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

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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 transcrition 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, $CD34^+$.

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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-fms (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, Walkersvill, 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 GARBAN 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 GARBAN. GARBAN 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 GARBAN 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 SI. *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 1×10^6 cells/ml and treated with Imatinib at a concentration of 2 μmol/l (generously provided by Dr. Elisabeth Buchdunger, Novartis, Basel, Swizterland) for 12 and 24 h (Horita *et al*, 2000). Deoxyspergualin (DSG) (generously provided by Nippon Kayaku, Tokyo, Japan), a HSPA8 inhibitor, was used to treat Mo7e and Mo7e-p210 cells *in vitro* for up to 72 h (100 μg/ml of DSG). DSG is modified by polyamine oxidase present in fetal bovine serum (Tepper *et al*, 1995), so 1 mmol/l aminoguanidine (Sigma, St. Louis, MO, USA), an inhibitor of the polyamine oxidase, was included in the culture. Viability and total cell counts were determined at various times by trypan blue exclusion. Proliferation, cell cycle analysis and apoptosis were determined when indicated.

Cell cycle and apoptosis analysis

For cell cycle analysis, 250 000 cells were cultured at a density of 1×10^6 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 SI. 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 chemilumincescence (Amershan 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 cytoplasmatic 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_006597) and *CCND1* (GeneBank: NM_053056) promoters region as described (Agirre *et al*, 2006; San Jose-Eneriz *et al*, 2006). 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; 22CpG) are shown in *Supplementary material* Table SI.

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 SI). 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 (Deininger *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 after 24 h (*Supplementary material* Fig S1). Gene expression changes were confirmed in a small number of genes by semi-quantitative RT-PCR (*Supplementary material* Table SV).

As the aim of our study was to determine new signal transduction pathways implicated in the pathogenesis of the disease and to search for new therapeutic targets, we focused on potential candidate genes. Microarray analysis indicated that expression of *CCND1* (Cyclin D1), known to be altered in different human neoplasias and involved in cell proliferation, as well as some regulators of the CCND1 activity, such as *HSPA8* (also known as HSP73 or HSC70), were significantly up-regulated in CD34$^+$ CML cells and were downregulated after treatment with Imatinib. 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

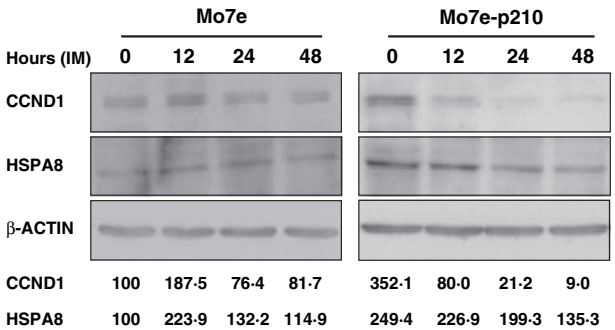

Fig 1. Effect of Imatinib on HSPA8 and CCND1 protein expression Mo7e and Mo7e-p210 cells were treated with Imatinib (2 μmol/l) for up to 48 h and the expression of HSPA8 and CCND1 proteins analysed by Western blot. The levels of β-actin were also analysed to ensure equal loading. Expression of each protein was densitometrically quantified with the use of Multi-Analyst v1·1 (Bio-Rad Laboratories Inc.). Values were normalized with β-actin expression. Protein expression in Mo7e was considered as 100%. IM: Imatinib. A representative example of three different experiments is shown.

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

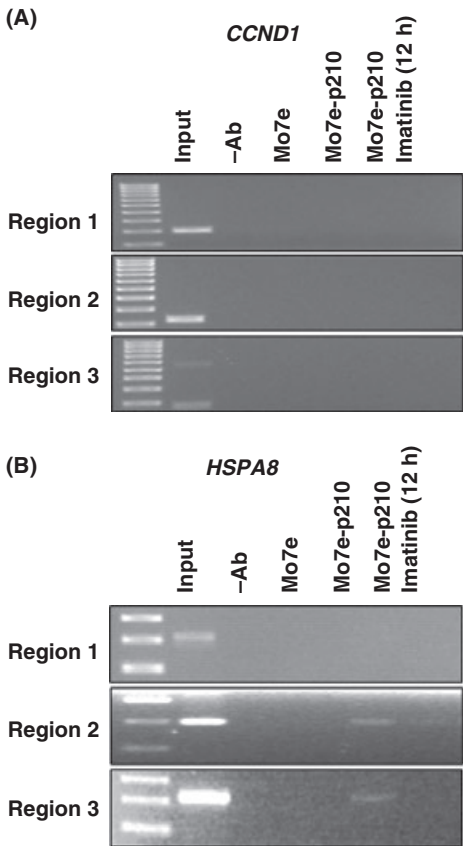

Fig 2. Transcriptional regulation of *CCND1* and *HSPA8* gene *CCND1* (A) and *HSPA8* (B) chromatin immunoprecipitation assays. Mo7e and Mo7e-p210 cell lines were cultured with and without Imatinib for 12 h. After fixation, cultures were processed for ChIP assays as described in the *Materials and methods*. PCR was performed using *CCND1*- and *HSPA8*-specific primers covering the promoter region that contains putative STAT5 binding sites. Input: total DNA collected after sonication (positive control); −Ab: DNA obtained from immunoprecipitation performed in the absence of anti-STAT5-specific antibody (negative control). A representative example of three different experiments is shown.

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 $3{\cdot}8 \times 10^6$ cells/ml (Mo7e-p210 without treatment) to $3{\cdot}2 \times 10^6$ 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-ABL-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 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

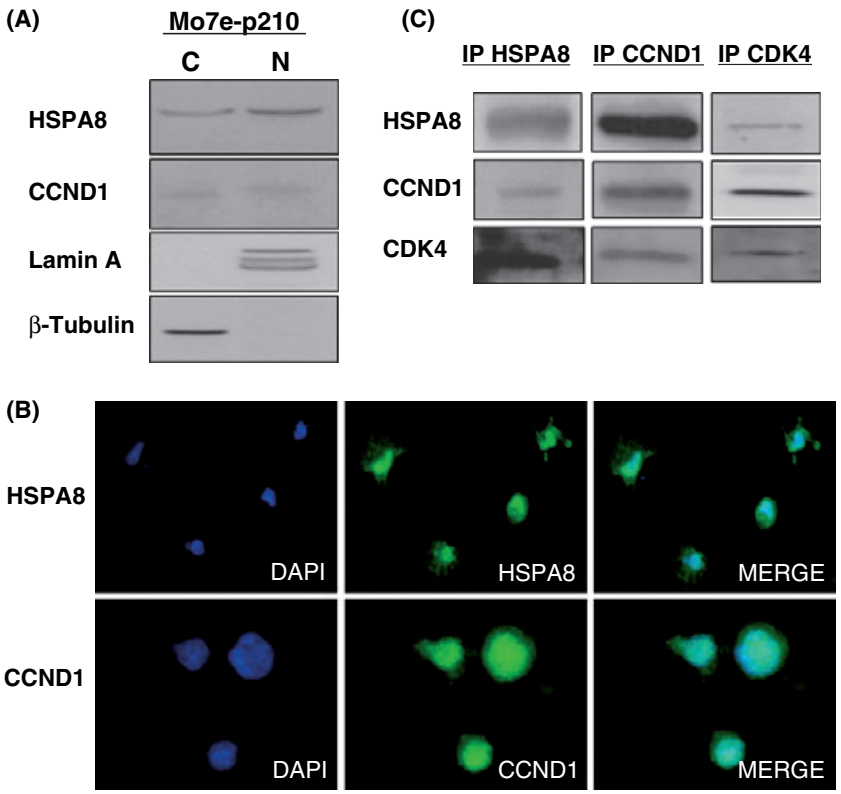

Fig 3. HSPA8 location and HSPA8-CCND1-CDK4 complex formation. (A) Analysis of the HSPA8 and CCND1 cell localization. Subcellular fractionation was performed using Mo7e-p210 cell line followed by Western blot against HSPA8 and CCND1. β-tubulin was used as cytoplasmatic control and lamin A as nuclear control. C: cytoplasmatic fraction; N: nuclear fraction. (B) Immunofluorescence staining for HSPA8 (green) and CCND1 (green) showing nuclear location. DAPI was used for nuclear staining. (C) HSPA8/CCND1/CDK4 complex formation: Cell extracts of Mo7e-p210 cell line were analysed by immunoprecipitation (IP) with specific antibodies for HSPA8, CCND1 and CDK4 and then subjected to Western blotting with the same antibodies used for IP. In A, B and C, a representative example of three different experiments is shown.

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-demethoxygeldanamycin) (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

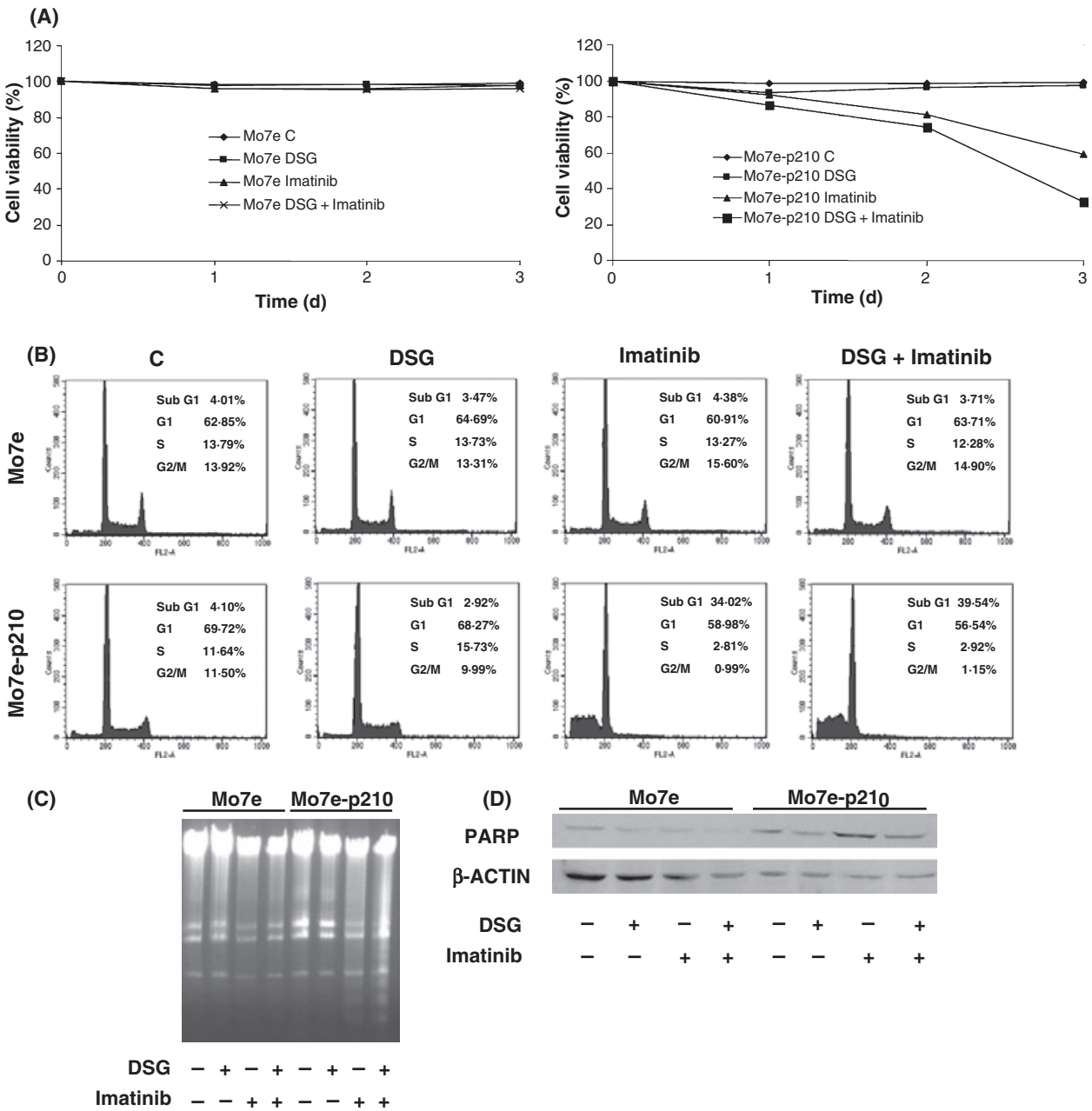

Fig 4. Effect of the specific HSPA8 inhibitor DSG on cell viability, proliferation and apoptosis of CML cells. Mo7e and Mo7e-p210 cell lines were treated for up to 96 h with DSG, Imatinib or DSG plus Imatinib and viability (A), cell cycle (B) and apoptosis (C) were measured. (A) Viability of Mo7e and 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.

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

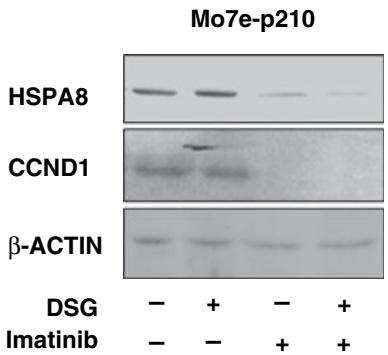

Fig 5. Effect of treatment with Imatinib and DSG on HSPA8 and CCND1 protein expression. Mo7e-p210 cell lines were treated with Imatinib and DSG as described in the *Materials and methods*. Cell extracts were analysed for the expression of HSPA8 and CCND1 proteins by Western Blot. The levels of β-actin were also analysed to ensure 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.

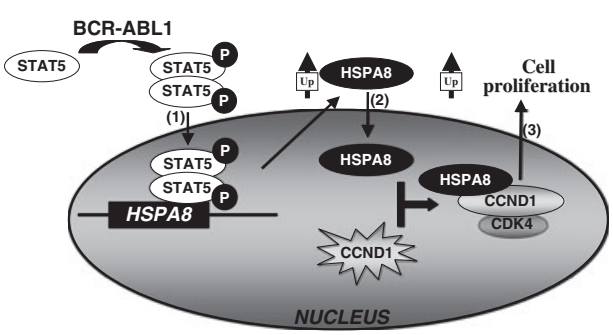

Fig 6. Potential model representing the participation of HSPA8 in cell proliferation in CML (see text for details). Phosphorylation of STAT5 by BCR-ABL1 induces dimerization, which allows STAT5 to translocate to the nucleus where it binds to consensus STAT5 binding sequences of *HSPA8* and therefore activates HSPA8 transcription (1), thus leading to an increase in HSPA8 protein level, which translocates to the nucleus (2). HSPA8 binding to CCND1 leads to stabilization of the CCND1/cdk4 complex inducing cell proliferation (3). Upregulation of HSPA8 in CML thus contributes to abnormal cell cycle proliferation in CML.

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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 SI. 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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