Acetylome and phosphoproteome modifications in imatinib resistant chronic myeloid leukaemia cells treated with valproic acid

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1. Introduction

Imatinib is an extremely effective therapy for chronic phase CML BCR/ABL positive, but patients may develop resistance, caused most frequently by point mutations within the kinase domain of BCR-ABL, or less often by amplification of the BCR/ABL genomic locus [1]. Mechanisms independent of BCR/ABL activity are also supposed to cause resistance. Novel ABL kinase inhibitors or dual SRC/ABL kinase inhibitors with higher potency against native and imatinib-resistant mutants of BCR/ABL have substantial clinical utility, but at least one mutation located in the p-loop of ATP domain of ABL (T315I) remains resistant to any kinase inhibitor until now in clinical use [2]. Thus, the search for alternative drugs effective in imatinib-resistant CML is still cogent [3]. Treatment of CML cells with histone deacetylase inhibitors (HDACi) of the class of hydroxamic acid analogues has been shown to promote proteasomal degradation of BCR/ABL [4], associated with apoptosis, and to result in anti-leukemic effect together with imatinib [5], even in T315I mutation positive cells [6]. These findings prompted the design of new clinical trials with agents inhibiting Heat Shock Protein 90 (HSP90) [7], as the pivotal role of this stress-activated chaperone protein was quite clear. HSP90 in fact, in combination with HSP70 [8], contributes to protect BCR/ABL from proteasomal degradation and this activity is inactivated by chaperon protein acetylation [6,9–11]. Although histones are the most prominent and abundant substrates for HDACs, they can de-acetylate non-histone substrates, situating themselves in cell cytoplasm, nucleus and in mitochondria [12]. Candidate substrates are at least two hundred “acetylatable” proteins, whose functions and localization are modulated by this post-translational modification. The mechanisms by which HDAC inhibitors may exert their broadly demonstrated antineoplastic activity could therefore lay not uniquely on their epigenetic effects [12,13]

We evaluated which protein modifications were involved in reversion of imatinib resistance by valproic acid (VPA), a feeble HDACi, belonging to the short chain fatty acid class [14,15]. We have recently shown that CML cells resistant to imatinib express a different protein phenotype respect to sensitive cells [16,17]. In this study, we support the suggestive observation of a substantial effect of HDACi/short chain fatty acids and imatinib in terms not only of inhibition of proliferation, but also BCR/ABL expression and induction of apoptosis [17,18]. We then present an analysis of the modifications of the acetylation and phosphorylation of proteins expressed after exposure to the combination of imatinib and VPA. To perform this analysis, we analysed human CML cell lines, with a clone resistant and a clone sensitive to ima-
tinib and utilized a two dimensional Western blot (WB) proteomic approach [19].

2. Materials and methods

2.1. Cells, drugs and culture conditions

BCR/ABL positive human cell lines K562, LAMA84 (sensitive S) and resistant R (kindly provided by Prof Gambacorti, University of Milano-Bicocca) [1,20,21] and BCR/ABL negative human cell line HEL60 (as negative control) were grown in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) in standard condi-
tions. BCR/ABL positive human cell line KBMs (parental P) and resistant R [22] (kindly provided by Dr Onida, University of Milano) were grown in IMDM medium supplemented with 10% FBS. LAMA 84 R, characterized by a 5.5 fold increase in BCR/ABL mRNA and protein over production [23], were continuously grown in the presence of imatinib 0.1 μM; KBMs R cells, characterized by presence of BCR/ABL with T315I mutation due to substitution of threonine-to-isoleucine at position 315 of ABL [24] were continuously grown in the presence of imatinib 1 μM. Before experiments, cells were washed thoroughly, starved for 6h and exposed for 48 h to different doses (0.2–1 μM in sterile PBS) of imatinib mesylate (ST571, kindly provided by Novartis Pharmaceuticals) and/or HDAC1 valeric acid (VPA) (Sanofi-Synthelabo) (2 mM in sterile RPMI, doses with demonstrated antileukemic activity [25]).

Mononuclear cells were obtained after informed consent, routinely provided by all participating patients, undergoing diagnostic and investigational procedures and previously approved by the legal Ethical Committee. In brief, cells from bone marrow aspirates of 3 patients with CML were isolated by density gradient centrifugation with Lympholyte (CÉDERLANE-Canada): samples were from 1 imatinib-sensitive patient in chronic phase (case #1, BCR–ABL FISH: 84% positive), 1 patient at diagnosis in chronic phase (case #2, BCR–ABL FISH: 98% positive), 1 imatinib-resistant patient in blast crisis (case #3, BCR–ABL FISH: 100% positive) and one normal donor. All cases did not show Bcr/Abl mutations at sequencing and no additional karyotypic abnormalities than [9; 22]. Primary cells were incubated for 24 h in the presence or the absence of imatinib (0.2 μM for normal donor and patient sensitive to imatinib, 1 μM for patient resistant to imatinib in blast crisis) and VPA (2 mM) as single drug or in combination. Drugs were added to cultures at the same time.

2.2. Cell viability and apoptosis

The number of viable LAMA S and R cells, KBMS P and R cells, K562 cells, HEL60 cells, normal donor peripheral blood and primary CML cells at 24 (primary) and 48 h (cell lines) was evaluated by dye reduction WST-1 test (WST-1 Cell Proliferation Reagent, Roche Applied Science). In the same samples, apoptosis was evaluated with Annexin-V–FLUOS Staining Kit (Roche Applied Science). All samples were tested in triplicate.

2.3. RNA extraction and BCR/ABL quantification

RNA extraction was performed by incubating with TriPure Isolation Reagent according to manufacturer’s recommendations (Roche Applied Science). Reverse transcription (RT) and Q-real-time PCR were performed using the QUANT–BCR–ABL p210 kit (Dia-Chem, Napoli, Italy) on Applied Biosystem 7300 Real Time PCR System (PE/Applied Biosystem). The cDNAs generated from RT reaction were ampli-
fied using specific primers and specific internal double-dye probe (FAM-TAMRA). Standard dilution of BCR/ABL fusion cDNAs was also present in the kit to allow quantification of specific transcripts.

2.4. Protein extraction, immunoprecipitation and Western blot analysis

Cells were washed once with ice-cold PBS containing 100 μM Na3VO4 and Protease Inhibitor Cocktail (Sigma) and lysed by incubating at 95 °C in Laemmli buffer (62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 0.005% blue bromophenol and 2% SDS). Protein concentration was determined by BCA reagent assay (Pierce). For immunoprecipitation, 500 μg of proteins for each sample were used with c-abl antibody (Santa Cruz Biotechnology, CA, USA) and protein G agarose beads (Amer-
shan Biosciences). Immunoprecipitated (IP) and total proteins were separated by SDS–PAGE on 9% polyacrylamide gel and then transferred onto PVDF membranes (Hybond-ECL; Amershams Biosciences, Germany). Membranes were incubated with primary antibodies (anti-Phosphotyrosine, Cell Signalling Technology-USA, 1:1000 in PBS containing 0.1% Tween 20 and 1% BSA (T-PBS)/1% BSA; anti-Imatinib A2/B3, Santa Cruz Biotechnology, CA, USA; 1:1000 in T-PBS/5% BSA; 16–18 h at 4 °C). Horseradish peroxidase (HRP) conjugate secondary antibodies anti-mouse IgG (Sigma) (1:5000 in T-PBS/5% BSA) and anti-rabbit IgG (Sigma) (1:5000 in T-PBS/2% BSA) for 45 min at room temperature were employed. Immunoblots were washed in T-PBS and then developed by ECL chemiluminescence detection (Amersham Bio-
sciences, Germany).

To verify equal loading of sample per lane, membranes were incubated with stripping buffer (62.5 mM Tris–HCl pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol; 30 min at 50 °C) and, afterwards, with a 1:1000 dilution in T-PBS/5% BSA of primary antibodies (c-abl and anti-HSP 90α) [4], Santa Cruz Biotechnology, CA, USA; anti-p38, Cell Signalling Technology-USA). HRP conjugate secondary antibody anti-rabbit IgG (Sigma) was employed.

2.5. 2D-electrophoresis and Western blot

Total cell proteins (100 μg) were separated by two-dimensional (2D) electrophoresis, as previously described [26], electroblotted on PVDF membranes and incubated with anti-pan-acetylated lysine (Santa Cruz Biotechnology, 1:1000 in T-PBS/1% BSA, 16–18 h at 4 °C) and anti-phospho-tyrosine (Cell Signalling Technology-USA, 1:1000 in T-PBS/1% BSA; 16–18 h at 4 °C) primary antibodies; HRP conjugate secondary antibody anti-mouse IgG (Sigma) and anti-rabbit IgG (Sigma) was used and immunoblots were developed by ECL. For mass-spectrometry analy-
sis, total cell protein extracts (200 μg) were separated by 2D electrophoresis and stained by Colloidal Comassie G250 (Biorad). 2D gels images were analysed by Image Master Platinum Ed. software (Amersham Biosciences) and proteins from Comassie blue stained gels were matched with corresponding spots in 2D immunoblotted gels [26,27].

Protein abundance were evaluated by comparing the Spot Staining Volume as calculated by Image Master Platinum Ed. Gels were run in triplicate for each sample and scatter plot (v/v) for the matching spot pools were obtained to verify the degree of reproducibility among three replicates of each sample (experiments n = 3) (see Supporting information-Fig 6).

2.6. MALDI–TOF mass spectrometry

Spots were excised from 2D Comassie blue stained gels, de-stained and sub-
jected to tryptic in-gel digestion with Sequencing Grade Modified Trypsin (Promega, WI) at 37 °C for 2 h. The resulting peptides were concentrated by Zip-Tip C18 (Millipore) and eluted directly by the Matrix solution (5 g/l 2.5-dihydroxybenzoic acid in 50% ACN, 0.15% TFA) on 384–400 μm Anchor Chip Target (Bruker Daltonics, Bremen, Germany). Mass spectra for peptide mass fingerprinting were acquired in reflecton positive ion mode on Ultraflex MALDI–TOF mass spectrome-
ter (matrix assisted laser desorption/ionization time-of-flight, Bruker Daltonics, Bremen, Germany), with an average of 100 laser shots/spectrum. Spectra were externally calibrated using a combination of four standard peptides: angiotensin II (1046.54 Da), substance P (1347.74 Da), bombesin (1619.82 Da) and ACTH18–39 Clip human (2465.20 Da), spotted on positions adjacent to the samples. Experimental peptide mass fingerprinting in the range of 600–3500 Da were compared with NCBI protein database by the software MASCOT (www.matrixscience.org). Confirmatory fragmentation analysis (MS/MS) was performed when needed. Investigation on protein function was conducted with NCBI Tools (Conserved Domain Database, CDD) or UniProt/TREMBL databases.

2.7. Statistical analysis

Mann–Whitney test was employed to determine the significance of differences between various experimental conditions.

In order to analyse reproducibility, gel similarities or experimental variations, Spotlynx analyses was performed by software Image Master Platinum Ed. (Amersham Biosciences) which calculates the linear dependence between spot values of one gel in comparison to another gel.

3. Results

3.1. Cell viability

We tested the ability of imatinib (0.2 μM for sensitive cells and 1 μM for resistant cells) and HDAC1 VPA 2 mM, as single drugs and combined, to reduce viability of CML cells K562, LAMA84 S and LAMA84 R, KBMS P and KBMS R, as well as of negative control HEL60 cells (Fig. 1A). Previously imatinib (range 0.125–1 μM), and VPA (range 0.125–2 mM) had been tested (data not shown). Imatinib induced a decrease of viability in sensitive cells (50% in LAMA S and 40% in KBMS P at 0.2 μM dose) and 25% in LAMA R at 1 μM dose, but it did not have effect on KBMS R and K562 cells. VPA as single drug inhibited K562, LAMA S and LAMA R cells, less KBMS R viability. When imatinib was added to VPA, a significant decrease in cell viability was achieved both in sensitive and resistant cells (Fig. 1A).

3.2. Cell apoptosis

We then tested the ability of imatinib to enhance apoptosis in combination with VPA (Fig. 1B). Imatinib (range 0.125–1 μM) and VPA (range 0.125–2 mM) were tested at escalating doses (data
Fig. 1. Effects of imatinib, VPA and imatinib plus VPA on cell viability and apoptosis. Viability by WST-1 test and apoptosis by Annexin-V-FLUOS Staining Kit in absence or in presence of drugs. (A and B) % of viable cells (A) and % of annexin V positive cells (B); (C and D) % of viable cells (C) and % of annexin V positive cells (D) in human CML primary cells. CTRL = control untreated cells; IM = imatinib; VPA = Valproic acid. Values are presented as mean of 3 experiments ± standard deviation. *P<0.05, n.s. = not significant.
Imatinib induced a significant increase of apoptosis in LAMA R and KBM5 P, but not in LAMA S, KBM5 R and K562. When cells were exposed to VPA, apoptosis in LAMA S, LAMA R, KBM5 P and K562 but not in KBM5 R was markedly induced. The combination of imatinib and VPA induced a significant increase of apoptosis in all cell lines respect to single drug treatment. As a negative control, viability and apoptosis were evaluated in HL60 cell line (Fig. 1A and B). No proper synergy was observed.

3.3. Cell viability and apoptosis in primary CML samples

We also analysed primary CML cases (Fig. 1C and D). After 24 h exposure, imatinib induced a significant decrease of viability in primary CML cells obtained from a case of imatinib-sensitive chronic phase (#1) and from a case of imatinib-resistant blast crisis (#3), but did not affect viability of marrow cells from another case of CML in chronic phase (#2) (Fig. 1C). Imatinib induced a significant
increase of apoptosis only in imatinib-sensitive chronic phase (#1) (Fig. 1D) but combination of imatinib and VPA induced a stronger inhibition of viability and enhancement of apoptosis in all 3 cases (Fig. 1C and D). Imatinib, VPA and imatinib plus VPA did not induce significant effects in normal donor cells (Fig. 1C and D). In all experiments, the percentage of cells in necrosis was <10% respect to total cells.

3.4. BCR/ABL expression and activation

3.4.1. Q-PCR analysis

We measured BCR/ABL mRNA by quantitative real time PCR (Fig. 2A) and evaluated the phosphorylation of BCR/ABL protein by Western blot (Fig. 2B). LAMA84 S cells, exposed to imatinib 0.2 µM only slightly decreased the number of copies of BCR/ABL mRNA, as expected by suboptimal dose, and VPA could reduce marginally BCR/ABL mRNA amount. When both low dose-imatinib and VPA were present for 48 h in culture, LAMA84 S cells produced a few copies of BCR/ABL mRNA. The mechanism of imatinib resistance in LAMA84 R cell line is amplification of BCR/ABL (about 15 copies of hybrid gene per chromosome), and our results of real-time PCR confirm it. In imatinib resistant LAMA84 cell clones, treatment with imatinib resulted in an increased transcription of BCR/ABL and the addition of sole VPA did not reduce BCR/ABL mRNA amount, while the combination of imatinib and VPA induced a significant decrease of BCR/ABL mRNA (Fig. 2A).

3.4.2. BCR/ABL protein analysis—Western blot

We immune-precipitated BCR/ABL protein and evaluated its phosphorylation status after treatment with imatinib and/or VPA for 48 h (Fig. 2B). Proteins’ loading was verified on the same membrane with anti-c-abl. In LAMA84 S, treatment with suboptimal doses of imatinib (0.2 µM) was able to reduce only marginally BCR/ABL protein phosphorylation (68% densitometry value respect to control phosphorylation), whereas no significant effect was seen with imatinib 1 µM in LAMA84 R cells. This concentration of imatinib has been shown previously to be sufficient for inhibiting BCR/ABL phosphorylation in LAMA R [18], but other authors obtained results consistent with ours in terms of active concentrations of imatinib [28]. This discrepancy is probably due to differential sensitivity of diverse clones of LAMA R cells in culture. VPA 2 mM slightly increased BCR/ABL activation in LAMA 84 S (119% densitometry value respect to control phosphorylation), but highly significantly in LAMA84 R (265% densitometry value respect to control phosphorylation). Although this increase in phosphorylation is difficult to explain, when cells were
exposed to combination of VPA and imatinib, BCR/ABL phosphorylation was profoundly inhibited in both S and R cell clones (37% in sensitive and 25% in resistant cells respect to control phosphorylation).

3.5. Acetylome analysis

3.5.1. 2D-electrophoresis and immunoblot anti-acetylated lysine

We analysed post-translational modifications of proteome in CML cell lines LAMA84 S and LAMA84 R after treatment with VPA in combination with imatinib for 48 h. We validated 2D electrophoresis and mass spectrometry (MS) analysis in terms of reproducibility, as we had already done in our previous work [16]. In the anti-pan acetylated WB blots (Fig. 3) we detected 118 clear spots for the imatinib-sensitive cells (Fig. 3A) and 76 for imatinib-resistant cells (Fig. 3E) corresponding to acetylated proteins. Imatinib exposure for 48 h did not influence proteins acetylation compared with untreated cells (135 spots in LAMA S (Fig. 3B) and 84 spots in LAMA R (Fig. 3F). In LAMA S cells treated for 48 h with VPA 2 mM the number of acetylated spots was 106 (Fig. 3C) and in LAMA R 112 (Fig. 3G). LAMA 84 S cells treated with combination of imatinib and VPA demonstrated 116 spots (Fig. 3D), while for LAMA84 R cells were detected 36 spots (Fig. 3H). When we measured global acetylation by densitometry (summing single spot densitometry value), we could not detect appreciable changes for LAMA S cells treated with VPA or imatinib, and with VPA plus imatinib, 94% \((\pm 1.3\) standard deviation \(- SD\)), 103\% \((\pm 1.05\) SD) and 101\% \((\pm 1.1\) SD) of control levels, respectively. On the contrary, global protein acetylation was increased in LAMA R cells by the treatment with VPA, 178\% \((\pm 1.9\) SD) respect to control untreated cells, according to increase of number spots. Imatinib was not able to increase the number and level of acetylated spots \(105\%, \pm 1.1\) SD but combination of imatinib and VPA lead a decrease of both global acetylation \(51\%, \pm 0.5\) SD.

3.5.2. Identification of single protein spots—immunoblot/Comassie gel match and mass spectrometry

Matching of Blue Comassie stained gels and immunoblotted gels was performed by direct superimposition and spots were picked when differently acetylated or expressed than those present on control gels [19]. When mass spectrometry was applied, the picked spots were analysed and 23 common proteins to LAMA S and LAMA R cells were identified and studied (Table 1). Ratio of densitometry values of correspondent acetylated spots in imatinib (IM), valproic acid (VPA) and imatinib plus valproic acid (IM + VPA) treated cells respect to untreated cells (C) was calculated.

Treatment with VPA decreased HSP90 expression in LAMA84 R cells (Fig. 4), but at the same time induced a robust acetylation (Fig. 3G) (ratio VPA treated cells/untreated cells of HSP90 acetylation \(= 16.17 – T a b l e\) 1). Treatment with imatinib plus VPA inhibited both HSP90 expression and acetylation (Fig. 3H, Fig. 4 and Table 1).

We also observed a significant decrease of hnRNP L expression in LAMA R after treatment with imatinib plus VPA (Fig. 4) and an acetylation increase particularly evident after combined treatment (ratio CTRL/IM + VPA = 5.15 – Table 1) (Fig. 3H).

3.6. Phosphoproteome analysis

3.6.1. 2D-electrophoresis and immunoblot anti-phosphorylated tyrosine

Phospho-proteome modifications of LAMA84 S and LAMA84 R are particularly complex to elaborate. We detected 164 spots in untreated LAMA S cells (Fig. 5A, a) and 150 spots in untreated LAMA R cells (Fig. 5A, e) corresponding to phosphorylated proteins. In LAMA S cells treated with imatinib for 48 h, the number of phosphorylated spots was 119 (Fig. 5A, b), while in LAMA R was 206, as expected for a reactive effect to drug exposure (Fig. 5A, f). VPA 2 mM influenced minimally phosphorylation (162 spots in LAMA S (Fig. 5A, c) and 145 in LAMA R (Fig. 5A, g). The combination of
Table 1
Modifications in acetylation of proteins, as identified by mass spectrometry after in vitro treatment with VPA and imatinib.

<table>
<thead>
<tr>
<th>ID</th>
<th>Protein name</th>
<th>Scorea</th>
<th>MWb</th>
<th>pFc</th>
<th>LAMA S</th>
<th>LAMA R</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi31615559</td>
<td>Chain B, X-ray crystal structure of cyclophilin A HIV-1</td>
<td>96</td>
<td>18156</td>
<td>8.34</td>
<td>1.01</td>
<td>0.08</td>
</tr>
<tr>
<td>gi2981764</td>
<td>Peptidylproli isomerase A (cyclophilin A)</td>
<td>124</td>
<td>18154</td>
<td>7.82</td>
<td>0.43</td>
<td>0.24</td>
</tr>
<tr>
<td>gi30582531</td>
<td>Cofilin 1 (non-muscle)</td>
<td>100</td>
<td>18775</td>
<td>8.22</td>
<td>1.38</td>
<td>1.94</td>
</tr>
<tr>
<td>gi5542166</td>
<td>Chain B, H platelet profilin</td>
<td>66</td>
<td>15056</td>
<td>8.47</td>
<td>0.63</td>
<td>0.66</td>
</tr>
<tr>
<td>gi5559846</td>
<td>Peroxiredoxin 1</td>
<td>190</td>
<td>22280</td>
<td>8.27</td>
<td>0.92</td>
<td>0.36</td>
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<tr>
<td>gi55598716</td>
<td>High-mobility group box 1</td>
<td>60</td>
<td>22249</td>
<td>9.61</td>
<td>0.52</td>
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<tr>
<td>gi17389815</td>
<td>Triosephosphate isomerase 1</td>
<td>203</td>
<td>26980</td>
<td>6.45</td>
<td>0.86</td>
<td>0.91</td>
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<td>gi75517570</td>
<td>hnRNP A1</td>
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<td>34317</td>
<td>9.19</td>
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<tr>
<td>gi55633947</td>
<td>Similar to hnRNP H3 isoform a</td>
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<td>gi46812638</td>
<td>hnRNP L</td>
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<td>60977</td>
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<tr>
<td>gi16876910</td>
<td>hnRNP F</td>
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<td>46097</td>
<td>5.38</td>
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<tr>
<td>gi4930291</td>
<td>Fructose bisphosphate aldolase A</td>
<td>228</td>
<td>39833</td>
<td>8.39</td>
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<tr>
<td>gi62897945</td>
<td>Enolase 1 variant</td>
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<td>47594</td>
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<tr>
<td>gi849693</td>
<td>Coproporphirinogen oxidase</td>
<td>62</td>
<td>40976</td>
<td>6.65</td>
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<tr>
<td>gi999893</td>
<td>Triosephosphate isomerase 1 (Tim)</td>
<td>190</td>
<td>26877</td>
<td>6.51</td>
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<td>gi5599292</td>
<td>Annexin A1</td>
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<td>6.57</td>
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<tr>
<td>gi54696794</td>
<td>Chaperonin containing TCP1, subunit 2 (beta)</td>
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<td>57878</td>
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<td>gi54696884</td>
<td>Stress-induced-phosphoprotein 1 (Hsp70/Hsp90-org protein)</td>
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<td>63381</td>
<td>6.4</td>
<td>0.4</td>
<td>0.7</td>
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<tr>
<td>gi55960357</td>
<td>Transgelin 2</td>
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<td>22590</td>
<td>8.41</td>
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<tr>
<td>gi2055427</td>
<td>KSRP</td>
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<td>gi54648253</td>
<td>KSRP</td>
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<td>73420</td>
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<tr>
<td>gi30582861</td>
<td>Nucleophosmin (nucleolar phosphoprotein B23, numatrin)</td>
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<td>32768</td>
<td>4.64</td>
<td>0.19</td>
<td>0.42</td>
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In the table are reported the ratios of densitometry values of phosphorylated spots identified for imatinib (IM), valproic acid (VPA) and IM + VPA treated cells and their corresponding untreated control. After matching of Blue Comassie stained gels and immunoblotted gels, spots were picked when significantly more acetylated or quantitatively different than those present on gels of control not treated cells see methods).

a Score – significance index calculated by MASCOT.
b MW – molecular weight.
c pI – isoelectric point.
d n.d. – not detected.

Fig. 4. Effect of VPA and imatinib plus VPA on expression of HSPs and hnRNP s in LAMA resistant cells. After 48 h of drug treatment, total extracted proteins were separated by 2D electrophoresis. Comassie stained gels were analysed by IMAGE MASTER Software. Spots were picked and identified by Mass Spectrometry analysis by MALDI-TOF. Proteins differently expressed after treatments are indicated.
Fig. 5. Effects of imatinib, VPA and imatinib plus VPA on phosphoproteome of LAMA S and R. (A) After 48 h of drug treatment, total extract proteins were separated by 2D electrophoresis and immunoblotted with anti-phosphotyrosine antibody. Proteins showing altered phosphorylation after treatments are indicated. (B and C) WB analysis, performed in LAMA R, with anti-phosphotyrosine (B) and anti-hnRNP A2/B1 (C). Equal loading of sample per lane was verified by stripping and incubating with anti-HSP90 (B) and anti-p38 (C) antibodies.
Table 2
Modifications in phosphorylation of proteins, as identified by mass spectrometry after in vitro treatment with VPA and imatinib.

<table>
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<th>ID</th>
<th>Protein name</th>
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<th>MW</th>
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<td>IM/C</td>
<td>VPA/C</td>
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<td>IM/C</td>
<td>VPA/C</td>
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<td>gi15215418</td>
<td>Heat shock 90kDa protein 1, beta</td>
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<td></td>
<td>0.3 n.d</td>
<td>n.d.</td>
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<td>gi62087882</td>
<td>Heat shock 70kDa protein 4 isoform 1</td>
<td>97</td>
<td>89015</td>
<td>5.44</td>
<td>n.d.</td>
<td>n.d.</td>
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<td>gi5729877</td>
<td>Heat shock 70kDa protein 8 isoform 1</td>
<td>123</td>
<td>71138</td>
<td>5.37</td>
<td>0.8</td>
<td>1.1</td>
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<tr>
<td>gi31542947</td>
<td>Heat shock 60kDa protein 1, beta</td>
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<td>gi15277503</td>
<td>ACTB protein</td>
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<tr>
<td>gi62897681</td>
<td>Calreticulin precursor variant</td>
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<td>47103</td>
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<td>gi62897409</td>
<td>Beta actin variant</td>
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<td>42122</td>
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<tr>
<td>gi75517570</td>
<td>hnRNP A</td>
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<td>hnRNP A2</td>
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<tr>
<td>gi7665942</td>
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<td>gi31645</td>
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<td>Lysophospholipase isomerase</td>
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<td>gi46360168</td>
<td>Prohibitin</td>
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<tr>
<td>gi30582861</td>
<td>Nucleophosmin (nucleolar phosphoprotein B23)</td>
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<td>32768</td>
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<td>0.9</td>
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In the table are reported the ratios of densitometry values of phosphorylated spots identified for imatinib (IM), valproic acid (VPA) and IM+VPA treated cells and their corresponding untreated control. After matching of Blue Comassie stained gels and immunoblotted gels, spots were picked when significantly more acetylated or quantitatively different than those present on gels of control not treated cells see methods).

a Score = significance index calculated by MASCOT.
b MW = molecular weight.
c pI = isoelectric point.
d n.d. = not detected.

imatinib and VPA markedly reduced the number of phosphorylated spots (147 spots in LAMA S (Fig. 5A, d) and 86 in LAMA R (Fig. 5A, h)).

Level of total phosphorylation was measured by densitometry and a marked decrease of phosphorylation, calculated respect to control gels, was observed in imatinib treated LAMA S cells (66%, ±0.9 SD), but also in VPA treated cells (73%, ±1.3 SD). In LAMA R cells, global phosphorylation was induced by imatinib (125%, ±1.1 SD) but not by VPA (109%, ±1.3 SD). Maximal inhibition of total phosphorylation was observed if this HDACi was combined with imatinib (41%, ±1.1 SD in LAMA S and 65%, ±0.9 SD in LAMA R).

3.6.2. Identification of single protein spots—immunoblot/Comassie gel match and mass spectrometry

Matching of gels was performed as above specified for acetylated and 15 proteins identified as differentially phosphorylated (Table 2). We identified HSP90, HSP70 and HSP60 which phosphorylation was induced by imatinib in LAMA R cells (Fig. 5A, f); treatment with VPA inhibited phosphorylation of HSP90 and HSP70 and decreased phosphorylation of HSP60 (Fig. 5A, g). Combination of IM and VPA maintained these proteins de-phosphorylated (Fig. 5A, h). To confirm the decrease of HSP90 phosphorylation in LAMA R, Western blot analysis was performed (Fig. 5B).

We observed, in an increase of hnRNP A1 and hnRNP A2 phosphorylation in LAMA R after treatment with VPA (Fig. 5A, g), but imatinib plus VPA inhibited phosphorylation of these proteins (Fig. 5A, h). At the same time, we observed that, in LAMA R cells, treatment with VPA and imatinib plus VPA induced a decrease of some hnRNP expression respect to untreated cells (Fig. 4). We investigated the modification of hnRNP A2 and hnRNP B1 expression by Western blot analysis and our results showed that in LAMA R single drugs did not induce modification of expression but a significant decrease was observed after treatment with imatinib plus VPA (Fig. 5C).

4. Discussion

Patients with CML have in the great majority of cases an excellent clinical, cytogenetic and molecular response to imatinib, but some may result refractory or loose clinical response because of innate or acquired resistance, caused by amplification of the BCR–ABL genomic locus or more frequently by point mutations within the kinase domain of BCR–ABL, which prevent imatinib binding [2]. Even if new tyrosine kinase inhibitors are in use (dasatinib [29, nilotinib [30, bosutinib [31]), still there is an unmet need to identify other molecular targets whose inactivation could contribute to the eradication of the CML clone/clones. In fact, different essential pathways like cytoskeleton organization [32], cell mitosis [33] and apoptosis [34] are modified by the presence of BCR/ABL protein. Nevertheless, modifications in cell metabolism determined by the development of imatinib resistance and the detailed molecular mechanisms involved in this event still remain unclear. Proteome analysis of CML cells is just starting to be published in the last few years and data are still scanty [16,35–37]. Our previous work has clarified proteome alterations in imatinib resistant versus sensitive LAMA84 chronic myeloid leukemia cells, and we have published the identification of four classes of proteins which were significantly different in resistant versus sensitive cells: (1) HSPs and chaperones, (2) nucleic acid binding/synthesis/stability, (3) structural proteins (4) cell signalling [16]. Imatinib has been combined with other drugs (i.e. HDAC inhibitors, bortezomib [38], interferon [39]) and some of these combinations have been successful in restoring imatinib sensitivity in CML. Especially co-treatment with imatinib and also other tyrosine kinase inhibitors (TKI) and HDACi (SAHA, LBH589) looks promising [4,5,14,40].

We observed, both in cell-lines and in primary CML cells, that VPA induced a decrease of viability and an increase of apoptosis. Combined treatment of suboptimal dose of imatinib and VPA was effective, confirming what already shown by other authors [17]. It is extremely intriguing that such activity of VPA is more pronounced in CML cells resistant to imatinib than in sensitive ones.

We hypothesized that the mechanism by which VPA restores sensitivity to the tyrosine kinase inhibitor may be mediated not only by chromatin remodelling and transcriptional regulation but also by acetylation and subsequent inactivation of HSP proteins [4] and by modification of critical protein expression (ribosomal shuttle proteins, cycle regulators, cytoskeleton proteins).

We therefore studied protein expression and post-translational modifications of proteome in LAMA84 R and LAMA84 S cells by 2D electrophoresis.

In LAMA R acetylation of some proteins was evidently induced by VPA: coflin-1, profilin, high mobility group box-1, copro-
porphyrinogen oxidase and Hsp90 β. Though total acetylation decreased globally after treatment with VPA plus imatinib, three proteins, hnRNP L, chaperonin containing TCP1 and KSRP, showed a significant increase in acetylation. Such acetylation could alter their function of shuttle and chaperonin proteins [12,13,41]. We found, in fact that VPA decreased expression and increased acetylation of HSP90 β (ratio acetylation VPA treated cells/untreated cells = 16.17 – Table 1). The involvement of HSP90 and indirectly of HSP70 in imatinib resistance had been demonstrated before [9,16]. Broad involvement of HSPs could in fact represent a common shared mechanism in tumour progression. Heat Shock Proteins are inactivated by acetylation and this impairs the interaction with the client protein BCR/ABL, promoting its proteasomal degradation [4,5,41]. In our opinion these findings are particularly relevant because indicate that VPA (similarly to SAHA) may interfere with chaperone protein-driven mechanism of imatinib resistance both by inhibiting cytoplasmic HSP90 expression and by acetylating it.

We observed, for the first time in our knowledge, a marked modulation of heterogeneous nuclear ribonucleoproteins (hnRNP) expression and post translational modifications following VPA treatment. hnRNP are involved in several RNA-related biological processes such as transcription, pre-mRNA processing, mature mRNA transport to the cytoplasm and translation [42]. In BCR/ABL-expressing cells, there is a marked increase in the levels of hnRNP [43]. BCR/ABL oncproteins are involved in post-transcriptional regulation via hnRN shuttle proteins. Transcriptional or post-transcriptional regulation might be the primary mechanism for controlling the levels and/or the activity of certain BCR/ABL effectors and might, in part, explain why the balance between proliferation, differentiation, and apoptosis is completely subverted in CML blast crisis [43]. Particularly, we had detected quantitative increase of 3 hnRNPs in LAMA 84 R respect to LAMA 84 S [16], whose importance and significance in stabilizing and shutting the message of BCR/ABL oncprotein seems fundamental to determine imatinib resistant phenotype. The hnRN K was expressed only in LAMA R [16] and after treatment with VPA and imatinib plus VPA the expression was inhibited. Consistently with our observations, hnRN K expression and sub cellular localization were recently shown to be directly related to Bcr/Abl presence and proliferation, clonogenic potential, and in vivo leukemicogenic activity of BCR/ABL-expressing myeloid cells [44].

We also investigated modifications of phosphoproteome in LAMA cell-lines after treatment with imatinib and VPA. We observed an expected increase in protein phosphorylation in imatinib-treated LAMA R cells. In cells transformed by BCR/ABL, hnRN A1 expression is markedly up-regulated [43]. We showed an inhibition of hnRN A1 expression and phosphorylation after treatment with imatinib plus VPA in LAMA R. HnRN A1 is a protein that shuttles continuously between the nucleus and the cytoplasm, where it dissociates from its mRNA cargo and is rapidly re-imported into the nucleus. It is tempting to speculate that also tyrosine phosphorylation could interfere with the assembly of multiprotein complexes targeting RNA-binding proteins for proteasome-dependent degradation.

In our opinion, the nature of the proteins (nucleus–cytoplasm shuttle and chaperonins) that we found post-translational modified in CML cells after VPA, and imatinib plus VPA, renders our findings particularly relevant and intriguing. VPA induced re-acquisition of sensitivity to imatinib is paralleled by modification of acetylation of both cytoplasmic and nuclear non-histone proteins, similarly to what has been shown for more potent HDACi like SAHA. We show here that not only HSPs, but that several cytoplasmic proteins essential for cell proliferation and mRNA stabilization are acetylated by VPA, and that combined treatment with imatinib, induces acetylation of hnRN L.

Although the effects of VPA in combination with imatinib have been observed earlier, our work constitutes the first attempt to analyze the effect of this HDACi on cellular proteome and the mechanism of cooperative effect.

Evidently, we identified proteins, HSPs and hnRNPs, whose induction of post-translational modifications parallels restoration of imatinib sensitivity. These proteins potentially play a relevant role in imatinib resistance and this role can be modulated by VPA as demonstrated by 2D Western blot. 

Conflict of interest

All authors have no conflict of interest to declare.

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Authors' contributions: FB, RP, DT and VS contributed equally to this work; FB and RP preformed experiments and wrote the results section; GF performed mass spectrometry analysis; VS and DT designed the study and wrote the manuscript; ES performed Western blot experiments and FS, AG and AB collected the samples from CML patients and organized the results.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2011.01.033.

References


