP-CML at diagnosis.

Feature	Low *BIM* expression ($n = 36$)	Normal *BIM* expression *P* ($n = 64$)	
Sex (M/F), %	57/43	59/41	NS
Age, median (int. r)	48 (37–59)	49 (37–60)	NS
Palpable spleen, %	68	64	NS
Median haemoglobin (g/L) (int. r)	120 (95.6–129.1)	110 (83.1–119.6)	NS
WBC $\times 10^9$/L, median (int. r)	162 (61.4–326.1)	143 (52.1–224.5)	NS
Median platelet count 10^9/L (int. r)	372 (280–650)	400 (265–619)	NS
Median peripheral blood blast as % WBC (int. r)	1 (0–4.2)	1 (0–3.8)	NS
Sokal score, %		NS	
High	35	27	
Low/intermediate	65	73	
Hasford score, %		NS	
High	19	26	
Low/intermediate	81	74	

(int. r) indicates interquartile range.

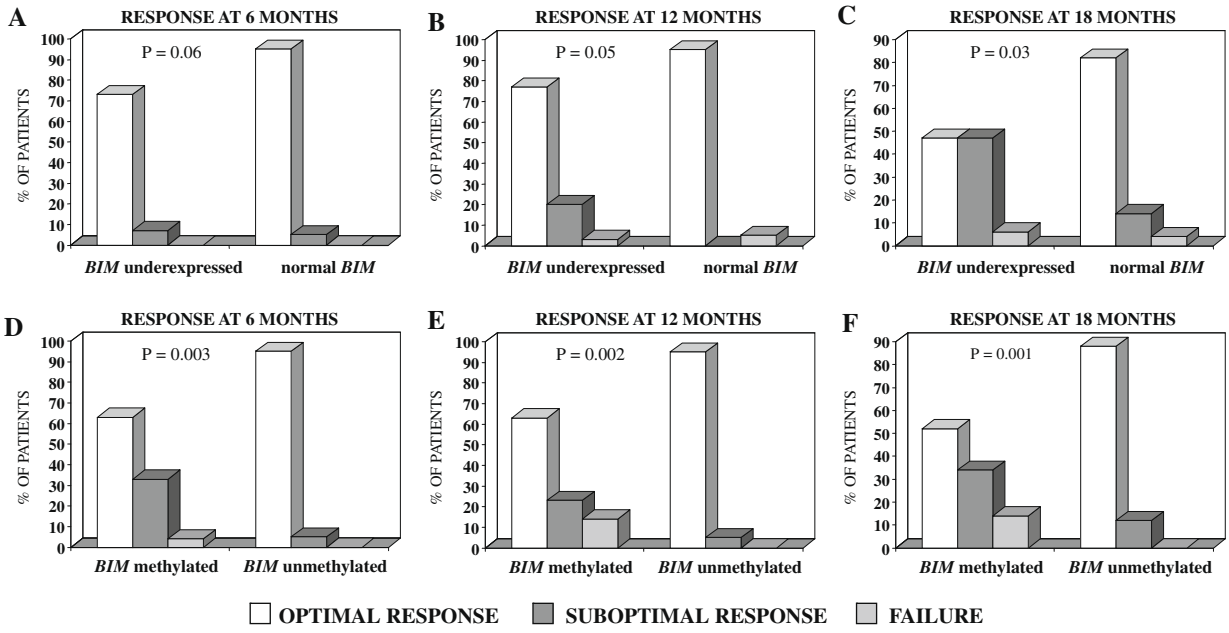

Fig. 1 – Expression and methylation of *BIM* in CML patients are associated with the response to imatinib. Response rate at 6, 12 and 18 months among CML patients treated with imatinib according to the expression of *BIM* (A–C) and methylation of the BIM promoter (D-F). Optimal response, suboptimal response and failure were defined as previously reported.[11] *P* represents the statistical comparison of the percentage of optimal responses between the groups of patients with normal or low expression of BIM and methylated patients versus non-methylated patients.

mal levels of *BIM* (7 ± 3 months, *P* = 0.03). Furthermore, after a median follow-up of 26 months, none of the patients with normal *BIM* expression had a progression of the disease while three patients (9%) with reduced expression of *BIM* lost complete cytogenetic response (Fig. 2). No statistically significant differences were found between both groups for any of the baseline parameters analysed before therapy and associated with prognosis (Table 1) or in the duration and dosage of imatinib therapy (data not shown).

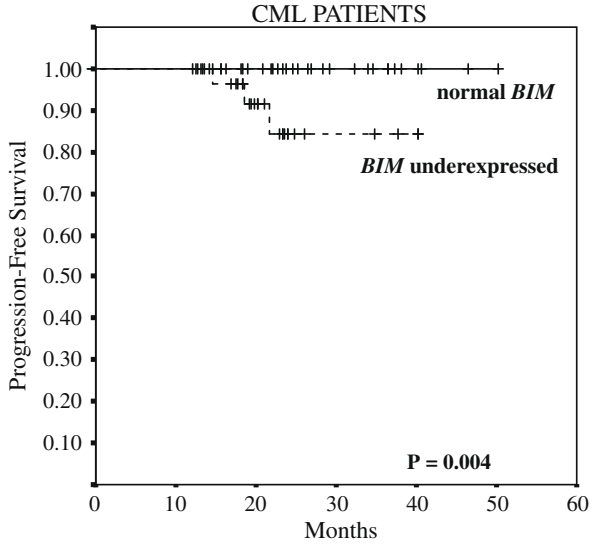

Fig. 2 – PFS in patients with CML treated with imatinib according to the *BIM* expression levels. PFS curve for all the patients enrolled in this study according to the *BIM* expression status. Solid line, patients with normal levels of *BIM* expression; dashed line, patients with down-regulation of *BIM* expression.

3.2. *Expression of BIM in CML cells is regulated by promoter hypermethylation*

Regulation of *BIM* includes transcriptional mechanisms as well as post-transcriptional mechanisms such as proteasome-dependent degradation.[16,17] We have recently described that *BIM* expression is regulated by both deletion and hypermethylation[14] in non-Hodgkin lymphoma and have also demonstrated that epigenetic events are important regulators of progression and clinical behaviour in CML.[18,19] Therefore, we analysed the methylation of the *BIM* promoter in CML. By MSP and bisulphite sequencing, the *BIM* promoter was found to be methylated in two of the three CML cell lines with low *BIM* expression (BV173 and KU812) and was found to be not methylated in CML cell lines with normal *BIM* (TCC-S and KYO-1) expression or in PB or BM cells from healthy donors (Fig. 3A, C, D and E). Treatment of CML cell lines with 4 μM of the de-methylating agent 5-aza-2′-deoxycytidine for 4 d restored expression of *BIM* mRNA in those cell lines that showed hypermethylation of the *BIM* promoter (Fig. 3F and G). Promoter methylation was not detected in K562 cell line despite low expression levels of *BIM* gene indicating a different mechanism of regulation of *BIM* expression.[7,8] These results at mRNA level were also confirmed at protein level by Western blot analysis showing that protein expression correlated with mRNA expression (Fig. 3B).

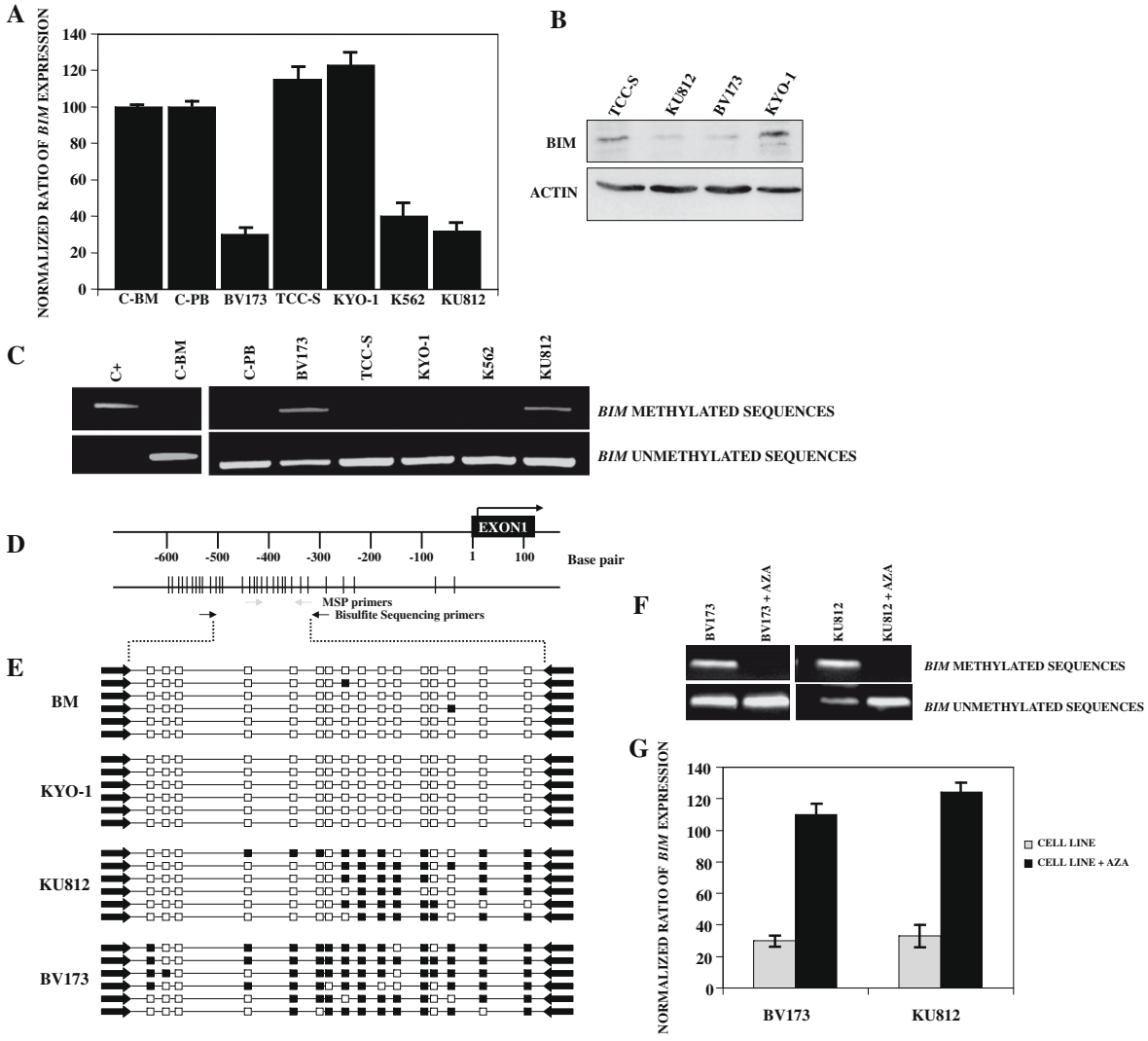

Fig. 3 – Analysis of *BIM* expression and promoter methylation in CML-derived cell lines. (A) Q-RT-PCR expression of *BIM* in CML-derived cell lines. Gene expression was normalised using the expression in BM-MNC from 30 healthy controls (Normalised ratio = 100%). (B) Western blot of BIM protein expression in CML-derived cell lines. β-Actin was used as the loading control. (C) MSP analysis of the *BIM* promoter in CML-derived cell lines. C+, positive methylated control; C-BM, marrow cells from healthy donors; C-PB, peripheral blood cells from healthy donors. (D) Schematic description of the *BIM* CpG island. Long black arrow indicates the *BIM* translation start site and each vertical bar represents a CpG dinucleotide. The grey arrows indicate the location of the MSP primers and the black arrows indicate the location of the bisulphite sequencing primers. (E) Bisulphite sequencing of the *BIM* CpG island. Each box indicates a CpG dinucleotide (white box: unmethylated, black box: methylated) and each line represents the analysis of 16 CpG dinucleotides of a single clone of *BIM*-analysed region. (F) MSP analysis of the *BIM* promoter in the BV173 and KU812 cell lines before and after treatment with 5-aza-2′-deoxycytidine demonstrating de-methylation of the promoter after treatment. (G) Expression of *BIM* mRNA in CML-derived cell lines demonstrating an up-regulation of gene expression after treatment with 4 μM of 5-aza-2′-deoxycytidine for 4 d. Gene expression was normalised as described above. (A,G) represent the mean of three different studies in triplicate; (B–F) a representative study of three different experiments is shown.

Hypermethylation of the *BIM* promoter was present in 44% of the patients in CP-CML and was correlated with down-regulation of *BIM* expression. Among CML patients, those with unmethylated *BIM* showed a mean N_{bim} of 82 ± 39%, similar to that found in healthy individuals, however, mean N_{bim} was significantly lower in those patients with hypermethylation of the *BIM* promoter (30 ± 47%, $P < 0.03$). Down-regulation of *BIM* was found in 82% (36/44) of CML patients with hypermethylated *BIM* and in 30% (17/54) of CML patients with unmethylated *BIM* ($P < 0.001$). Our results extend recent studies where reduced expression of *BIM* was described in a small group of CML patients at different stages of the disease[20] and suggest that down-regulation of *BIM* is present in a significant percentage of patients at diagnosis mainly, although not exclusively, due to epigenetic silencing of the promoter. As expected, cytogenetic and molecular responses were signifi-

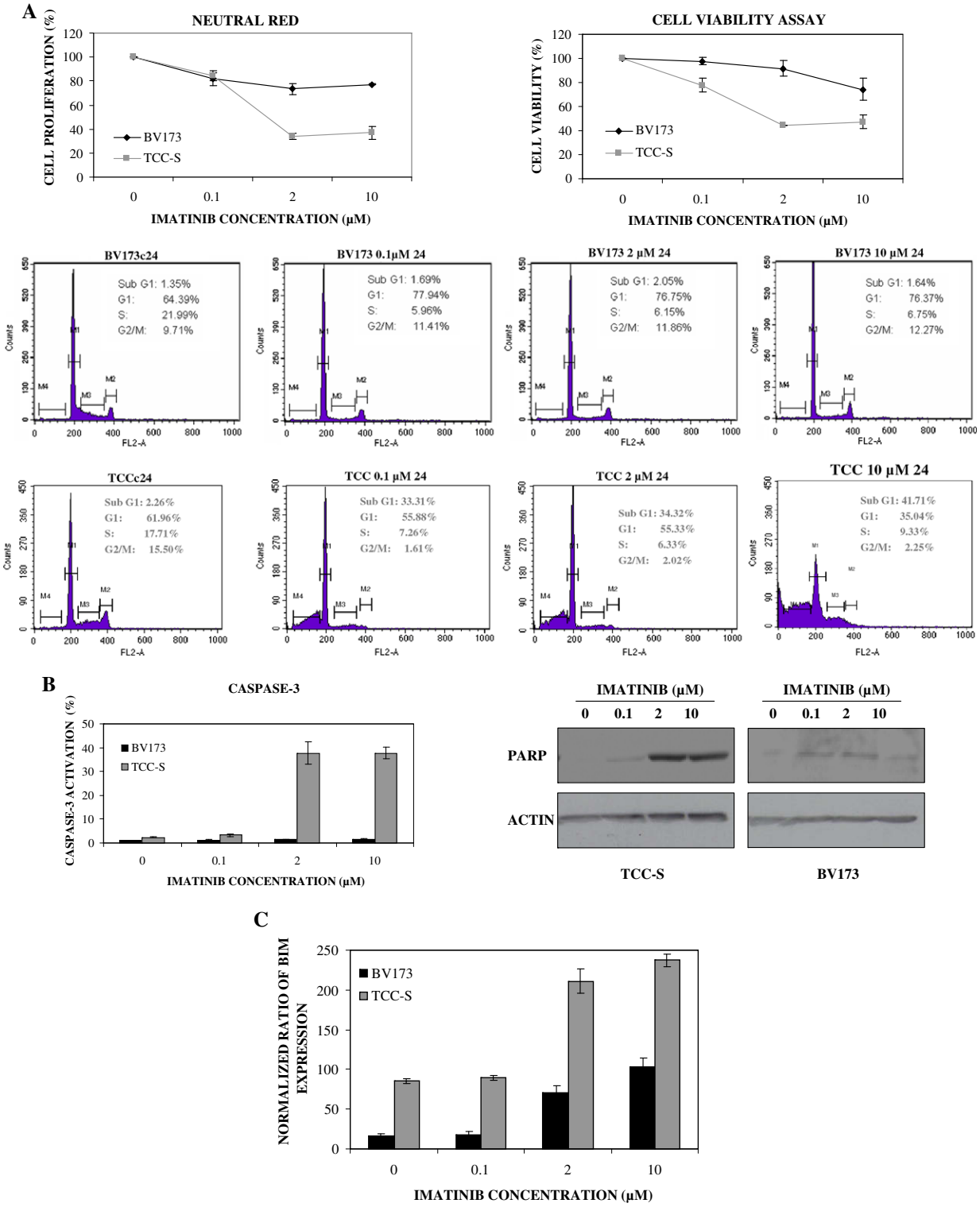

Fig. 4 – Effect of imatinib treatment in CML-derived cell lines with different expression of *BIM*. Cell cycle (FACS), proliferation and apoptosis were analysed in BV173 and TCC-S cell lines after treatment for 24 h with different concentrations of imatinib (0.1–10 μM) as described in Section (A,B). (C) *BIM* mRNA expression was analysed before and after imatinib treatment. Gene expression was normalised using the expression in BM-MNC from 30 healthy donors (Normalised ratio = 100%). (D) Expression of BIM protein by Western blot analysis in CML-derived cell lines after treatment with imatinib using β-actin as the loading control. (E) Methylation of the *BIM* promoter in BV-173 treated with imatinib (0.1–10 μM). C+, positive methylated control; C-BM, marrow cells from healthy donors. (A–C) The mean ± SEM of three different experiments is shown except for PARP and cell cycle analysis by FACS where a representative example of three different experiments is shown. (D,E) A representative example of three different experiments is shown.

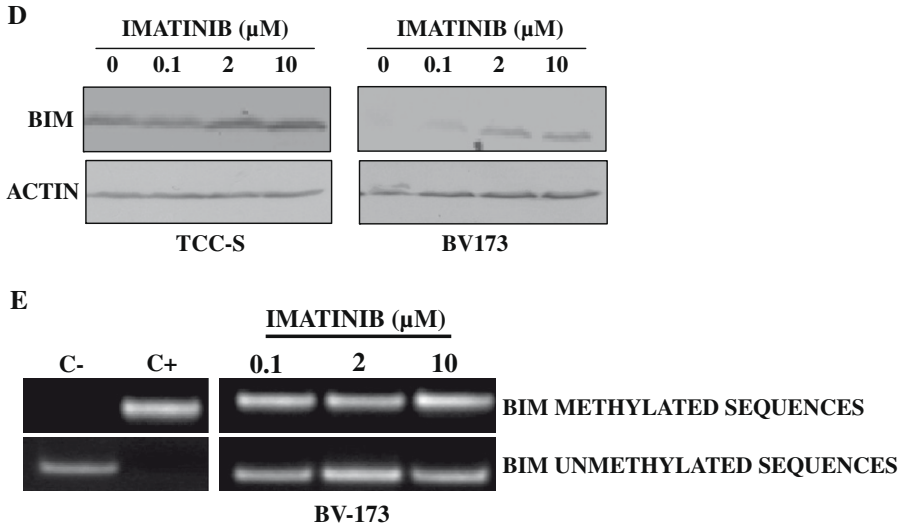

Fig. 4 (*continued*)

cantly worse among patients with abnormal methylation of *BIM* promoter (Fig. 1).

3.3. *Down-regulation of BIM expression in CML cells is responsible for reduced imatinib-induced cell killing*

To examine if BIM expression was required for imatinib-induced apoptosis in CML cells, we treated two different CML cell lines with different concentrations of imatinib (0.1, 2 and 10 μM). While the TCC-S showed normal levels of *BIM* expression (the promoter is unmethylated), the promoter of *BIM* was hypermethylated and the expression of *BIM* was down-regulated in the BV173 cell line (Fig. 3). imatinib treatment had a very limited effect on proliferation, viability and apoptosis of BV173 cells even at higher doses (Fig. 4A and B). Conversely, viability of TCC-S cells was significantly decreased after treatment with imatinib. Cell cycle analysis revealed an increase in apoptosis and a decrease in the percentage of cells in the G2/M phase and S phase after imatinib treatment in TCC-S cells (Fig. 4A) and an increase in cell apoptosis as demonstrated by the increase in the amount of active caspase-3. Similar results were obtained with other specific techniques of apoptosis such as the expression of the 85-kDa fragment of PARP by Western blot analysis (Fig. 4B). These results clearly demonstrate that BV173 cells are more resistant to imatinib treatment than TCC-S cells and suggest that down-regulation of *BIM* may protect BV173 cells from imatinib-induced inhibition of cell proliferation and apoptosis. Treatment with imatinib induced an up-regulation of *BIM* expression both at the mRNA and protein levels in both the cell lines (Fig. 4C and D) but higher doses of imatinib were required to increase the *BIM* expression in BV173 cells with a maximum up-regulation that was lower than the one observed in the TCC-S cell line (Fig. 4C and D). Up-regulation of *BIM* expression in BV173 was not due to promoter de-methylation as demonstrated by the analysis of the methylation status of the *BIM* promoter (Fig. 4E).

To demonstrate that down-regulation of BIM was in fact responsible for the resistance to imatinib, TCC-S cells were infected with two different specific siRNAs against *BIM* (viability was 89% and transfection efficiency was 88% by FACS using BLOCK IT Fluorescent Oligo, Fig. S1) and were treated with imatinib as described. Both *BIM* siRNAs efficiently decreased *BIM* expression as detected by Q-RT-PCR (Fig. 5A) and by Western blot analysis (Fig. 5B). Down-regulation of *BIM* expression in TCC-S cells significantly reduced apoptosis and inhibition of proliferation induced by imatinib treatment ($P < 0.05$) in comparison with TCC-S cells with normal BIM expression (Fig. 5C and D). Treatment of TCC-S cells either non-transfected, only nucleofected or nucleofected with the Silencer Select Negative Control-1 siRNA with imatinib-induced apoptosis and inhibition of cell cycle and proliferation as expected (Fig. 5C and D). These results clearly indicate that down-regulation of BIM protects cells from imatinib-induced apoptosis and inhibition of cell proliferation.

3.4. *Combination of imatinib with a de-methylating agent: a new therapeutic strategy for patients with CML and hypermethylation of the BIM promoter*

Based on the previous results, we reasoned that a combination of imatinib and a de-methylating agent in patients with hypermethylation of the *BIM* promoter could be beneficial. To test this hypothesis we treated the TCC-S and BV173 cell lines with different concentrations of 5-aza-2′-deoxycytidine (0.5, 1 and 2 μM) alone or in combination with imatinib for 24 h. As expected, treatment with 5-aza-2′-deoxycytidine induced expression of *BIM* at the mRNA and protein levels in the BV173 cell line which was associated with the de-methylation of the *BIM* promoter (Fig. 6A–C) but had a very modest effect on *BIM* expression in the TCC-S cell line (unmethylated cell line).

While treatment with 5-aza-2′-deoxycytidine induced a decrease in cell proliferation, viability and cell cycle of both cell lines, the effect was significantly higher in BV173 cells (methylated BIM) (Fig. 6D). Treatment with 5-aza-2′-deoxycytidine also induced a higher increase in cell apoptosis of BV173 rather than of TCC-S (Fig. 6E). The effect of 5-aza-2′-deoxycyt-

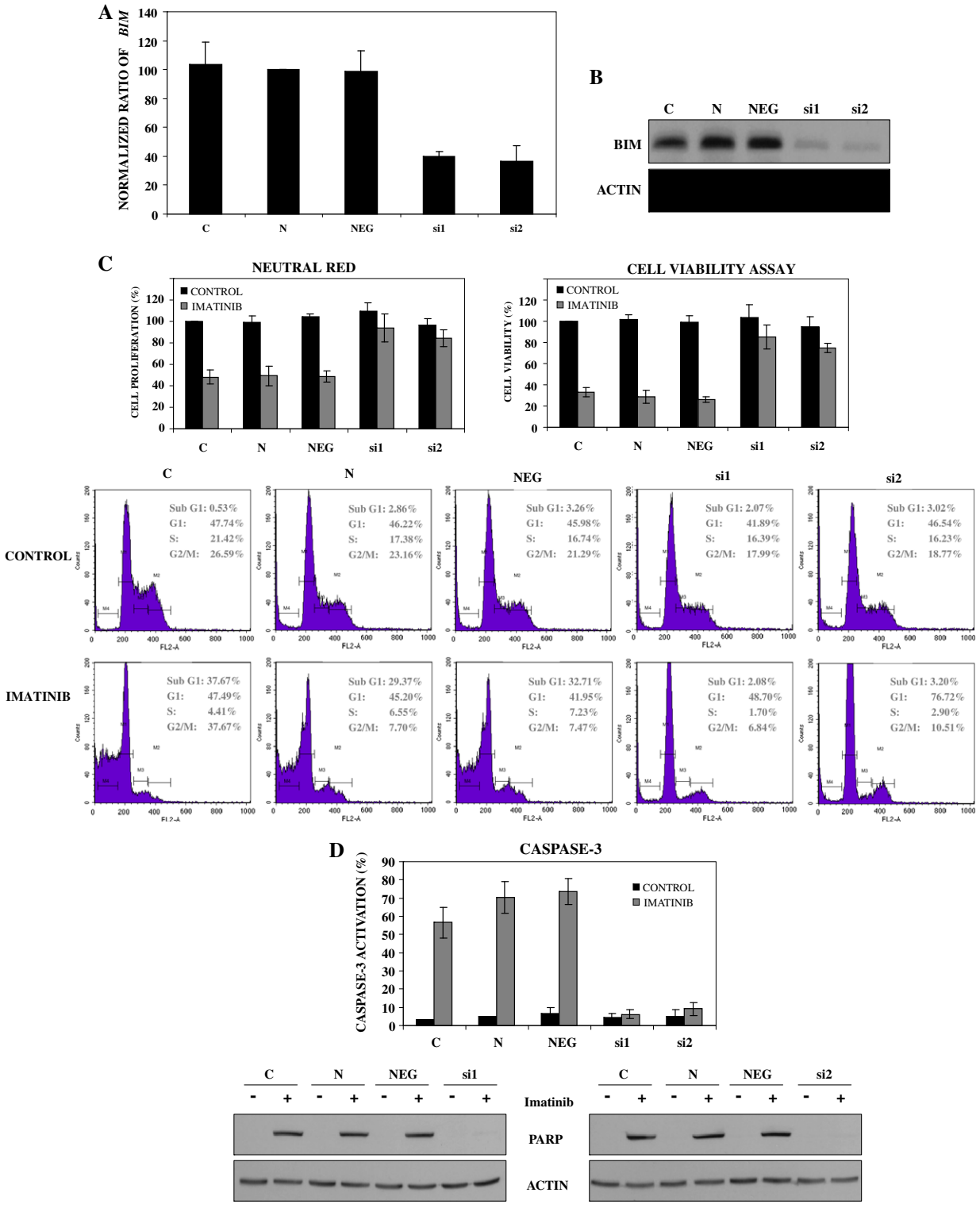

Fig. 5 – Down-regulation of BIM expression reduces imatinib-induced apoptosis and inhibition of cell proliferation. *BIM* mRNA expression was analysed by Q-RT-PCR (A) and Western blot analysis (B) 24 h after transfection with *BIM* siRNA. Gene expression was normalised by the expression in cells that were only nucleofected (Normalised ratio = 100%). Cell proliferation (C), cell cycle (C) and apoptosis (D) were analysed in TCC-S cells transfected with two specific *BIM* siRNAs and treated with imatinib for 24. C, cells without nucleofection; N, cells nucleofected; NEG, cells nucleofected with the Silencer Select Negative Control-1 siRNA; si1, cells nucleofected with the *BIM* Silencer Select siRNA s195012; si2, cells nucleofected with the *BIM* Silencer Select siRNA s19474. (A, C, D) The mean ± SEM of three different experiments is shown. (B) A representative example of three different experiments is shown.

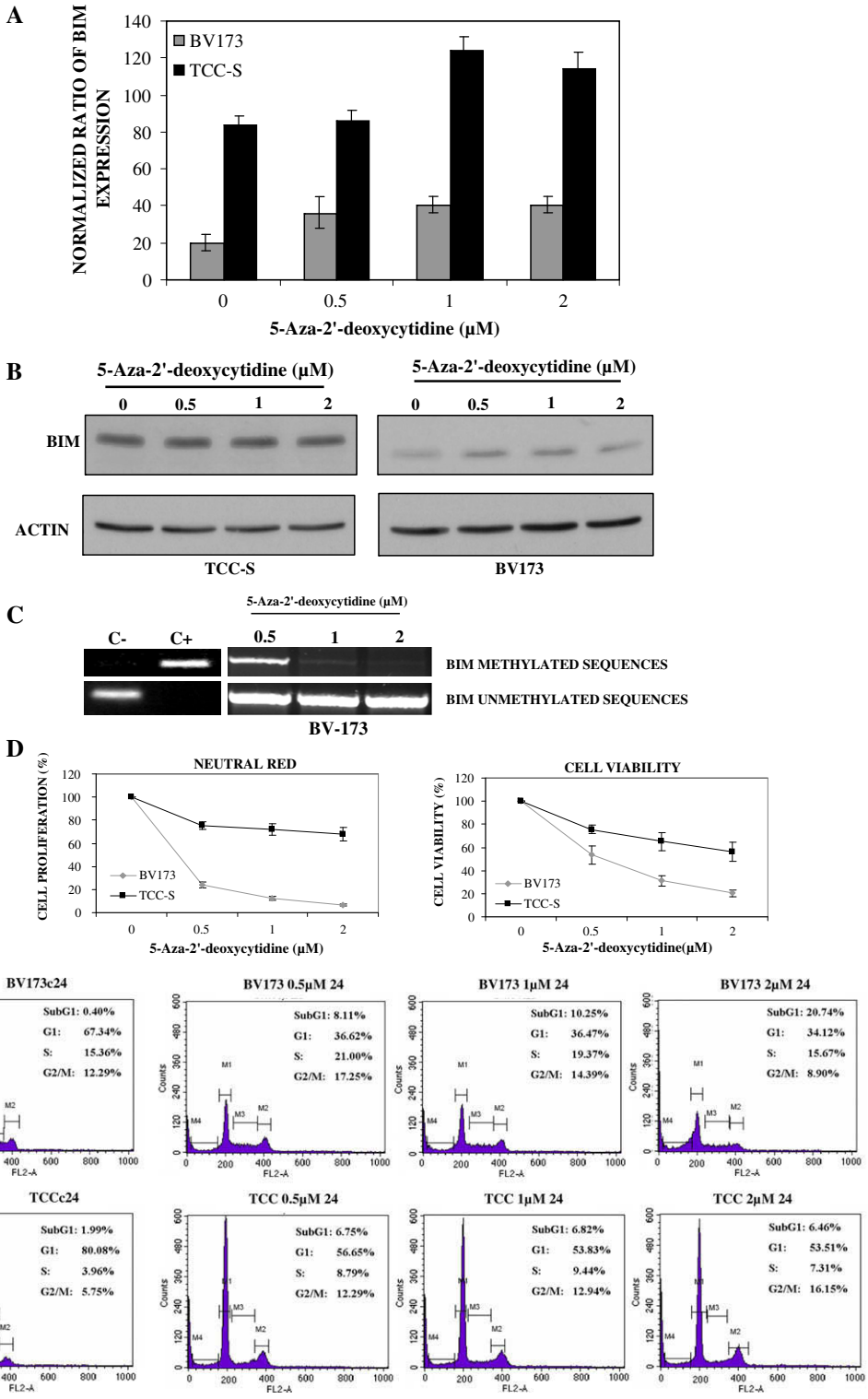

Fig. 6 – Effect of 5-aza-2'-deoxycytidine treatment in CML-derived cell lines with different expression of *BIM*. Expression of *BIM* mRNA (A) and protein (B) in BV173 and TCC-S cell lines after 0.5, 1 and 2 μM 5-aza-2′-deoxycytidine treatment for 24 h. Gene expression was normalised with expression in normal cells (Normalised ratio = 100%). β-actin was used as the loading control. (C) MSP analysis of *BIM* promoter in the BV173 cell line after treatment with 5-aza-2′-deoxycytidine. C+, positive methylated control; C-BM, marrow cells from healthy donors; BV173 0.5, 1, 2: BV173 cell line treated with 0.5, 1 or 2 μM of 5-aza-2′-deoxycytidine. Effect of 5-aza-2'-deoxycytidine treatment on cell proliferation (D), viability (D), cell cycle (D) and apoptosis (E) of BV173 and TCC-S cell lines. (A,D,E) The mean ± SEM of three different experiments is shown. (B,C) A representative example of three different experiments is shown.

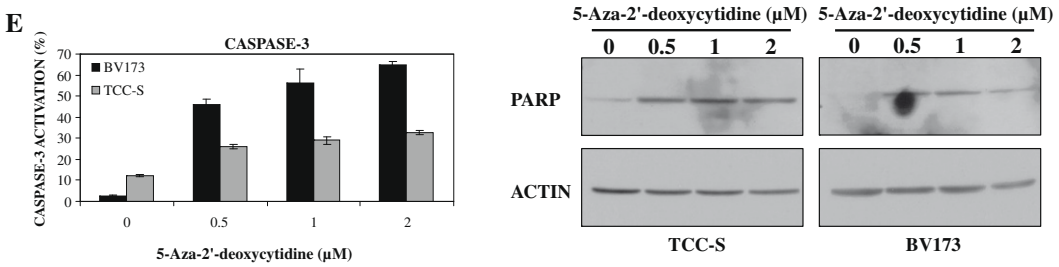

Fig. 6 (*continued*)

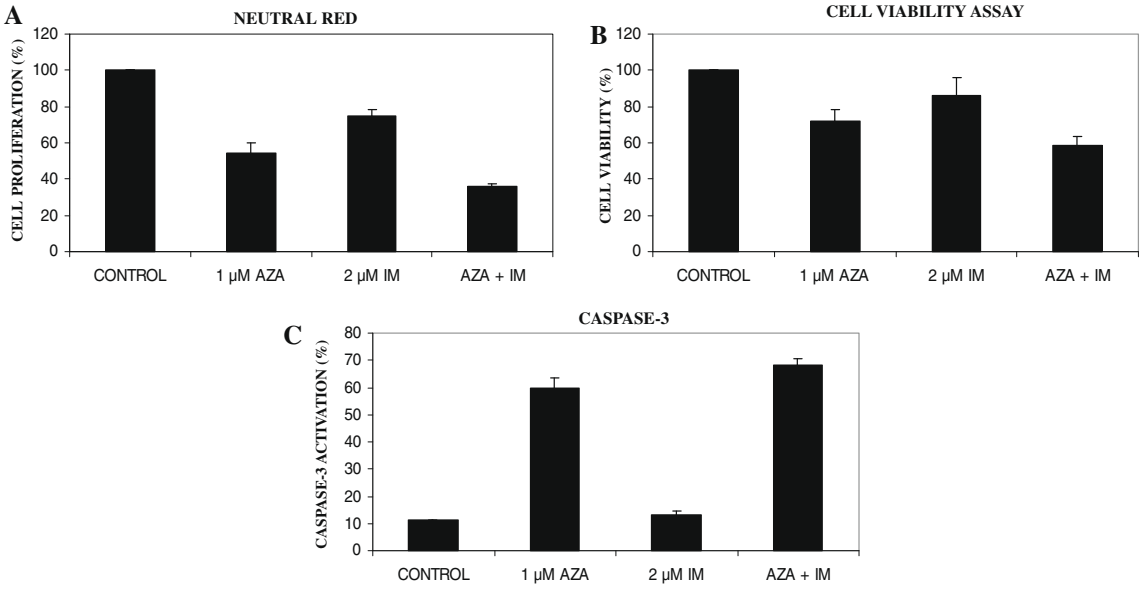

Fig. 7 – Effect of the combination of imatinib with 5-aza-2′-deoxycytidine in CML cell lines. BV173 cells were treated with 2 μM of imatinib, 1 μM of 5-aza-2′-deoxycytidine or with the combination of 5-aza-2′-deoxycytidine and imatinib, and cell proliferation (A–B) and apoptosis (C) were measured. CONTROL, untreated cells; AZA, 5-aza-2′-deoxycytidine; IM, imatinib.

idine on proliferation and apoptosis was dose dependent. Finally, treatment of the BV173 cell line with the combination of 5-aza-2′-deoxycytidine (1 μM) and imatinib (2 μM) for 24 h led to a additive effect inducing a further decrease in cell proliferation and viability and an increase in apoptosis in comparison with imatinib alone (Fig. 7). These results suggest that de-methylation and expression of *BIM* contribute to imatinib-induced CML apoptosis and inhibition of cell proliferation mainly in those cases where hypermethylation of the *BIM* promoter is present and that the combination of a demethylating agent with imatinib may be useful in patients with hypermethylation and down-regulation of *BIM*.

4. Discussion

Although recent studies have shown down-regulation of *BIM* mRNA in CML[5,6] and that imatinib killing of CML cells required BIM expression,[8] in this study, we further demonstrate that abnormal expression of *BIM* in CML cells depends on the abnormal hypermethylation of the promoter and that decreased expression and hypermethylation of the promoter are not only associated with reduced responses to imatinib treatment in patients with CML but also that expression of *BIM* is required for imatinib-induced apoptosis and inhibition of proliferation in CML.

Several mechanisms of regulation of BIM function have been described: first, mRNA expression is down-regulated by cytokines in mouse IL-3-dependent Baf-3, FL5.12 and 32D cells through the Ras/MAPK and PI3-K pathways, independently[21]; second, subcellular localisation of BIM is controlled by IL-3 in FDC-P1 cells, another mouse IL-3-dependent line, and by exposure to UV light in 293 cells[22]; third, NGF phosphorylates BIM through the MEK/MAPK pathway in neuronally differentiated PC-12 cells[23]; fourth, we have recently described that in non-Hodgkin lymphoma cell lines, BIM expression is regulated by both deletion and hypermethylation[14]; and finally, proteasome-dependent degradation is involved in the regulation of BIM expression in serum-deprived fibroblasts and macrophage colony-stimulating factor-dependent osteoclasts.[17] These somewhat confusing results suggest that the function of BIM may be regulated in different ways in certain situations and that the relative contribution of each of these mechanisms may differ between cell types. Although our results clearly indicate that the regulation of *BIM* expression is mostly epigenetically mediated, the lack of methylation in K562 cells as well as the fact that

up-regulation of *BIM* expression in BV173 after treatment with imatinib was not due to promoter de-methylation (Fig. 4E) suggests that other mechanisms may be implicated in the regulation of *BIM* in CML. Along this line, a recent study demonstrated that down-regulation of BIM in CML cell lines depends on the BCR-ABL1-induced phosphorylation of FoxO3a that prevents transcriptional activation of BIM and that treatment with imatinib induces activation of BIM transcription mediated by FoxO3a.[7]

Abnormal methylation of cell cycle, apoptosis and tumour suppressor genes has been implicated in the pathogenesis of ALL.[15,24–27] Although epigenetic regulation of many of these genes was examined in our CML samples, hypermethylation of gene promoters was not observed (data not shown). The fact that we did not find an abnormal hypermethylation of the promoters of genes implicated in cell cycle, apoptosis and tumour suppressor genes in patients with CP-CML suggests that epigenetic regulation of *BIM* expression is a specific finding that may be involved in the resistance of CML cells to apoptosis.

Treatment of CML has been notably improved by the development of imatinib mesylate, a potent tyrosine-kinase inhibitor that blocks the kinase activity of BCR-ABL1, thus inhibiting proliferation of Ph^+ progenitors.[28] However, both the persistence of molecular disease in a percentage of imatinib-treated patients, and the observation that imatinib discontinuation usually results in a rapid loss of the response, indicate that the cure of CML with imatinib alone is unlikely.[29,30] The most prevalent mechanism of resistance to imatinib is the development of mutations in c-abl.[31] However, depending on the assay used 10–50% of patients with resistance lack detectable *BCR-ABL1* mutations[28] and less than 20% of the primary resistances are due to point mutations raising the possibility that additional mechanisms might play a role in this resistance. Recently, Kuroda et al.[8] demonstrated that BCR-ABL1-transformed haematopoietic cells lacking BIM are resistant to imatinib-induced killing. Similar resistance was observed in cells engineered to overexpress BCL-2 or BCL-xL. Our results in patients with newly diagnosed CML indicate that decreased expression of *BIM* is responsible for the lack of optimal response in a significant percentage of the patients who do not obtain a complete molecular response and along with the in vitro studies demonstrating that the combination of imatinib with the de-methylating agent 5-aza-2′-deoxycytidine notably improves the efficacy of treatment of CML cells showing hypermethylation of *BIM* promoter, provide the bases for combination therapy in these patients.

In fact, the antileukaemic effect of decitabine – the de-methylating agent 5-aza-2′-deoxycytidine – in all phases of CML has been demonstrated[32] and a Phase II study of decitabine in combination with imatinib has been carried out in patients in advanced phase CML with promising results[33] further suggesting that treating patients with hypermethylation of *BIM* with a de-methylating agent in combination with imatinib could increase the response rate.

In conclusion, our results suggest a role for *BIM* in the pathogenesis of CML and establish methylation and expression of *BIM* as the prognostic factors for imatinib-treated CML patients. Our study also indicates that *BIM* expression in CML is at least partially regulated by epigenetic mechanisms such as gene promoter methylation and that *BIM* expression is required for imatinib-induced apoptosis of CML cells. Thus, the combination of imatinib with a de-methylating agent could be a new therapeutic strategy for patients with CML and hypermethylation of the *BIM* promoter.

Authorship and disclosures

Author's contribution: E.S.J.-E., J.R.-G., X.A. and F.P. designed and performed the research and collected and interpreted the data and wrote the manuscript; A.J.-V., L.C., V.M., V.A. and L.G. performed the experiments, and collected and interpreted the data and performed the statistical analysis. V.F., F.C. and J.A.M.-C., contributed vital new reagents or analytical tools and A.H. and A.T. reviewed the manuscript and interpreted the data. All authors reviewed and approved the final manuscript.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.04.005.

REFERENCES

1. Horita M, Andreu EJ, Benito A, Arbona C, Sanz C, Benet I, et al. Blockade of the Bcr-Abl kinase activity induces apoptosis of chronic myelogenous leukemia cells by suppressing signal transducer and activator of transcription 5-dependent expression of Bcl-xL. *J Exp Med* 2000;**191**:977–84.
2. Bedi A, Zehnbauer BA, Barber JP, Sharkis SJ, Jones RJ. Inhibition of apoptosis by BCR-ABL in chronic myeloid leukemia. *Blood* 1994;**83**:2038–44.
3. Amarante-Mendes GP, McGahon AJ, Nishioka WK, Afar DE, Witte ON, Green DR. Bcl-2-independent Bcr-Abl-mediated resistance to apoptosis: protection is correlated with up regulation of Bcl-xL. *Oncogene* 1998;**16**:1383–90.
4. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, et al. Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J* 1998;**17**:384–95.
5. Aichberger KJ, Mayerhofer M, Krauth MT, Vales A, Kondo R, Derdak S, et al. Low-level expression of proapoptotic Bcl-2-

interacting mediator in leukemic cells in patients with chronic myeloid leukemia: role of BCR/ABL, characterization of underlying signaling pathways, and reexpression by novel pharmacologic compounds. *Cancer Res* 2005;**65**:9436–44.
6. Kuribara R, Honda H, Matsui H, Shinjyo T, Inukai T, Sugita K, et al. Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors. *Mol Cell Biol* 2004;**24**:6172–83.
7. Essafi A, Fernandez de Mattos S, Hassen YA, Soeiro I, Mufti GJ, Thomas NS, et al. Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene* 2005;**24**:2317–29.
8. Kuroda J, Puthalakath H, Cragg MS, Kelly PN, Bouillet P, Huang DC, et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance due to their loss is overcome by a BH3 mimetic. *Proc Natl Acad Sci USA* 2006;**103**:14907–12.
9. Sokal JE, Cox EB, Baccarani M, Tura S, Gomez GA, Robertson JE, et al. Prognostic discrimination in "good-risk" chronic granulocytic leukemia. *Blood* 1984;**63**:789–99.
10. Hasford J, Pfirrmann M, Hehlmann R, Allan NC, Baccarani M, Kluin-Nelemans JC, et al. A new prognostic score for survival of patients with chronic myeloid leukemia treated with interferon alfa. Writing Committee for the Collaborative CML Prognostic Factors Project Group. *J Natl Cancer Inst* 1998;**90**:850–8.
11. Baccarani M, Saglio G, Goldman J, Hochhaus A, Simonsson B, Appelbaum F, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood* 2006;**108**:1809–20.
12. Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. *Blood* 2006;**108**:28–37.
13. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, Barrios M, et al. Transcriptional silencing of the Dickkopfs-3 (Dkk-3) gene by CpG hypermethylation in acute lymphoblastic leukaemia. *Br J Cancer* 2004;**91**:707–13.
14. Mestre-Escorihuela C, Rubio-Moscardo F, Richter JA, Siebert R, Climent J, Fresquet V, et al. Homozygous deletions localize novel tumor suppressor genes in B-cell lymphomas. *Blood* 2007;**109**:271–80.
15. Agirre X, Roman-Gomez J, Jimenez-Velasco A, Garate L, Montiel-Duarte C, Navarro G, et al. ASPP1, a common activator of TP53, is inactivated by aberrant methylation of its promoter in acute lymphoblastic leukemia. *Oncogene* 2006;**25**:1862–70.
16. Luciano F, Jacquel A, Colosetti P, Herrant M, Cagnol S, Pages G, et al. Phosphorylation of Bim-EL by Erk1/2 on serine 69 promotes its degradation via the proteasome pathway and regulates its proapoptotic function. *Oncogene* 2003;**22**:6785–93.
17. Ley R, Balmanno K, Hadfield K, Weston C, Cook SJ. Activation of the ERK1/2 signaling pathway promotes phosphorylation and proteasome-dependent degradation of the BH3-only protein, Bim. *J Biol Chem* 2003;**278**:18811–6.
18. Roman-Gomez J, Castillejo JA, Jimenez A, Cervante F, Boque C, Hermosisn L, et al. Cadherin-13, a mediator of calcium-dependent cell–cell adhesion, is silenced by methylation in chronic myeloid leukemia and correlates with pretreatment risk profile and cytogenetic response to interferon alfa. *J Clin Oncol* 2003;**21**:1472–9.
19. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Cervantes F, Sanchez J, Garate L, et al. Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene* 2005;**24**:7213–23.
20. Aichberger KJ, Mayerhofer M, Krauth MT, Skvara H, Florian S, Sonneck K, et al. Identification of mcl-1 as a BCR/ABL-dependent target in chronic myeloid leukemia (CML): evidence for cooperative antileukemic effects of imatinib and mcl-1 antisense oligonucleotides. *Blood* 2005;**105**:3303–11.
21. Shinjyo T, Kuribara R, Inukai T, Hosoi H, Kinoshita T, Miyajima A, et al. Downregulation of Bim, a proapoptotic relative of Bcl-2, is a pivotal step in cytokine-initiated survival signaling in murine hematopoietic progenitors. *Mol Cell Biol* 2001;**21**:854–64.
22. Lei K, Davis RJ. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci USA* 2003;**100**:2432–77.
23. Biswas SC, Greene LA. Nerve growth factor (NGF) down-regulates the Bcl-2 homology 3 (BH3) domain-only protein Bim and suppresses its proapoptotic activity by phosphorylation. *J Biol Chem* 2002;**277**:49511–6.
24. Roman-Gomez J, Cordeu L, Agirre X, Jimenez-Velasco A, San Jose-Eneriz E, Garate L, et al. Epigenetic regulation of Wnt-signaling pathway in acute lymphoblastic leukemia. *Blood* 2007;**109**:3462–9.
25. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, Garate L, et al. Promoter hypermethylation and global hypomethylation are independent epigenetic events in lymphoid leukemogenesis with opposing effects on clinical outcome. *Leukemia* 2006;**20**:1445–8.
26. San Jose-Eneriz E, Agirre X, Roman-Gomez J, Cordeu L, Garate L, Jimenez-Velasco A, et al. Downregulation of DBC1 expression in acute lymphoblastic leukaemia is mediated by aberrant methylation of its promoter. *Br J Haematol* 2006;**134**:137–44.
27. Roman-Gomez J, Jimenez-Velasco A, Agirre X, Prosper F, Heiniger A, Torres A. Lack of CpG island methylator phenotype defines a clinical subtype of T-cell acute lymphoblastic leukemia associated with good prognosis. *J Clin Oncol* 2005;**23**:7043–9.
28. Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 2005;**105**:2640–53.
29. Druker BJ, Guilhot F, O'Brien SG, Gathmann I, Kantarjian H, Gattermann N, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *New Engl J Med* 2006;**355**:2408–17.
30. Rousselot P, Huguet F, Rea D, Legros L, Cayuela JM, Maarek O, et al. Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood* 2007;**109**:58–60.
31. Shah NP, Sawyers CL. Mechanisms of resistance to STI571 in Philadelphia chromosome-associated leukemias. *Oncogene* 2003;**22**:7389–95.
32. Kantarjian HM, O'Brien S, Cortes J, Giles FJ, Faderl S, Issa JP, et al. Results of decitabine (5-aza-2'deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. *Cancer* 2003;**98**:522–8.
33. Oki Y, Kantarjian HM, Gharibyan V, Jones D, O'Brien S, Verstovsek S, et al. Phase II study of low-dose decitabine in combination with imatinib mesylate in patients with accelerated or myeloid blastic phase of chronic myelogenous leukemia. *Cancer* 2007;**109**:899–906.