BCR-ABL1-induced expression of HSPA8 promotes cell survival in chronic myeloid leukaemia

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Received 11 December 2007; accepted for publication 19 March 2008
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Supported in part by grants from Gobierno de Navarra, Department of Education, Beca Ortiz de Landázuri 2006, Department of Health-Gobierno de Navarra, Junta de Andalucía PI 0004/2007, Fondo de Investigaciones Sanitarias FIS 03/0661, 06/0003 and RETIC C03/10. This project was funded through the “UTE project CIMA”.

Summary
In order to determine new signal transduction pathways implicated in chronic myeloid leukaemia (CML), we performed a gene expression profile comparison between CD34+ cells from CML patients and healthy donors. Functional studies were performed using the Mo7e and Mo7e-p210 cell lines. Expression of CCND1 (Cyclin D1), as well as the chaperone HSPA8, which is important for regulation of CCND1, were significantly upregulated in CD34+ CML cells. Upregulation of HSPA8 was dependent, at least in part, on STAT5 (signal transducer and activator of transcription 5)-dependent transcriptional activation, as demonstrated by chromatin immunoprecipitation. The presence of HSPA8 in the nuclear protein fraction as well as its binding to CCND1 suggests that it may contribute to stabilization of the CCND1/CDK4 complex, which, in turn, may participate in proliferation of CML cells. Treatment of CML cells with the specific HSPA8 inhibitor 15-deoxyspergualin induced inhibition of CML cell viability but did not induce apoptosis. In conclusion, our studies suggest that STAT5-mediated activation of HSPA8 induces nuclear translocation and activation of the CCND1/CDK4 complex leading to increased proliferation of CML cells, deciphering a new pathway implicated in CML and supporting a potential role of chaperone inhibitors in the treatment of CML.

Keywords: chronic myeloid leukaemia, signal transduction, HSPA8, CD34+.
Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of the haematopoietic stem cell characterized by the presence of the Philadelphia chromosome (Ph) generated by the reciprocal translocation between the long arms of chromosomes 9 and 22 t(9;22) (q34;q11). This translocation produces the fusion of the genes BCR (chromosome 22) and ABL1 (chromosome 9), generating the BCR-ABL1 oncogene that causes the disease (Daley & Baltimore, 1988; Daley et al., 1990; Li et al., 1999). The p210 BCR-ABL1 fusion oncoprotein present in the majority of CML patients, has a constitutive tyrosine kinase (TK) activity that results in activation of a number of signal transduction pathways that contribute to the abnormal regulation of cell cycle, adhesion and apoptosis observed in CML cells (Horita et al., 2000; Andreu et al., 2005).

The development of Imatinib Mesylate (previously known as STI571) has impacted the treatment of CML disease (Savage & Antman, 2002). Imatinib, a 2-phenylaminopyrimidine, is a highly specific inhibitor of several tyrosine kinases, such as BCR-ABL1 (Druker et al., 1996), platelet-derived growth factor receptor (PDGFR), c-KIT, ARG (Okuda et al., 2001) or c-tons (macrophage colony-stimulating factor receptor) (Dewar et al., 2005). Besides its clinical efficacy, the highly specific inhibition of BCR-ABL1 tyrosine kinase activity makes this compound a basic tool to study the mechanism of action of BCR-ABL1 and the biology of CML.

Recent studies have addressed the genetic abnormalities associated with expression of the BCR-ABL1 oncogene using high throughput techniques, such as gene expression microarrays (Jena et al., 2002; Nowicki et al., 2003; Tipping et al., 2003; Hakansson et al., 2004; Janssen et al., 2005; Kronenwett et al., 2005). Most of these studies have used either cells lines (Tipping et al., 2003; Hakansson et al., 2004), or mononuclear cells from patients with CML (Nowicki et al., 2003; Janssen et al., 2005) and only a recent study focussed on the expression profile of stem and progenitor CML cells (CD34+ cells) (Kronenwett et al., 2005). Recent findings suggesting that Imatinib does not eradicate all leukaemia stem cells, even in the best responders, and that these cells could remain a potential source of relapse in chronic phase or advanced phase of the disease (Goldman & Gordon, 2006) (Bhatia et al., 2003; Jorgensen et al., 2006) support the need for new studies aimed at determining pathways dependent or independent of BCR-ABL1 that can lead to the development of more effective therapies.

The current study compared the gene expression profile of CD34+ cells from CML patients and healthy donors in order to determine pathways implicated in the pathogenesis of CML. Based on the results of the microarray analysis, we defined a new signal transduction pathway implicated in the abnormal proliferation of CML cells, suggesting that the chaperone HSPA8 and CCND1 contribute to the abnormal behaviour of CML cells and represent an interesting target for new therapies.

Materials and methods

Cell lines and human samples

Human-derived Mo7e (a megakaryoblastic leukaemia cell line without BCR-ABL1 fusion), Mo7e-p210 cells (Mo7e cells transfected with p210 isoform of BCR-ABL1) and the chronic myeloid leukaemia cell lines TCC-S, KU812, BV173 and KYO were cultured as described (Horita et al., 2000; San Jose-Eneriz et al., 2006). Peripheral blood (PB) from CML patients at diagnosis (n = 3) and from healthy donors (n = 2) were obtained by leucaphaeresis as described (Horita et al., 2000). Bone marrow samples were obtained from patients with CML at diagnosis and healthy volunteer donors. Samples were obtained after informed consent and using guidelines approved by the Ethics Committee for the Use of Human Subjects at the University of Navarra. CML patients were 100% Philadelphia chromosome positive by conventional cytogenetic analysis. CD34+ cells were enriched using the MACS CD34+ isolation kit (Miltenyi Biotec, Cologne, Germany) and the AutoMACS selection device as previously described (Horita et al., 2000; Andreu et al., 2005). After immune selection, CD34+ cells (purity always above 90%) were cultured in serum-free media (BIT-9500, Stem Cell Technologies; Vancouver, Canada) supplemented with 200 pg/ml stem cell factor (SCF), 50 ng/ml granulocyte colony-stimulating factor (G-CSF) (both from Amgen; Thousand Oaks, CA, USA), 200 pg/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; ImmuneX; Seattle, WA, USA), 1 ng/ml interleukin 6 (IL-6), 50 pg/ml leukaemia inhibitory factor (LIF), 200 pg/ml macrophage inflammatory protein 1α (MIP1α) (all from R&D Systems, Minneapolis, MN, USA), 0.1 mmol/l 2-mercaptoethanol and penicillin/streptomycin (BioWhitaker, Walkersville, MD, USA).

RNA isolation

Total RNA from cells was isolated using the Trizol reagent (Life Technologies) and purified with the Rneasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. RNA levels, quality and purity were assessed with the use of the RNA 6000 Nano assay on the Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA). None of the samples showed RNA degradation or contamination with genomic DNA.

Oligonucleotide microarray analysis and validation

CD34+ cells from PB of CML patients at diagnosis (n = 3) and from healthy donors (n = 2) were used for the microarray analysis. RNA isolation, labelling and hybridization to the HG-U133 A GeneChip Oligonucleotide Microarray (Affymetrix, Santa Clara, CA, USA) were performed as previously described (Gutierrez et al., 2005). All arrays were visually examined for searching possible irregularities. Data normalization was
performed with the Affymetrix Microarray Suite software version 5.0 (MAS5.0) according to the manufacturer’s protocol (Affymetrix). All samples had a scaling factor lower than threefold and a 3′/5′ of GAPDH probe set <2.5 (Appendix S1).

Unsupervised cluster analysis. Hierarchical clustering based on the average-linkage method with the centred correlation metric was carried out using Cluster and Treeview software (Page, 1996) and Garran software (Genomic Analysis for Rapid Biological ANnotation) (Martinez-Cruz et al, 2003).

Supervised analysis. In order to identify genes with statistically significant changes in expression between both groups, we used two different algorithms to increase the readability of the study: SAM (Significant Analysis of Microarrays) (Tusher et al, 2001) and Garran. Garran analysis was based on genes where the differences between the two groups using a t-test showed a P value <0.05 or 0.01. In the case of SAM, all data were permuted over 100 cycles by using the two-class (unpaired) format. Classification of the genes according to the Gene Ontology and matching of the gene products in the Boehringer Mannheim chart of Biological Pathways and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was made using the Garran software.

For validation of the microarray data, semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to analyse the expression of some of the genes that were differentially expressed based on the array information. All primers were designed with the software Oligo 4.0 (Molecular Biology Insights, Inc., Cascade, USA). The primers used, the annealing temperature, cycle number and the size of PCR products are shown in Supplementary material Table S1. ABL1 was used as internal control. Expression of each gene was densitometrically quantified with the use of Quantity One (Bio-Rad Laboratories Inc., Hercules, CA, USA). Values were normalized with ABL1 expression.

In vitro treatment with Imatinib and Deoxyspergualin
Mo7e, Mo7e-p210 cell lines and CD34+ cells were cultured at a density of 1 × 10⁶ cells/ml, washed twice with phosphate-buffered saline (PBS) and resuspended in 0.2% Tween-20 in PBS and 0.5 mg/ml Rnase A (Sigma) and incubated for 30 min at 37°C. Subsequently, cells were stained with 25 μg/ml of propidium iodide (Sigma) and analysed using a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Apoptosis was analysed by DNA fragmentation using the QiAmp DNA Mini Kit (Qiagen, Hilden, Germany). Equal amounts of DNA were separated on 1% agarose gels containing 0.5 μg/ml of ethidium bromide. The detection of the 85 kDa fragment of poly(ADP-ribose)polymerase (PARP) that results from caspase-3 cleavage was also used as a marker for apoptosis.

Cell cycle and apoptosis analysis
For cell cycle analysis, 250 000 cells were cultured at a density of 1 × 10⁶ cells/ml, washed twice with phosphate-buffered saline (PBS) and resuspended in 0.2% Tween-20 in PBS and 0.5 mg/ml Rnase A (Sigma) and incubated for 30 min at 37°C. Subsequently, cells were stained with 25 μg/ml of propidium iodide (Sigma) and analysed using a BD FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Apoptosis was analysed by DNA fragmentation using the QiAmp DNA Mini Kit (Qiagen, Hilden, Germany). Equal amounts of DNA were separated on 1% agarose gels containing 0.5 μg/ml of ethidium bromide. The detection of the 85 kDa fragment of poly(ADP-ribose)polymerase (PARP) that results from caspase-3 cleavage was also used as a marker for apoptosis.

Quantitative real-time PCR (Q-RT-PCR)
Reverse transcription reactions and Q-RT-PCR were carried out as described previously (Agirre et al, 2006). We used Assays-On-Demand (Applied Biosystems, Foster City, CA, USA) for CCND1 (Hs00765553-m1) and 18S (Hs99999901_s1) gene expression analysis. Primers (HSPA8-D and HSPA8-R) and probe (HSPA8-S) for HSPA8 expression analysis were designed using the program Primer Express 2.0 (Applied Biosystems) and are shown in Supplementary material Table S1. For the mRNA analysis of each gene, a patron curve was generated using cDNA obtained from PB or BM of healthy donors. Data were interpolated in the curve and then the level of expression of each gene was normalized with the level of expression of the internal control 18S. Finally, data were compared among them.

Western blot, immunoprecipitation analysis and subcellular fractionation
Proteins extracted from cell lines after treatments with Imatinib or DSG were analysed by polyacrylamide gel electrophoresis, and the protein bands were electrophoretically transferred onto nitrocellulose membranes as described before (Roman-Gomez et al, 2004). The membranes, after being blocked, were incubated with primary antibodies against HSPA8 (Abcam, Cambridge, UK), CCND1 (Calbiochem, San Diego, CA, USA), CDK4 (BD Pharmingen, San Diego, CA, USA), PARP (Promega Corp., Madison, WI, USA), β-Actin (Sigma), β-tubulin (Sigma) or Lamin A (Cell Signaling) and then with alkaline phosphatase-conjugated secondary antibodies. Bound antibodies were revealed by a chemiluminiscent reagent (Tropix, Bedford, MA, USA) and detected using HyperfilmTM enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). β-Actin was used as a loading control.

For immunoprecipitation, 500 μg of protein extracts were precleared with Sephadex G-10 (Amersham Biosciences, Uppsala, Sweden) immunoprecipitated with 2 μg of antibody
anti-HSPA8 (Abcam), anti-CCND1 (Calbiochem) or anti-CDK4 (BD Pharmingen) and 20 μl of protein A/G (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoprecipitates were washed five times with Triton Buffer and then with cold 0.5 mol/l LiCl. Subsequently, immunoprecipitates were eluted with 8 mol/l Urea and 0.2 mol/l dithiothreitol, electrophoresed and transferred to nitrocellulose membranes. Membranes were incubated with the same primary antibodies used for immunoprecipitation as described above. Subcellular fractionation was performed as previously described (Ishida et al, 2002) using β-tubulin as cytoplasmic control and lamin A as nuclear control.

### Bisulphite sequencing

We used bisulphite sequencing technique to analyse and determine the methylation status of HSPA8 (GeneBank: NM_053056) and CCND1 (GeneBank: NM_053056) promoters region as described (Agirre et al, 2006; San Jose-Enériz et al, 2003). Primers and PCR conditions used for amplification of the HSPA8 fragment (Primers HSPA8-BS1, HSPA8-BS2; 56 CpG dinucleotides) and CCND1 fragment (Primers CCND1-BS1, CCND1-BS2; 22 CpG) are shown in Supplementary material Table S1.

### Chromatin immunoprecipitation (ChIP)

Cell extracts from Mo7e and Mo7e-p210 cell lines with and without Imatinib treatment were subjected to chromatin immunoprecipitation in order to assess the interactions between STAT5 (signal transducer and activator of transcription 5) and the HSPA8 and CCND1 promoters. The ChIP assay was performed as previously described (Ballestar et al, 2003). Three primer sets were designed in order to include all the possible STAT5 binding sites in the HSPA8 (HSPA8-1D, HSPA8-1R, HSPA8-2D, HSPA8-2R, HSPA8-3D and HSPA8-3R) and CCND1 promoters (CCND1-1D, CCND1-1R, CCND1-2D, CCND1-2R, CCND1-3D and CCND1-3R) (Supplementary material Table S1). The sensitivity of the PCR amplification was evaluated on serial dilutions of total DNA collected after sonication (input fraction). PCR amplifications were carried out with 36 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. PCR products were run in 1.8% agarose gels and visualized with ethidium bromide.

### Immunofluorescence

Mo7e and Mo7e-p210 cells were fixed in paraformaldehyde for 10 min at 4°C. Cytospins were permeabilized with 0.1% Triton in PBS for 15 min and washed three times with PBS. After blocking non-specific binding sites with 1% bovine serum albumin (BSA) for 4 h, slides were incubated overnight at 4°C in a humidified chamber with 150 μl of the corresponding primary antibody at a concentration of 10 μg/ml diluted in 1% BSA: anti-HSPA8 (Abcam), anti-CCND1 (Calbiochem) and anti-β-tubulin (Sigma-Aldrich, Steinheim, Germany). Slides were then washed three times with 0.1% Tween-20 in PBS for 5 min each and incubated for 1 h with fluorescein isothiocyanate-conjugated anti-mouse IgM (Sigma-Aldrich) or Alexa Fluor 488 rabbit anti-mouse IgG (Invitrogen Life Technologies, Paisley, UK) or Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA, USA), all diluted 1:1000 in 1% BSA. Slides were counterstained with 4',6-diamino-2-phenylindole in PBS:glycerol (1:1) and observed under a fluorescence microscope. In the case of double immunostaining, both primary antibodies were incubated together.

### Results

Expression of CCND1 and HSPA8 is up-regulated in CD34+ CML cells

The comparison between the gene expression profile of CD34+ cells of CML patients and healthy donors showed that 1151 genes were differentially expressed in CML progenitor cells (P < 0.05) (Supplementary material Table SII). Most of these genes are implicated in metabolism, gene transcription, signal transduction, transport, developmental processes and cell proliferation, as expected, based on the known pathways affected by the expression of the BCR-ABL1 oncogene (Deaninger et al, 2000). In vitro treatment of CD34+ CML cells with Imatinib for 12 and 24 h induced changes in 772 and 730 genes respectively (Supplementary material Tables SIII and SIV), although only 113 genes were found to be regulated at both 12 and 24 h. Interestingly, dendrogram analysis clustered CML untreated samples with samples treated for 12 h with Imatinib and healthy donors samples with CML samples treated for 24 h with Imatinib, suggesting that treatment with Imatinib induces a healthy normal gene profile in CD34+ CML cells. To confirm these results, we analysed the mRNA expression of CCND1 and HSPA8 in a new group of samples from patients with CML (BM CD34+ cells n = 10 and BM mononuclear cells n = 25) as well as CML cell lines (KYO, KU812, BV173, TCC-S and Mo7e-p210). In agreement with the results observed in the microarray analysis, HSPA8 was overexpressed in cell lines, BM CD34+ cells and BM mononuclear cells of CML patient samples (mean...
expression of HSPA8 in bone marrow of healthy donors and CML cells was 86.36 ± 3.26% and 275.24 ± 83.2% respectively; \( P = 0.02 \). Similarly, CCND1 was also overexpressed in cell lines and CML patient samples (mean expression of CCND1 in bone marrow of healthy donors and CML cells was 66.33 ± 34.81% and 119.32 ± 31.1%, respectively; \( P = 0.05 \)). Protein expression of both HSPA8 and CCND1 was higher in Mo7e-p210 in comparison with Mo7e, and there was a significant increase in expression of both proteins in BCR-ABL1 cells (Fig 1).

**BCR-ABL1 mediates regulation of CCND1 and HSPA8 expression**

To demonstrate that upregulation of CCND1 and HSPA8 proteins was dependent on BCR-ABL1, the Mo7e and Mo7e-p210 cell lines were treated with Imatinib. As expected, Imatinib treatment induced inhibition of BCR-ABL1 phosphorylation and cell cycle and induced apoptosis of CML cells (Supplementary material Fig S2). This treatment was also associated with a decreased expression of CCND1 and HSPA8 protein expression in the Mo7e-p210 cell line but not in parental Mo7e cell line (Fig 1). Similarly, the upregulated HSPA8 mRNA level observed in Mo7e-p210 cells was downregulated after treatment in vitro with Imatinib in accordance with the results obtained at the protein level (not shown). Treatment with Imatinib also induced a significant downregulation of CCND1 mRNA expression in Mo7e-p210 cell lines (not shown).

We next examined the potential mechanisms of BCR-ABL1-mediated regulation of HSPA8 and CCND1 mRNA levels. As we have previously demonstrated that abnormal hypomethylation of gene promoters plays a role in the altered expression of some genes in CML and more so in the progression of the disease (Roman-Gomez et al, 2005, 2006), we decided to examine the methylation status of HSPA8 and CCND1 promoters in Mo7e and Mo7e-p210 cell lines. Bisulphite sequencing analysis did not show changes in the methylation status of the HSPA8 and CCND1 promoters, indicating that promoter hypomethylation was not the mechanism of transcriptional regulation of HSPA8 (Supplementary material Fig S3A) and CCND1 (Supplementary material Fig S3B).

As HSPA8 and CCND1 present a number of STAT5 binding consensus sequences in the upstream promoter region and we have previously demonstrated that BCR-ABL1 induces STAT5 phosphorylation and transcriptional activation (Horita et al, 2000), we reasoned that expression of HSPA8 and CCND1 could be regulated at the transcription level as a direct consequence of STAT5 activation and direct interaction with the HSPA8 and CCND1 promoters. ChiP analysis demonstrated the binding of STAT5 in the HSPA8 promoter region in Mo7e-p210 but not in the wild-type Mo7e cell line. This interaction was abrogated after treatment with Imatinib (Fig 2B). No interaction with STAT5 was observed when we analysed the promoter of CCND1 by ChiP (Fig 2A). These results along with the reduced expression of HSPA8 in Mo7e-p210 after treatment with Imatinib, suggest a transcriptional regulation of HSPA8 mediated by BCR-ABL1 activation of STAT5.

**HSPA8 is located into the nucleus and associates with CCND1/CDK4**

In order to elucidate the potential role of HSPA8 and CCND1 in the pathogenesis of CML, and based on the fact that HSPA8 is a chaperone that has been associated with CCND1 (Diehl et al, 2003), we analysed the location of HSPA8 by subcellular fractionation followed by Western blot of HSPA8 and CCND1 in the Mo7e-p210 cell line. As shown in Fig 3A, HSPA8 and CCND1 proteins were present in the nuclear fraction in Mo7e-p210 cells, which was further confirmed by immunofluorescence (Fig 3B). Furthermore, the formation of a complex between HSPA8, CCND1 and CDK4 was demonstrated by immunoprecipitation with antibodies against HSPA8, CCND1 or CDK4 followed by Western blot analysis, suggesting a possible role of HSPA8 in the stabilization of the nuclear complex between HSPA8/CCND1/CDK4 (Fig 3C).

**Deoxyspergualin, an inhibitor of HSPA8, inhibits proliferation and decreases viability of CML cells in combination with Imatinib**

Finally, to demonstrate that upregulation of HSPA8 contributes to abnormal viability of CML cells, Mo7e and Mo7e-p210 were treated with the HSPA8 inhibitor DSG (Nadler et al, 1995; Tepper et al, 1995) alone or in combination with Imatinib. A dose–response study indicated the optimal dose of DSG to inhibit proliferation of BCR-ABL1 cells to be 100 μg/ml (Supplementary material Fig S4). As expected, treatment with DSG, Imatinib or both did not have any effect...
on viability of Mo7e cells. However, viability of Mo7e-p210 cells was significantly reduced after treatment with Imatinib and DSG (33%) in comparison with Imatinib alone (57%) (Fig 4A). Although DSG did not induce a decreased viability in Mo7e-p210 cells, there was a mild reduction in the number of total cells after treatment with DSG from 3.8 x 10⁶ cells/ml (Mo7e-p210 without treatment) to 3.2 x 10⁶ cells/ml (Mo7e-p210 treated with DSG) (Supplementary Material Fig S5). This suggests that inhibition of HSPA8 could have an effect on proliferation of CML but does not induce apoptosis or decrease cell viability. Similar results were obtained when we analysed cell cycle by FACS where a small decrease in the percentage of Mo7e-p210 cells in the G2/M phase was observed with DSG alone (Fig 4B) while an increase in cell death was observed when DSG and Imatinib were used in combination (Fig 4B). Treatment with DSG alone did not induce an increase in cell apoptosis as indicated by DNA fragmentation and caspase cleavage of PARP (Fig 4C and D).

While treatment with Imatinib induced a downregulation of HSPA8 and CCND1 protein expression in Mo7e-p210 cells, the combination of Imatinib and DSG did not have any additional effect on protein expression, suggesting that DSG may acts modifying HSPA8 activity (Fig 5).

Discussion

Despite the clinical success obtained with the use of specific inhibitors of BCR-ABL1 such as Imatinib (Druker et al, 2006), Dasatinib (Quintas-Cardama et al, 2006) and Nilotinib (Kantarjian et al, 2006) and the high percentage of CML patients that achieve a complete cytogenetic response or even a complete molecular response the malignant clone is unlikely to be eliminated by these treatments (Graham et al, 2002; Bhatia et al, 2003; Elrick et al, 2005). It has been recently demonstrated that Imatinib does not inactivate all BCR-ABL1-activated signaling pathways, suggesting that some of these pathways can be essential for leukaemic progenitor cell survival (Hu et al, 2006). This implies that persistent malignant progenitors can be a potential source of relapse in CML patients and that there is a need to improve our understanding of the signal transduction pathways implicated in the biology of CML in order to provide new targets for therapy. The results of the microarray analysis shown here identified a new signaling pathway that is altered in CML patients and involved in cell cycle and cell survival, such as the HSPA8-CCND1 signaling pathway that is altered in CML patients and involved in cell cycle and cell survival, such as the HSPA8-CCND1 identified in the microarray analysis and confirmed in a larger number of patients with chronic phase CML at diagnosis in which expression of HSPA8 and CCND1 was up-regulated both in CD34⁺ BM cells (progenitor cells) as well as BM mononuclear cells.

Two experimental evidences support the relationship between the BCR-ABL1 kinase activity and the upregulation of HSPA8 and CCND1: firstly, both proteins were up-regulated in CML cell lines and patient samples; and secondly, the treatment with Imatinib significantly reduced the expression of both proteins. We provide new insights into the mechanism that mediates the increased expression of HSPA8 and CCND1. In accordance with previous studies from our group and others demonstrating that BCR-ABL1 phosphorylation of STAT5 is one of the mechanisms that contribute to abnormal regulation of apoptosis in CML (Horita et al, 2000; Weisberg & Griffin, 2000), our work further suggests that BCR-ABL1 can also increase cell proliferation by inducing STAT5-mediated transcriptional upregulation of HSPA8 (Fig 2B). Recent studies in a breast cancer models indicated that CCND1 is regulated by STAT5 (Joung et al, 2005) which was not the case in CML (Fig 2A), differences that could probably be explained based on the different models. It has been recently published that another Signal Transducer and Activator of Transcription, STAT3, regulates transcription of
CCND1, so we cannot exclude that activation of STAT3 would be responsible for the upregulation of CCND1 (Leslie et al., 2006) expression in CML.

HSPA8 and CCND1 are interesting targets for the development of new cancer therapies in CML for several reasons: Up-regulation of CCND1 has been described to play an important role in a number of tumours such as breast cancer (Stendahl et al., 2004), colorectal (Kristt et al., 2000), brain gliomas (Tan et al., 2004), thyroid (Nakashima et al., 2004), urothelial bladder (Mhawech et al., 2004), oesophageal (Wu et al., 2004) or endometrial (Moreno-Bueno et al., 2004) cancer making the CCND1/CDK4 complex an attractive target (Grillo et al., 2006; Landis et al., 2006; Yu et al., 2006). Further, the interaction between BCR-ABL1 and CCND1 has been previously described in advanced phase of the disease with an increased expression in accelerated phase (de Groot et al., 2000; Liu et al., 2004).

The involvement of chaperones in regulation of survival and resistance to apoptosis in different tumours (Jameel et al., 1992; Chant et al., 1995; Ogata et al., 2000; Becker et al., 2004; Atkins et al., 2005), including a recently described role of HSP70 overexpression in resistance to Imatinib in CML cells (Pocaly et al., 2007), has stimulated the development of inhibitors of heat shock protein (HSP), such as the Hsp90 inhibitor geldanamycin and its analog 17-AAG (17-allylamino-17-deethoxygeldanamycin) (Guo et al., 2005), two drugs that are actually undergoing phase I and II clinical trials in different tumours (Drysdale et al., 2006). On the other hand, chaperone molecules have been identified, such as leukaemia-associated antigens (Greiner et al., 2003), providing the ground for development of an HSP-based vaccine that has shown its efficacy in several tumour models (Hoos & Levey, 2003). Similarly, autologous vaccine of leucocyte-derived heat shock protein 70-peptide complexes (Hsp70PC) and autologous heat shock protein-peptide vaccine AG-858, based on Hsp 70, have been used against CML in combination with Imatinib with immunological and clinical responses (Li et al., 2005). The Hsp70PC vaccine contains similar amounts of HSP70 and HSPA8 proteins, suggesting that both proteins could be possible targets in CML. Besides, HSP70 and HSP90 have already been related to BCR-ABL1, being the tyrosine kinase a client protein for these heat shock proteins.

But, could HSPA8 become a therapeutic target? The use of specific inhibitors of HSPA8, such as DSG, may have advantages over the use of patient-specific vaccines. DSG interacts specifically with HSPA8, binding exactly with the...
Glu-Glu-Val-Asp (EEVD) regulatory domain inhibiting its functions and the binding of proteins to HSPA8 (Nadler et al., 1992, 1995, 1998), so it is predicted to compete with protein or peptide binding, thereby affecting protein trafficking (Nadeau et al., 1994). In fact it has been shown that DSG inhibits the localization of HSP70 into the nucleus and also decreases nuclear translocation of the transcription factor nuclear factor (NF)κB (Nadler et al., 1995). Experiments performed in our laboratory suggest that DSG does not completely inhibit binding of proteins to HSPA8 in CML cells. In fact, after

![Graphs and Diagrams](image)

**Fig 4.** Effect of the specific HSPA8 inhibitor DSG on cell viability, proliferation and apoptosis of CML cells. Mo7e and Mo7e-p210 cell lines were treated for up to 96 h with DSG, Imatinib or DSG plus Imatinib and viability (A), cell cycle (B) and apoptosis (C) were measured. (A) Viability of Mo7e and Mo7e-p210 cell lines was measured by trypan blue dye exclusion for up to 4 days. (B) Cell cycle was analysed by FACS using propidium iodide, as described in the Materials and methods. (C): untreated control cells; DSG: cells treated with the inhibitor DSG; Imatinib: cells treated with the inhibitor Imatinib; DSG + Imatinib: cells treated with the combination of both DSG and Imatinib; SubG1: percentage of cells with subdiploid DNA content; G1: percentage of cells in the phase G1 of cell cycle; S: percentage of cells in phase S of cell cycle; G2/M: percentage of cells in phase G2/M of cell cycle. (C and D) Apoptosis was measured by DNA fragmentation (C) or by Western blot against PARP. (C) Untreated control cells; DSG: cells treated with the inhibitor DSG; Imatinib: cells treated with the inhibitor Imatinib; DSG + Imatinib: cells treated with the combination of both DSG and Imatinib. The levels of β-Actin were also analysed to assure equal loading.
treatment with DSG, CCND1/HSPA8 complexes can be observed (data not shown) suggesting that DSG may reduce the amount of complexes by decreasing the efficiency of interaction or by competition with the interacting proteins. It is important to stress that DSG by itself was not able to decrease cell viability of BCR-ABL1 cells. As BCR-ABL1 continues to be active, promoting other survival pathways, the combination of Imatinib plus DSG was required to decrease cell viability and to induce apoptosis of CML cells, indicating an additive effect between both inhibitors. These results lend support to the potential use of both agents to inhibit leukaemia progenitors and maybe to overcome the resistance of the malignant stem and progenitor cells. In any case, the development of new HSPA8 inhibitors would be necessary to further prove the benefit of inhibiting HSPA8.

In conclusion, our study supports the role of HSPA8 and CCND1 in the abnormal proliferation of CML cells and establishes a new pathway (Fig 6) where BCR-ABL1 induces the expression of HSPA8 at the level of transcription. HSPA8 then binds to CCND1. This in turn could lead to the stabilization of CCND1/CDK4 complexes in the nucleus and to the activation of the cell cycle, as has been demonstrated in other models (Diehl et al., 2003). This multicomplex is present in patients with CML and participates in the abnormal proliferation that characterizes leukaemia cells.

Acknowledgements

We thank Dr. Consuelo del Cañizo, Dr. José Rifón and Dr. Javier Pérez Calvo for providing the samples of patients with CML at diagnoses; Dr Kaori Kusama for kindly providing a specific inhibitor of HSPA8 15-deoxyspergualin (DSG).

References


### Supplementary Material

The following supplementary material is available for this article online:

**Appendix S1.** Array design.

**Figure S1.** Dendrogram of healthy donors, CML and CML samples treated with 12 and 24 h of Imatinib.

**Figure S2.** Imatinib inhibition of c-abl tyrosine kinase activity in CML cell lines.

**Figure S3.** Transcriptional regulation of *HSPA8* and *CCND1* gene.

**Figure S4.** Effect of the specific HSPA8 inhibitor DSG on cell viability, proliferation and apoptosis of CML cells.

**Figure S5.** Effect of DSG on proliferation of CML cells.

**Table S1.** Primers and PCRs conditions.

**Table SII.** Total analysis of genes differently expressed between CD34+ cells of CML patients and healthy donors.

**Table SIII.** Total analysis of genes differently expressed between CD34+ cells of CML patients before and after 12 h with Imatinib.

**Table SIV.** Total analysis of genes differently expressed between CD34+ cells of CML patients before and after 24 h with Imatinib.

**Table SV.** Expression of selected genes by RT-PCR in comparison with microarray data between CD34+ cells of CML patients and healthy donor samples and between CML patients samples at baseline and after treatment with Imatinib for 24 h.

The material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2141.2008.07221.x

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