Identification of Differentially Expressed Proteins in Imatinib Mesylate-resistant Chronic Myelogenous Cells

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Resistance to imatinib mesylate (also known as Gleevec, Glivec, and STI571) often becomes a barrier to the treatment of chronic myelogenous leukemia (CML). In order to identify markers of the action of imatinib mesylate, we used a mass spectrometry approach to compare protein expression profiles in human leukemia cells (K562) and in imatinib mesylate-resistant human leukemia cells (K562-R) in the presence and absence of imatinib mesylate. We identified 118 differentially regulated proteins in these two leukemia cell-lines, with and without a 1 μM imatinib mesylate challenge. Nine proteins of unknown function were discovered. This is the first comprehensive report regarding differential protein expression in imatinib mesylate-treated CML cells.

Keywords: Chronic myelogenous leukemia, CML, Gleevec, Glivec, Imatinib mesylate, Proteomics

Introduction

The Philadelphia chromosome (Ph) is the molecular hallmark of chronic myelogenous leukemia (CML), and results from a reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11)]. The Ph translocation results in the formation of oncogenic *Bcr-Abl* fusion proteins, which produce a constitutively active and deregulated protein tyrosine kinase (PTK). The acquired oncogenic activity of *Bcr-Abl* fusion proteins drives a series of inappropriate hematopoietic cell proliferations, and thereby contributes to leukemic transformation (Warmuth *et al.*, 1999; Druker *et al.*, 2002). Multiple signaling/survival pathways originating from this constitutive expression of *Bcr-Abl* PTK appear to be involved in this oncogenesis. Notable among these are the PI3-kinase pathway (Calabretta and Skorski, 1996), a variety of CRKL-linked signaling processes (ten Hoeve *et al.*, 1994), and the Jak-STAT-pathway (Carlesso *et al.*, 1996). In addition to the above-mentioned signaling pathways, a variety of signaling molecules, including mitogens, anti-apoptotic proteins, hematopoietic factors, and cytoskeletal components have been demonstrated to be either phosphorylated or activated by *Bcr-Abl* (Cambier *et al.*, 1998; Mayerhofer *et al.*, 2002). Recently, CML therapy was greatly improved by the introduction of imatinib mesylate (Gleevec or Glivec or STI571 in other names, Novartis Inc., Basel), which is a selective tyrosine kinase inhibitor of *Bcr-Abl*, *Abl*, *Arg*, c-*Kit*, and platelet-derived growth factor receptor. Imatinib mesylate functions as a competitive inhibitor of ATP at the ATP-binding site formed by the tyrosine kinase domains of *Bcr-Abl*, by interfering with cell growth and thereby, inducing cell death in *Bcr-Abl*-harboring leukemic cells (Radford, 2002).

Despite the high efficacies of current hematologic and cytogenetic responses, CML relapses have been observed in a low proportion of CML patients on continued imatinib

mesylate therapy. Intense research has delineated mechanisms by which imatinib mesylate resistance occurs. These mechanisms include the acquisition of genetic alterations, i.e., *Bcr-Abl* gene amplification, point mutations at position 315 (Thr to Ile), and increases in the levels of *Bcr-Abl* protein, P-glycoprotein, α1 acid glycoprotein (Larghero *et al.*, 2003; Mahon *et al.*, 2003).

It is currently accepted that imatinib mesylate inhibits the PTK action of *Bcr-Abl*, and that this eventually blocks the activation of pathways downstream of *Bcr-Abl*, thereby suppressing cell proliferation (Fang *et al.*, 2000). However, the signaling pathways downstream of *Bcr-Abl* responsible for apoptotic resistance have yet to be defined clearly, and thus, the effects of imatinib mesylate have not been established.

In this study, we employed a proteomic approach, employing two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) in order to identify differentially expressed proteins involved in the actions of imatinib mesylate with regard to *Bcr-Abl* in human CML cells. Such candidate proteins may also serve as potential molecular markers of therapeutic efficacy, and facilitate the development of new drugs. In addition to imatinib mesylate-sensitive CML cells, we investigated the proteomic patterns of imatinib mesylate-resistant CML cells created in our laboratory. Using both imatinib mesylate-sensitive and imatinib mesylate-resistant CML cell lines, we investigated cellular protein expressions in response to imatinib mesylate challenge. Finally, we identified and functionally assigned 118 proteins. These functions included; energy transduction, protein synthesis, signal transduction, regulation, differentiation, apoptosis, and cellular defense.

Materials and Methods

Cell culture The K562 human CML cell-line used in this study was obtained from the Korean Cell Line Bank (Seoul), and is sensitive to 1 μM imatinib mesylate. These cells were grown in RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G, and were maintained in a 37°C, 5% CO_2, fully humidified incubator, passaged twice weekly, and prepared for experimental procedures when in the log phase of growth.

Establishment of an imatinib mesylate-resistant CML cell line The imatinib mesylate-resistant K562 (K562-R) cell line was derived from the parental K562 cell line in our laboratory, by subculture in progressively higher imatinib mesylate concentrations, as previously described (Nimmanapalli *et al.*, 2002). K562 cells were cultured, with continuous exposure to imatinib mesylate, in stepwise increments of 100 nM from 0.1 to 1 μM every 10 days. Viable imatinib mesylate-resistant K562 cells were maintained under selection pressure in a medium containing 1 μM imatinib mesylate.

Fluorescence in Situ Hybridization analysis for the Bcr-Abl fusion gene in K562 cells Fluorescence in Situ Hybridization (FISH) was performed on K562-R cell interphase nuclei, as described previously (Lee *et al.*, 2003). FISH analyses were carried out according to the manufacturer's instructions using commercially available FISH probes manufactured by Vysis (Downers Grove, USA). Cells were counterstained with a mixture of 10 ml of 4',6-diamino-2-phenylindole, p-phenylenediamine in phosphate-buffered saline and glycerol (DAPI II) and Vectashield antifade in a ratio of 1 : 100. Analyses were performed using an Olympus fluorescence microscope (Olympus America, Melville, USA) attached to an imaging system (Quips XL Genetics Workstation; Vysis) equipped with a triple-bandpass filter. Representative cell images were captured using a computer-based imaging system. Two hundred nuclei were counted; interphase cell analyses were performed according to the manufacturer's instructions.

Western blot analysis Immunoblot analysis for Bcr-Abl protein was carried out as previously described (Fang *et al.*, 2000; Lee *et al.*, 2003), using an enhanced-chemiluminescence (ECL) detection system (Amersham Corp., Arlington Heights, USA). Monoclonal antibody to Bcr-Abl protein was purchased from Calbiochem (Cambridge, UK).

Preparation of cell lysates K562 and K562-R cells were seeded at 4×10^5 cells/ml, and incubated with 1 μM imatinib mesylate for 48 hours, and these are hence referred to as K+ and R+ cells (where + = 1 μM imatinib mesylate treated), whereas untreated K562 and K562-R cells are referred to as K- and R- cells. K+, K-, R+, and R-cells were washed with Ca^{2+}/Mg^{2+}-free PBS, and suspended in 1 ml of lysis buffer containing 9 M urea, 2% CHAPS, 60 mM dithiothreitol (DTT), 0.5% pharmalyte (pH 3-10), and 1X complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, USA). Cells were then disrupted twice by ultrasonication (2 × 5 seconds) on ice, and separated by centrifugation at 15,000 *g* for 30 min at 4°C. Protein concentrations were determined using Bradford reagents.

Cell proliferation assay Viable cell numbers were determined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Cell Titer 96 Aqueous, Promega, Madison) (Fig. 1). K562 and K562-R cells were plated at a density of 2×10^4 cells in 100 μl of culture medium with/without 1iM imatinib mesylate per well of 96-well plates. Plates were incubated for the numbers of days indicated above and then 20 μl of MTS was added to each well. One hour after the addition of MTS, the plates were read at 490 nm using a microplate reader (Multiskan Ascent, Labsystems, Helsinki).

Two-dimensional gel electrophoresis Isoelectric focusing (IEF) was performed using pre-cast immobilized pH gradient strips (24 cm, pH 3-10, linear, Amersham Biosciences, Uppsala, Sweden). Five hundred μg of proteins were solubilized in rehydration buffer (9 M urea, 2% CHAPS, 60 mM DTT, 0.5% pharmalyte, pH 3-10, 0.002% bromophenol blue) and protein samples were loaded on IPG strips and rehydrated overnight. IEF gel was run for a total of
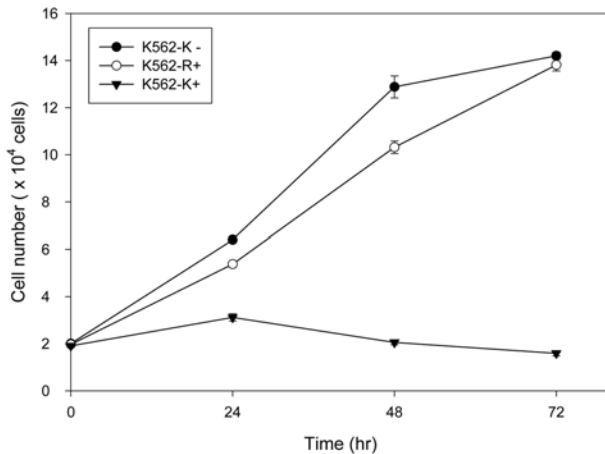
Fig. 1. The effect of imatinib mesylate on the proliferation activities of K562 and K562-R cells. The K562 cells were cultured in the presence or absence of 1 μM imatinib mesylate. K562-R cells were also cultured in the presence of 1 μM imatinib mesylate. Cell proliferation was detected by MTS reduction to its formazan product by determining absorbance at 490 nm. Results are expressed as mean OD's at 490 nm of quadruplicate cultures (directly proportional to viable cell number). Error bars represent standard errors. K562-K-, K562-K+, and K562-R+ represent K562 cells incubated with no imatinib mesylate, incubated with 1 μM imatinib mesylate, and K562-R cells incubated with 1 μM imatinib mesylate, respectively.

36 kVh during which the voltage was linearly increased from 100 to 8000 V over 6 h and then, maintained for 3 h at 8000 V. After IEF, strips were first equilibrated for 15 min in a reducing solution (50 mM Tris HCl, pH 8.8, 6 M urea, 30%(v/v) glycerol, 2% (w/v) SDS, 1% (w/v) DTT), and then for a further 15 min in an alkylating solution, which was identical in make-up to the reducing solution except that 2.5% (w/v) iodoacetamide was substituted for DTT (Gorg et al., 2000; Barnes and Kim, 2004).

Second gel electrophoresis was performed using a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protocol, using the Ettan DALT 6 System (Amersham Biosciences, Uppsala). SDS-PAGE was run on 12% polyacrylamide gel, and gels were visualized by silver staining, according to the protocol described by Rabilloud et al. (1999). Stained gels were analyzed using Phoretix 2D image analyzing software (Non-Linear Dynamics Inc., Durham, USA). Two-dimensional gel electrophoresis was carried out triplicate for each sample and reproducible spots were then selected for identification.

In gel digestion and mass spectrometry Gel spots were excised, destained by reduction using a solution of 30 mM potassium ferricyanide/100 mM sodium thiosulfate, and washed with water. The gel pieces were then incubated with 0.2 M NH$_4$HCO$_3$ for 20 min, dehydrated, shrunk with 100% acetonitrile twice, and dried by vacuum centrifugation.

For "in-gel" digestion with trypsin, gel pieces were rehydrated in digestion buffer containing 0.05 M NH$_4$HCO$_3$ and 10 ng/μl of modified porcine trypsin (Promega, Madison, USA) at 4°C for 30-45 min. Excess supernatant was then removed, and the gel pieces

were covered with 30 μl of 0.05 M NH$_4$HCO$_3$ buffer. Digestion was performed overnight at 37°C, and after "in-gel" tryptic digestion, tryptic peptides were extracted from the gel particles (Rosenfeld et al., 1992; Shevchenko et al., 1996). Samples were desalted using a GELoader tip (Eppendorf AG, Hamburg, Germany) and packed with POROS 20 R2 resin (Applied Biosystems Inc., Foster City, USA). Peptide binding and washing were realized in 0.1% trifluoroacetic acid (TFA) in water. To produce the MALDI sample matrix, α-cyano-4-hydroxy cinnamic acid was dissolved in a solution containing 70% acetonitrile and 0.1% TFA at concentration of 5 g/l. Elution was performed with 1 μl of sample matrix and the eluted peptides were directly spotted on the target plate. Protein identification was carried out using peptide mass fingerprinting (PMF) using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-PRO MALDI-TOF mass spectrometer, Applied Biosystems Inc., Foster City, USA) (Scheler et al., 1998). Mass spectra were registered in reflectron positive ion mode. Mass accuracy was set at 50 ppm for PMF analysis. Database searches for PMF were performed using the MASCOT search program, which was developed by Matrix Science Ltd. (access is available on http://www.matrix.science. com), at the NCBI database (http://www. ncbi.nlm.nih.gov/entrez) and using the ExPASy Molecular Biology Server at the SWISS-PROT database (http://www.expasy.org).

Results

Effect of imatinib mesylate on K562 cell growth Imatinib mesylate at 1 μM affected K562 cell but not K562-R cell growth, verifying the resistance of K562-R cells to imatinib mesylate at this concentration. After imatinib mesylate treatment, K562 cells underwent the death process. Therefore, we performed MTS assays, to determine the survival rates of 1 μM imatinib mesylate treated K562 cells (Fig. 1). The assays revealed that the population of viable K562 cells after 48 hours of 1 μM imatinib mesylate treatment was only 16% of that of untreated K562 cells. 48 hrs was selected because it showed this dramatic difference between the survival rates of imatinib mesylate treated and untreated cells (Fig. 1), and thus maximize differential protein expressions due to imatinib mesylate.

Fluorescence in Situ Hybridization analysis for the Bcr-Abl fusion gene in imatinib mesylate-resistant K562 cells FISH analyses were performed on K562-R cell interphase nuclei. All probes were directly labeled with spectrum green or spectrum orange, and thus, Bcr and Abl gene localizations in K562-R cells were detected as green and orange signals, respectively, and Bcr-Abl fusion genes as yellow signals (Fig. 2).

FISH analyses were carried out to detect interphase nuclei among lots of 200 cells. 100% of the K562-R cells showed yellow signals representing reciprocal translocations between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11)]. Moreover, all observed interphase nuclei contained four or more copies of the Ph chromosome.
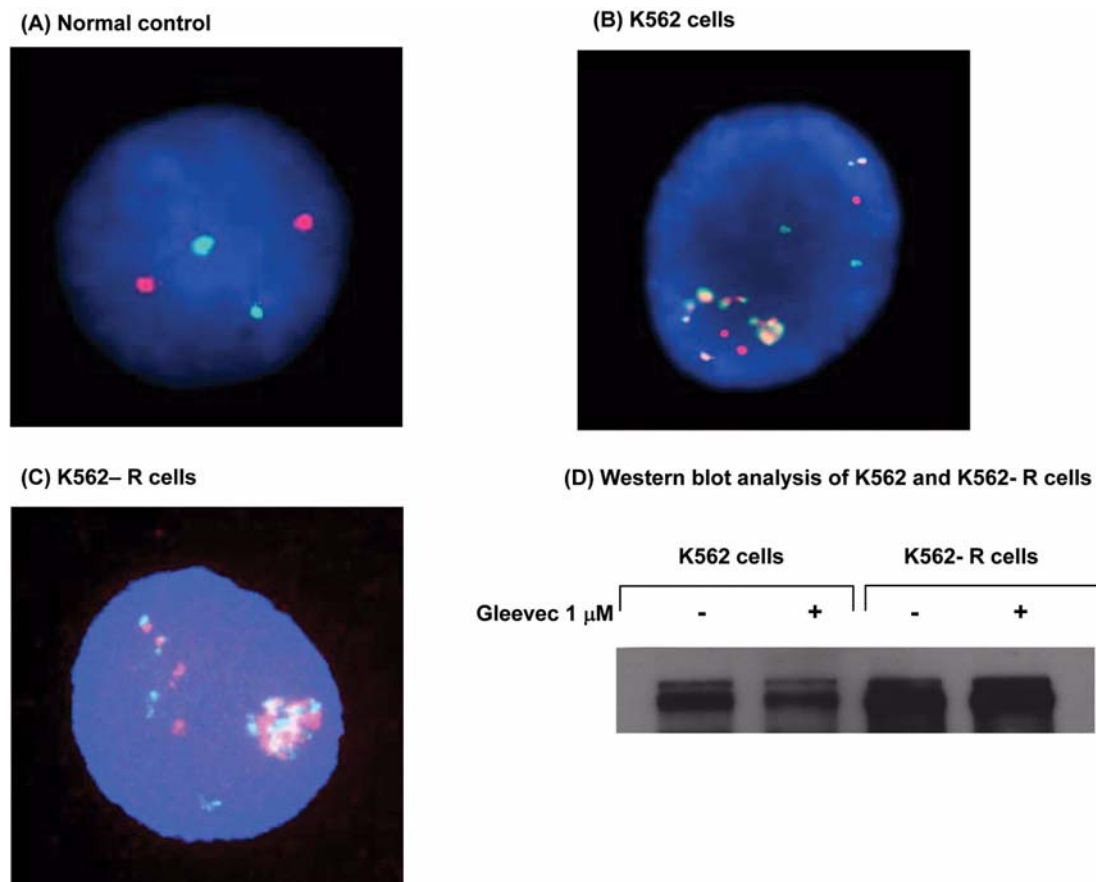
Fig. 2. FISH and Western blot analyses of *BCR-ABL* gene amplification in imatinib mesylate-sensitive and resistant K562 cells. FISH analyses are shown. The localizations of the *BCR* and *ABL* genes in the interphase nuclei of K562 and K562-R cells were detected as green and orange signals, respectively, whereas *BCR-ABL* fusion genes appeared as yellow signals due to color overlap. The several clustered yellow signals shown indicate amplification of the *BCR-ABL* fusion gene. The yellow color is not present in mononuclear cells from peripheral blood. The yellow signal was present in K562 cells but signals were amplified in K562-R cells. Western blot was carried for Bcr-Abl fusion protein in K562 and K562-R cells treated with/without 1 mM imatinib mesylate, and Bcr-Abl protein was found to be markedly amplified in K562-R cells. FISH analyses are shown in (A) normal control, (B) K562 cells, (C) K562-R cells. Western blot (D) shows K562 and K562-R cells.

Analysis of proteomic patterns in CML cell lines In order to identify proteins differentially expressed in K562 and K562-R cells due to exposure to 1 μM imatinib mesylate, we performed proteomic analyses using 2-DE and MALDI-TOF MS. Both cell lysates were prepared for 2-DE after 48 hours of treatment with 1 μM imatinib mesylate. Protein spots on gels were identified by PMF using a database search. Four typical K+, K-, R+, and R- cell 2-DE gels are shown in Fig. 3. Multiple (n>5) cell preparations were carried out at each condition for 2-DE. Spots which appeared consistently were marked with circles for MALDI-TOF MS identification.

The identified proteins were assigned to these broad classes, according to their known or putative functions: (1) Energy transduction; (2) Protein synthesis (DNA/RNA processing, synthesis, transcription, amino acid metabolism); (3) Signal transduction, regulation, differentiation, and apoptosis; (4) Cellular defense; and (5) Other/Unknown functions.

Proteomic patterns of K562 cells treated or not treated with imatinib mesylate Imatinib mesylate treatment caused K562 cells to undergo the death process, and we undertook to identify proteins that were differentially expressed by this treatment. The results obtained indicated that 38 proteins were differentially expressed in K562 cells by imatinib mesylate, as shown in Table 1. Compared with K- cells, 11 proteins were consistently over-expressed K+ cells, while 27 proteins were down-regulated. The following were up-regulated by more than 300% compared to expression in K- cells; ATP synthetase coupling factor B and flavin reductase (ATP production - a broad class, designating "any involvement in ATP production," also, all other broad experimental classes are defined in a similar manner) (Barile *et al.*, 2000), pulmonary surfactant-associated protein (cellular defense) (Floros and Hoover, 1998), Yippee-like protein 6 (unknown function), Kunitz-type protease inhibitor 3 (protease inhibitor
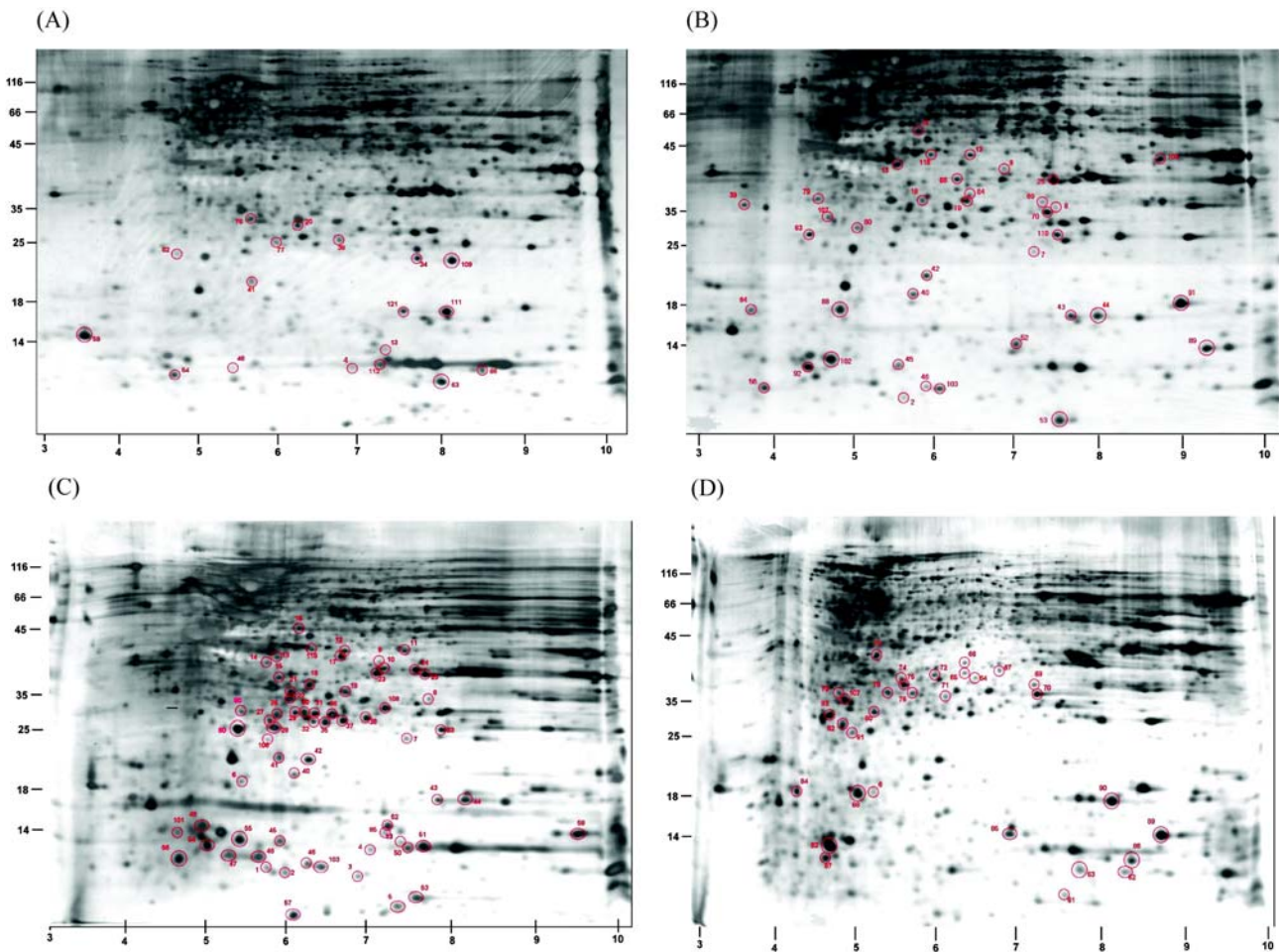
Fig. 3. Two-dimensional gel analysis of K562 and K562-R proteins after treatment with/without 1 μM imatinib mesylate Fig. 3 (A) K562 cells treated with 1 μM imatinib mesylate (K+ cells), Fig. 3 (B) untreated K562 cells (K- cells), Fig. 3 (C) K562-R cells treated with 1 μM imatinib mesylate (R+ cells), and Fig. 3 (D) untreated K562-R cells (R- cells). 2-DE gels were silver-stained and the marked proteins represent matching proteins in the two gels compared that showed different expression levels after exposure to 1 μM imatinib mesylate for 48 hours. Only up- and down-regulated spots listed in Tables 1, 2, and 3 are labeled. Their respective counterparts spots are not labeled for clarity. The numbers marked on the gels match the protein numbers in Tables 1, 2, and 3. Numbers on the vertical axis represent molecular weights in kDa while those on the horizontal axis are p*I* values.

unknown), and WAP four-disulfide core domain protein 3 (protease inhibitor). Whereas, the following were down-regulated by more than three-fold versus their expressions in the K- cell control; Tropomyosin β chain (cytoskeleton) (Schiaffino and Reggiani, 1996), Ubiquitin-protein ligase E3 Mdm2 (cell cycle regulation) (Goetz *et al.*, 2001), Rho GDP-dissociation inhibitor 1 (signal transduction) (Huber *et al.*, 1994), phosphorylase B kinase γ catalytic chain (signal transduction) (Brieger *et al.*, 2004) and an unknown protein for IMAGE 4908447 (unknown).

Six proteins involved in signal transduction were identified: phosphatidylinositol transfer protein α and β isoform (Larijani *et al.*, 2003), serine/threonine protein kinase 13 (Ebos *et al.*, 2002), Rho GDP-dissociation inhibitor 1 (Huber *et al.*, 1994), phosphorylase B kinase γ catalytic chain (Brieger *et al.*, 2004), and 14-3-3 protein epsilon (Henriksson *et al.*, 2000). All of these were down-regulated in K+ versus K-, which

implies that imatinib mesylate treatment reduces signal transduction pathways downstream of *Bcr-Abl* PTK.

Interestingly, five proteins of unknown function were isolated during the differential proteomic analysis: Yippee-like protein 6, an unknown protein IMAGE 4908447, acidic leucine-rich nuclear phosphoprotein 32 family member B, and UPF0203 protein 15E1.1. Functional investigations are required on these proteins to determine whether they can be used as biomarkers for CML therapy.

Proteomic patterns of K562-R cells treated or not treated with imatinib mesylate Imatinib mesylate at 1 μM was fatal to K562 cells, whereas K562-R cells remained viable, even after prolonged exposure (48 hours) (Fig. 1). It was anticipated that the differential proteomic expressions of K562-R cells in the presence and absence of imatinib mesylate might provide protein expression data regarding the

Table 1. Identification of proteins showing expressional changes in K562 cells after 1 μM imatinib mesylate treatment

Gel match No.[a]	Protein	Function	Database Accession No[b]	Ratio[c] (K+/K-)	MW	pI	Sequence Coverage (%)
Energy transduction							
112	ATP synthase coupling factor B	ATP production	Q99766	**8.651***[d]	20300	6.65	17
109	Flavin reductase	ATP production	P30043	**3.965***	22000	7.31	40.5
69	Phosphoglycerate mutase A	glycolysis	gi/4505753	-2.654	29000	6.75	57.7
43	NADH-ubiquinone oxidoreductase 19 kDa subunit	energy transduction	P51970	-1.756	19973	7.93	60.8
Protein synthesis (DNA/RNA processing, synthesis, transcription, amino acid metabolism), chaperone, protein folding							
86	Exosome complex exonuclease RRP40	RNA processing	Q9NQT5	1.874	29600	8.39	15.6
77	Methyl-transferase-like protein 4	methyl-transferase	Q8TCB7	1.516	28000	5.32	19.6
39	Tropomyosin β chain	cytoskeleton	P07951	**-3.084***	28484	4.61	23
8	Axonemal dynein light intermediate polypeptide 1	motor protein	O14645	-2.411	28650	6.96	17.1
52	Zinc finger protein 18 fragment	transcription factor RNA synthesis	P17022	-2.051	6456	9.15	57.1
19	Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B)	RNA synthesis	Q99729	-1.974	30588	7.69	31.6
40	Prefoldin subunit 3	chaperon, transport	Q15765	-1.657	21448	6.63	26.5
46	Trefoil factor 2	structural component	Q03403	-1.522	11989	5.21	32.1
Signal transduction, regulation, differentiation and apoptosis							
41	Cyclin-dependent kinase 6 inhibitor	cell cycle regulation	P42773	2.085	18127	6.05	38.1
25	CASP8 and FADD-like apoptosis regulator precursor	apoptosis	O15519	-1.716	34384	8.12	18
110	Phosphatidylinositol transfer protein β isoform	signal transduction	P48739	-1.684	31400	6.44	24.4
18	Phosphatidylinositol transfer protein α isoform	signal transduction	Q00169	-1.507	31675	6.13	14.5
105	Serine/threonine protein kinase 13	signal transduction	Q9UQB9	-1.501	35600	8.96	19.4
16	Ubiquitin-protein ligase E3 Mdm2	cell cycle regulation	Q00987	**Infinite decrease**	35900	4.34	16.6
7	Rho GDP-dissociation inhibitor 1	signal transduction	P52565	**-3.498***	23200	5.03	19.6
118	Phosphorylase B kinase γ catalytic chain	signal transduction	Q16816	**-3.066***	44900	6.39	17.9
103	Interleukin 1 family member 7	cytokine	Q9NZH6	-2.851	21900	6.53	21
42	Somatotropin	growth control	P01241	-2.029	19801	5.92	26.3
80	14-3-3 protein epsilon (protein kinase C inhibitor protein 1)	signal transduction	P62262	-1.852	29000	4.63	16.1
cellular defense							
20	Pulmonary surfactant associated protein	glycoprotein cellular defense	P35247	**6.855***	35472	6.97	30.4
70	T-cell surface glycoprotein CD1b	cellular defense	P29016	-1.923	35000	5.88	16.1
91	C5a anaphylatoxin chemotatic receptor	cellular defense	P21730	-1.714	8273	8.93	64.9

effects of imatinib mesylate on non-PTK action.

Our results indicated that 46 proteins were differentially expressed under these conditions, as shown in Table 2. Compared to the R- cells, 30 of these 46 proteins were consistently up-regulated and 16 were consistently down-regulated in R+ cells. The following proteins were up-regulated in by more than 300% in R+ cells versus R- cells; NADH-ubiquinone oxidoreductase 19 kDa subunit (energy

Table 1. Continued

Gel match No.[a]	Protein	Function	Database Accession No[b]	Ratio[c] (K+/K-)	MW	pI	Sequence Coverage (%)
other functions							
63	Yippee-like protein 6	unknown	Q96NS1	**17.329***	13300	8.68	50
4	Kunitz-type protease inhibitor 3 fragment	protease inhibitor unknown	P49223	**6.213***	7407	6.36	32.8
13	WAP four-disulfide core domain protein 3	protease inhibitor unknown	Q8IUB2	**3.77***	22100	7.66	30
38	DGCR6L protein	cell migration	Q9BY27	1.969	24932	7	13.7
59	Coagulation factor X	glycoprotein	P00742	1.69	15725	4.59	48.2
66	Unknown (protein for IMAGE 4908447)	unknown	gi/19684029	**-3.713***	32000	9.13	22
79	Acidic leucine-rich nuclear phosphoprotein 32 family member B	unknown	Q92688	-2.936	29000	3.94	12.7
89	Metallothionein-IV	metal binding protein/ unknown	P47944	-2.467	6418	8.38	37.1
56	UPF0203 protein 15E1.1	unknown	O43715	-1.902	8785	5.37	56.6
53	Metallothionein-IR	metal binding protein/ unknown	Q93083	-1.871	6062	8.38	83.6
64	Follistatin	glycoprotein	P19883	-1.785	35000	5.31	32.7
83	Macropain subunit delta	endopeptidase	gi/296734	-1.554	12000	6.81	66

[a] Gel match numbers were arbitrarily assigned by the Phoretix 2D image analysis program.
[b] Accession numbers represent entries in the SWISS-PROT and NCBI databases.
[c] Ratios are relative values presenting fold-changes of protein spots in 2-DE gels. K+ = K562 cells treated with 1 μM imatinib mesylate for 48 hours. K- = K562 cells untreated for 48 hours. Ratios represent intensities of K+ versus K- in 2-DE gels. + (plus) and - (minus) ratio values represent up-regulated and down-regulated by imatinib mesylate, respectively.
[d] * and bold values represent points at which mean fold change was 3 or -3.

transduction) (Gomez-Diaz *et al.*, 1997), mitochondrial import inner membrane translocase subunit TIM9A (protein transport) (Koehler *et al.*, 2000), tropomyosin 1a-chain (cytoskeleton) (Schiaffino and Reggiani, 1996), tumor suppressor p53 (transcription factor) (Goetz *et al.*, 2001), heterogeneous nuclear ribonucleoprotein A/B (RNA synthesis) (Ostrowski *et al.*, 2000), B-cell lymphoma/leukemia 11A (transcription factor) (Brieger *et al.*, 2004), zinc finger protein 79 (RNA synthesis) (Wang *et al.*, 2001), glycine N-methyltransferase (amino acid metabolism) (Ogawa *et al.*, 1998), fatty acid-binding protein (lipid transport), Ras-related protein Rab-36 (protein transport) (Burstein *et al.*, 1992), regulator of G-protein signaling 16 (signal transduction) (Moratz *et al.*, 2000), guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-11 subunit (signal transduction) (Billadeau *et al.*, 2000), calsenilin (potassium channel/apoptosis) (Sanz *et al.*, 2002), HLA Class II histocompatibility antigen DRB3-2 b chain (MHC related) (Curti *et al.*, 2002), Kunitz-type protease inhibitor 3 (protease inhibitor unknown), inter-alpha-trypsin inhibitor light (glycoprotein), and metallothionein-IR (metal binding protein/unknown). Whereas, the following were down-regulated more than three-fold in R+ versus R-cells; phosphoglycerate mutase A (glycolysis) (Adam *et al.*, 1995), tropomyosin a 4 chain (Schiaffino and Reggiani,

1996), tumor necrosis factor receptor superfamily member 25 (apoptosis) (Mukhopadhyay *et al.*, 2002), complement component C9a (cellular defense), T-cell surface glycoprotein CD 1b (cellular defense), complement factor 1 (cellular defense), acidic leucine-rich nuclear phosphoprotein 32 family member B (unknown), follistatin (glycoprotein), and macropain subunit delta (endopeptidase).

Four proteins associated with signal transduction were identified: regulator of G-protein signaling 16 (Moratz *et al.*, 2000), guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-11 subunit (Billadeau *et al.*, 2000), serine/threonine-protein kinase 22C (Ebos *et al.*, 2002), and Rho GDP-dissociation inhibitor 1 (Huber *et al.*, 1994; Larijani *et al.*, 2003). The first three of these proteins were up-regulated in R+ versus R-, and the forth was down-regulated, which differs from the result in the previous section, in which all six signal transduction proteins were down-regulated in K+ versus K-cells.

Interestingly, five proteins of unknown function were isolated during the analysis: RING finger protein 152, acidic leucine-rich nuclear phosphoprotein 32 family member B, 14-3-3 protein eta (protein AS1), Yippee-like protein 5, and hypothetical protein FLJ31482. In particular, the acidic leucine-rich nuclear phosphoprotein 32 family member B was

Table 2. Identification of proteins showing expressional changes in K562-R cells after 1 μM imatinib mesylate treatment

Gel match No.[a]	Protein	Function	Database Accession No[b]	Ratio[c] (R+/R-)	MW	pI	Sequence Coverage (%)
Energy transduction							
43	NADH-ubiquinone oxido-reductase 19 kDa subunit	energy transduction	P51970	**3.827***[d]	19973	7.93	60.8
49	Cytochrome c oxidase polypeptide Va	energy transduction	P20674	1.638	12513	4.88	50.5
69	Phosphoglycerate mutase A	glycolysis	gi/4505753	**-4.221***	29000	6.75	57.7
Protein synthesis (DNA/RNA processing, synthesis, transcription, amino acid metabolism) chaperone, protein folding							
5	Mitochondrial import Inner membrane translocase subunit (TIM9A)	protein transport	Q9Y5J7	**22.352***	10377	6.71	32
113	Tropomyosin 1 α-chain	cytoskeleton	P09493	**7.616***	32700	4.69	21.8
10	Tumor suppressor p53	transcription factor	P04637	**6.873***	43600	6.33	16
19	Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B)	RNA synthesis	Q99729	**6.39***	30588	7.69	31.6
21	B-cell lymphoma/leukemia 11A	transcription factor	Q9H165	**5.429***	30251	6.82	24.5
55	Zinc finger protein 79 fragment	RNA synthesis	Q15937	**4.53***	11999	8.96	83.1
23	Glycine N-methyltransferase	amino acid metabolism	Q14749	**3.654***	32611	6.58	26.5
57	Fatty acid-binding protein	lipid transport	O15540	**3.484***	14757	5.41	71.8
24	Ras-related protein Rab-36	protein transport	O95755	**3.205***	36322	8.05	30
32	Short stature homeobox protein	transcription factor	O15266	1.719	25500	6.34	20
82	Tropomyosin α 4 chain	cytoskeleton	P07226	-6.755*	28500	4.67	18.1
72	Doublesex and mab-3 related transcription factor 2	transcription factor	Q9Y5R5	-2.007	24000	5.07	16.8
Signal transduction, regulation, differentiation and apoptosis							
31	Regulator of G-protein signaling 16	signal transduction	O15492	**5.843***	22767	6.32	37.6
1	Guanine nucleotide-binding protein G(I)/G(S)/G(O) gamma-11 subunit	signal transduction	P61952	**4.974***	8480	5.47	26
27	Calsenilin	potassium channel/ apoptosis	Q9Y2W7	**4.175***	29231	5.23	24.6
41	Cyclin-dependent kinase 6 inhibitor	cell cycle	P42773	**3.752***	18127	6.05	38.1
25	CASP8 and FADD-like apoptosis regulator	apoptosis	O15519	1.602	34384	8.12	18
22	Serine/threonine-protein kinase 22C	signal transduction	Q96PN8	1.535	30101	6.25	14.9
62	Tumor necrosis factor receptor superfamily member 25	apoptosis	Q93038	**-12.131***	13300	8.68	50
107	Rho GDP-dissociation inhibitor 1	signal transduction	P52565	-2.441	23200	5.03	19.6
81	Hepatoma-derived growth factor (HDGF)	growth factor	P51858	-1.958	27000	4.7	14.2
80	14-3-3 protein epsilon (protein kinase C inhibitor protein-1)	many functions/cell signaling	P62262	-1.634	29000	4.63	16.1

down-regulated both in K+ vs. K- and R+ vs. R- proteomic patterns. It appears that this protein is down-regulated by imatinib mesylate or imatinib mesylate-related pathways; its function has not been established.

It is important that the functions of these five newly discovered unknown proteins be further investigated, in order to determine whether or not they can be used as biomarkers for CML therapy.

Proteomic patterns of K562 and K562-R cells treated with imatinib mesylate The proteomic patterns of K562 and K562-R cells were investigated after 1mM imatinib mesylate

Table 2. Continued

Gel match No.[a]	Protein	Function	Database Accession No[b]	Ratio[c] (R+/R-)	MW	pI	Sequence Coverage (%)
Cellular defense							
108	HLA Class II histocompatibility Antigen, DRB3-2 β chain	MHC related	P01913	**6.868***	27000	6.83	12.7
36	Extracellular superoxide dismutase	antioxidant/cellular defense	P08294	2.821	24162	6.32	26.6
26	Peroxiredoxin 6	antioxidant/cellular defense	P30041	2.104	24903	6.02	19.3
78	Complement component C9a	cellular defense/degradation	P02748	**-22.883***	28000	4.59	24.6
70	T-cell surface glycoprotein CD 1b	cellular defense	P29016	**-3.998***	35000	5.88	16.1
67	Complement factor 1	cellular defense/degradation	P05156	**-3.324***	35000	6.69	30.3
other functions							
4	Kunitz-type protease inhibitor 3 fragment	protease inhibitor/unknown	P49223	**5.054***	7407	6.36	32.8
47	Inter-alpha-trypsin inhibitor light	glycoprotein	P02760	**3.864***	15974	4.89	22.4
53	Metallothionein-IR	metal binding protein/unknown	Q93083	**3.81***	6062.2	8.38	83.6
33	RING finger protein 152	unknown	Q8N8N0	**3.679***	22357	8.2	17.7
38	DGCR6L protein	cell migration	Q9BY27	**3.522***	24932	7	13.7
50	Thyrotropin β chain	glycoprotein hormone	P01222	2.759	12900	8	51.8
46	Trefoil factor 2	structural component	Q03403	2.011	11989	5.21	32.1
29	Regulatory factor X-associated protein	MHC related	O00287	1.959	28232	5.41	19.1
51	Metallothionein-IA	metal binding protein/unknown	P04731	1.701	6133	8.38	91.8
79	Acidic leucine-rich nuclear phosphoprotein 32 family member B	unknown	Q92688	**-20.773***	29000	3.94	12.7
64	Follistatin	glycoprotein/FSH inhibitor	P19883	**-14.342***	35000	5.31	32.7
83	Macropain subunit delta	endopeptidase	gi/41150733	**-4.613***	12000	6.81	66
75	14-3-3 protein eta (protein AS1)	unknown	Q04917	-2.882	28000	4.76	20.4
63	Yippee-like protein 5	unknown	Q96NS1	-2.302	13300	8.68	50
71	Hypothetical protein FLJ31482	unknown (similar to ATP dependent RNA helicase)	Q96N29	-1.868	29000	5.28	21.6

[a]Gel match numbers were arbitrarily assigned by the Phoretix 2D image analysis program.
[b]Accession numbers are entries in the SWISS-PROT database and the NCBI database.
[c]Ratios are fold-changes of protein spots in 2-DE gels. R+ = K562-R cells treated with 1 μM imatinib mesylate for 48 hours. R- = K562-R cells left untreated for 48 hours. Ratios represent intensities of R+ versus R- in 2-DE gels. + (plus) and − (minus) ratio values represent up-regulated and down-regulated by imatinib mesylate, respectively.
[d]* bold values represent points at which the mean fold change was 3 or -3.

treatment (K+ vs. R+ proteomic analysis). K562-R cells were viable in 1 μM imatinib mesylate, whereas this concentration was fatal to K562 cells. This analysis was designed to complement the examination described in the previous section.

The K- cell in the K+/K- proteomic analysis was identical to the R+ cell with regard to the K+/R+ proteomic analysis. Both the K- and R+ cells survived under both control experimental conditions, wherein the K- cells were left untreated, and the R+ cells were treated with 1 μM imatinib mesylate. The expected results from the two controls were that there would be no imatinib mesylate-induced effects in the K- cell, whereas the R+ cell would undergo imatinib

mesylate-mediated non-PTK action. Therefore, it was also expected that a comparison between the untreated K562 cells versus the imatinib mesylate-treated K562-R cells as controls, might provide additional information regarding protein expression resulting from the effects of imatinib mesylate on non-PTK action.

The K+ vs. R+ proteomic analysis revealed that a total of 34 proteins were differentially expressed under these conditions, as shown in Table 3. Compared to R+ cells, six proteins were consistently over-expressed and 28 proteins were consistently down-regulated in K+ cells. Tropomyosin α-4, chain (cytoskeleton) (Schiaffino and Reggiani, 1996),

Table 3. Identification of the proteins showing expressional differences in K562 vsrsus K562-R-cells exposed to 1 μM imatinib mesylate

Gel match No.[a]	Protein	Function	Database Accession No[b]	Ratio[c] (K+/R-)	MW	pI	Sequence Coverage (%)
Protein synthesis (DNA/RNA processing, synthesis, transcription, amino acid metabolism) chaperone, protein folding							
82	Tropomyosin α 4 chain	cytoskeleton	P07226	**9.8***[d]	28500	4.67	18.1
101	Ras-related protein Rab-3B	protein transport	P20337	**-3.052***	24700	4.85	18.7
106	Ras-related protein Ral-A	protein transport	P11233	-2.638	24400	7	14.6
8	Axonemal dynein light intermediate polypeptide 1	motor protein	O14645	-2.409	28650	6.96	17.1
52	Zinc finger protein 18 fragment	RNA synthesis	P17022	-2.122	6456	9.15	57.1
24	Ras-related protein Rab-36	protein transport	O95755	-1.997	36322	8.05	30
29	Regulatory factor X-associated protein	transcription factor	O00287	-1.866	28232	5.41	19.1
10	Tumor suppressor p53	transcription factor	P04637	-1.842	43600	6.33	16
21	B-cell lymphoma/leukemia 11A	transcription factor	Q9H165	-1.825	30251	6.82	24.5
40	Prefoldin subunit 3	chaperone transport	P61758	-1.743	21448	6.63	26.5
19	hnRNP A/B	RNA synthesis	Q99729	-1.599	30588	7.69	31.6
Signal transduction, regulation, differentiation and apoptosis							
41	Cyclin-dependent kinase 6 inhibitor	cell cycle	P42773	**-3.944***	18127	6.05	38.1
103	Interleukin 1 family member 7	cytokine	Q9NZH6	**-3.095***	21900	6.53	21
42	Somatotropin	growth control	P01241	-2.946	19801	5.92	26.3
18	Phosphatidylinositol transfer protein α isoform	signal transduction	Q00169	-2.5	31675	6.13	14.5
35	Tumor necrosis factor receptor superfamily member 25	apoptosis	Q93038	-2.364	23197	6.71	20.6
22	Serine/threonine-protein kinase 22C	signal transduction	Q96PN8	-1.757	30101	6.25	14.9
121	Proline-serine-threonine phosphatase interacting protein 2	signal transduction	Q9H939	**3.461***	38700	8.73	24.3
111	Regulator of G-protein signaling 4	signal transduction	P06493	2.393	23200	8.69	30.2
118	Phosphorylase B kinase gamma catalytic chain	signal transduction	Q16816	-1.723	44900	6.39	17.9
1	Guanine nuclestide-binding protein G(I)/G(S)/G(O) gamma-11 subunit	signal transduction	P50152	-1.644	8480	5.47	26
80	14-3-3 protein epsilon (protein kinase C inhibitor protein1)	signal transduction	P62262	-1.546	29000	4.63	16.1
cellular defense							
20	Pulmonary surfactant-associated protein	glycoprotein cellular defense	P35247	1.575	35472	6.97	30.4
108	HLA Class II histocompatibility Antigen	MHC related	P01913	**-3.019***	27000	6.83	12.7
26	Peroxiredoxin 6	antioxidant cellular defense	P30041	-2.275	24903	6.02	19.3
60	Complement C1r subcomponent	cellular defense degradation	P00736	-1.571	27095	5.36	26.4

proline-serine-threonine phosphatase interacting protein 2 (signal transduction) (Bai *et al.*, 2001), Yippee-like protein 5 (unknown) and Kunitz-type protease inhibitor 3 (protease inhibitor unknown) were up-regulated by more than 300% in K+ cells, whereas, Ras-related protein Rab-3B (protein transport) (Burstein *et al.*, 1992), cyclin-dependent kinase-6 inhibitor (cell cycle) (Gesbert *et al.*, 2000), interleukin 1 family member 7 (cytokine), HLA Class II histocompatibility antigen (MHC related) (Curti *et al.*, 2002), inter-alpha-trypsin

inhibitor light chain (glycoprotein), and WAP four-disulfide core domain protein 3 (protease inhibitor unknown) were down-regulated more than three-fold in K+ versus R+ cells.

Interestingly, two proteins of unknown function were isolated during this differential proteomic analysis: Yippee-like protein 5 and M025-like protein. Yippee-like protein 5 was highly up-regulated (about 6 fold) in K+ versus R+, but down-regulated (2.3 fold) in R+ versus R- cells. Thus, it appears that Yippee-like protein 5 is strongly related to

Table 3. Identification of the proteins showing expressional differences in K562 vsrsus K562-R-cells exposed to 1 μM imatinib mesylate

Gel match No.[a]	Protein	Function	Database Accession No[b]	Ratio[c] (K+/R-)	MW	pI	Sequence Coverage (%)
other functions							
63	Yippee-like protein 5	unknown	Q96NS1	**5.677***	133000	8.68	50
4	Kunitz-type protease inhibitor 3 fragment	protease inhibitor unknown	P49223	**3.705***	7407	6.36	32.8
47	Inter-alpha-trypsin inhibitor light chain	glycoprotein	P02760	**-7.084***	15974	4.89	22.4
44	WAP four-disulfide core domain protein 3	protease inhibitor unknown	Q8IUB2	**-3.155***	22079	7.66	34.8
46	Trefoil factor 2	structural component	Q03403	-2.967	11989	5.21	32.1
11	M025-like protein	unknown	Q9H9S4	-2.931	38728	7.68	19.8
53	Metallothionein-IR	metal binding protein/ unkown	Q93083	-2.602	6062.21	8.38	83.6
17	Plasminogen	blood coagulation	P00747	-1.561	41641	7.74	59.5

[a]Gel match numbers were arbitrarily assigned by the Phoretix 2D image analysis program.
[b]Accession numbers are entries in the SWISS-PROT and NCBI databases.
[c]Ratios are presented by fold-changes in 2-DE gels. K+ = K562 cells treated with 1 μM imatinib mesylate for 48 hours. R+ = K562-R cells treated with 1 μM imatinib mesylate for 48 hours. Ratios represent K+ versus R+ for spots in 2-DE gels. + (plus) and − (minus) represent up-regulated and down-regulated fold changes, respectively.
[d]* and bold values represents points at which the mean fold change was 3 or -3.

imatinib mesylate treatment in CML cells. The functions of these two proteins require further investigation, in order to determine whether they are useful biomarker proteins for CML therapy.

Discussion

Based on 2-DE results, this study demonstrates that 27 proteins were down-regulated in K562 cells treated with 1 μM imatinib mesylate (K+ cells) versus K- cells (Table 1). Moreover, 10 of these 27 down-regulated proteins could be linked to signal transduction pathways and cellular regulation (Table 1). The down-regulation of phosphatidylinositol transfer protein and Rho GDP-dissociation inhibitor could be linked to the Ras signal transduction pathway (Huber *et al.*, 1994; Larijani *et al.*, 2003), and serine/threonine protein kinase 13, which is associated with protein phosphorylation, has been tentatively implicated in a candidate mechanism for imatinib mesylate (Heriche and Chambaz, 1998; Lai *et al.*, 2002). All signal transduction proteins were down-regulated in K+ versus K- by proteomic analysis.

Therefore, it seems that the constitutively expressed *Bcr-Abl* PTK, and its downstream signal transduction pathway, are suppressed by imatinib mesylate treatment, via the blockage of *Bcr-Abl* PTK activity (Park *et al.*, in press).

During the K+ *vs.* K- proteomic analysis (Table 1), the expressions of two proteins were found to altered by more than 1000%. These two proteins were ubiquitin-protein ligase E3 Mdm2 (infinite decrease; K+ vs. K-) (Goetz *et al.*, 2001)

and Yippee-like protein 6 (17-fold increase; K+ vs. K-). Mdm2 and Yippee-like protein 6 might be useful as protein biomarkers of imatinib mesylate treatment, and might represent the transformed and untransformed features of K562 cells, respectively. Mdm2 is a key regulator of cell growth and death. Therefore, it plays a pivotal role in the transformation of normal cells to cancerous cells. The Mdm2 gene is amplified in many sarcomas and in other human tumors. High concentrations of Mdm2 reduce p53 (tumor suppressor) activity by catalyzing its ubiquitination, which targets the p53 gene for proteosomal degradation (Goetz *et al.*, 2001). Imatinib mesylate treatment was found to have a tremendous effect on reducing Mdm2 levels. Therefore, treatment may enable p53 activity recovery. On the other hand, Yippee-like protein 6 is a member of the Yippee and Yippee-like proteins, a novel family containing a putative zinc finger-like metal binding domain. The Yippee and Yippee-like proteins are ubiquitously and differentially expressed during different stages of eukaryotic cell development (Roxstrom-Lindquist and Faye, 2001). Moreover, its high degree of sequence conservation among a wide range of eukaryotes suggests that Yippee-like protein 6 plays a role as an important transcription factor for normal cell growth. Therefore, further investigations are needed to determine how the molecular mechanisms responsible for the action of imatinib mesylate are related to the infinitely reduced expression level of ubiquitin-protein ligase E3 Mdm2 and the highly increased expression level of Yippee-like protein 6 in K562 cells.

K562-R cells are resistant to 1 μM imatinib mesylate, and imatinib mesylate treatment has no apparent effect on PTK

activity in these cells. Proteomic analysis of R+ *vs.* R- (Table 2) surprisingly showed that imatinib mesylate caused an extraordinarily change in the expressions of five proteins after imatinib mesylate treatment, namely, mitochondrial import inner membrane translocase subunit (a 22.5 fold increase) (Koehler, 2000), tumor necrosis factor receptor superfamily member 25 (12 fold decrease), complement component C9a (22.9 fold decrease), acidic leucine-rich nuclear phosphoprotein 32 family member B (20.8 fold decrease), and follistatin (14.3 fold decrease). Furthermore, in total 46 proteins were found to have been differentially expressed by this R+ *vs.* R- proteomic analysis (Table 2), which was the highest number observed for the three proteomic analyses, i.e., K+ *vs.* K-, R+ *vs.* R-, and R+ *vs.* K+). The above five proteins, with markedly different expressions, may provide hints concerning the further characterization of the mechanisms underlying the action of imatinib mesylate.

Several proteins of unknown function were identified four proteins in the K+ vs. K- proteomic analysis (Table 1) (Yippee-like protein 6, unknown protein for IMAGE 4908447, acidic leucine-rich nuclear phosphoprotein 32 family member B, and UPF0203 protein 15E1.1), five proteins in the R+ *vs.* R- proteomic analysis (Table 2) (RING finger protein 152, acidic leucine-rich nuclear phosphoprotein 32 family member B, 14-3-3 protein eta, Yippee-like protein 5, and hypothetical protein FLJ31482), and two proteins in the K+ *vs.* R+ proteomic analysis (Table 3) (Yippee-like protein 5 and M025-like protein). Three proteins (macropain subunit delta, Yippee-like protein 5, and acidic leucine-rich nuclear phosphoprotein 32 family member B) were identified twice in three proteomic analyses (K+/K-, K+/R-, and K+/R-) and thus, these three proteins might constitute priority targets for further functional research. The newly discovered nine proteins with unknown function require further investigation with regard to their roles in CML therapy, in order to ascertain whether they constitute useful drug targets or biomarkers.

The proteins identified during the current study represent the first comprehensive documentation of differential protein expression as a result of imatinib mesylate treatment in human CML cells. These proteomic patterns may facilitate in-depth study of the effects of imatinib mesylate, and thus, could be useful for monitoring response to imatinib mesylate therapy. In addition, further analyses of proteins identified with an unknown function should provide new insights into the anti-oncogenic events associated with imatinib mesylate, and also on the status of tyrosine phosphorylation due to *Bcr-Abl* PTK in downstream signal transduction pathways.

Resistance to imatinib mesylate could be represented by a state of tyrosine phosphorylation in the downstream signal transduction pathwqys of Bcr-Abl protein. To identify proteins affected by Bcr-Abl protein, it might be effective to identify differentially tyrosine phosphorylated proteins after imatinib mesylate treatment. Our effects will hopefully lead to a through understanding of resistance to and the mode of action of imatinib mesylate in CML cells.

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