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Proteomic analysis identifies differentially expressed proteins in AML1/ETO acute myeloid leukemia cells treated with DNMT inhibitors azacitidine and decitabine

Francesca Buchi, Elena Spinelli, Erico Masala, Antonella Gozzini, Alessandro Sanna, Alberto Bosi, Germano Ferrari, Valeria Santini*

Functional Unit of Hematology, University of Florence, Florence, Italy

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ABSTRACT

Azacitidine and decitabine are DNA methyltransferase inhibitors used to treat myelodysplastic syndromes and acute myeloid leukemias. To further characterize different mechanisms between these two agents, cellular extracts from leukemic cells untreated or treated with either drug were analyzed using 2D electrophoresis. Numerous differentially expressed proteins were identified with MALDI-TOF/TOF-MS. Cyclophilin A, Catalase, Nucleophosmin and PCNA were decreased exclusively by azacitidine, TCP1 and hnRNP A2/B1 by both drugs; alpha-Enolase and Peroxiredoxin-1 by decitabine. Interestingly, the expression of the proinflammatory protein Cyclophilin A, also suggested as marker of cell necrosis, was stimulated by decitabine. Finally, a comprehensive pathway analysis of data highlighted a relationship between the identified proteins and potential effectors.

1. Introduction

5′-Azacitidine and 5′-aza-deoxycytidine – decitabine are ring analogs of cytosine and deoxy-cytosine, differing from the natural nucleoside because of nitrogen *in lieu* of carbon in position 5 of the pyrimidine. The efficacy of azacitidine and decitabine as antineoplastic agents, widely used for treatment of myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) of the elderly, is attributed to two distinct mechanisms: cytotoxicity and induction of DNA hypomethylation [1,2]. Azacitidine is transformed into the active nucleotide, 5-aza-2′-deoxycytidine-5′-triphosphate, through ATP-dependent phosphorylation by uridine-cytidine kinase and subsequent activity of ribonucleotide reductase. Previous and recent incorporation studies in leukemic cells have shown that the distribution of AZA in nucleic acids is 65:35, RNA:DNA [3,4]. Decitabine, quite differently, incorporates primarily into DNA after deoxycytidine kinase mediated phosphorylation. Regional hypomethylation is thought to be the principal and shared responsible for the clinical efficacy of DNA methyltransferase (DNMT) inhibitors. In fact, once incorporated into DNA, by covalently trapping DNMT, both these drugs deplete the treated cells of functionally active DNMT 1, resulting in reversible but profound hypomethylation and re-expression of silenced oncosuppressor genes.

There are few direct comparisons of the two agents *in vivo* or *in vitro*, but their difference in metabolism and DNA incorporation suggests possible diverse mechanisms of action and biological effects [3,5,6].

We and others have previously demonstrated that AML1/ETO positive leukemic cells are preferentially sensitive to epigenetic drugs [7,8] and specifically to DNMT inhibitors [9–11]. We utilized a two-dimensional electrophoresis system, followed by MALDI-TOF/MS to analyze the regulation of protein expression in AML1/ETO positive cells, to further define the mechanism of action of azacitidine and decitabine, as well as possible differences in their biological activity in this particularly suitable cellular model.

2. Materials and methods

2.1. Cells and culture conditions

Kasumi-1 cells (a human AML1/ETO-positive cell line derived from an acute myeloblastic leukemia) were plated at 3×10^5 cell/ml in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), 4 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells from human bone marrow aspirates were obtained after informed consent (previously approved by the local Ethical Committee), provided by 2 AML patients with *t*(8; 21), undergoing routine diagnostic and investigational procedures. In all the experiments, mononuclear cells isolated by density gradient centrifugation with Lympholyte (CEDERLANE-Canada), and Kasumi-1 cells were incubated in the presence or absence of freshly prepared 5-azacitidine (Sigma–Aldrich) 1.0 μM and decitabine (Sigma–Aldrich) 0.5 μM for 24 h. Equipotent concentrations of drugs were chosen on the basis of their hypomethylating activity as previously published [3,12].

* Corresponding author at: Functional Unit of Hematology, University of Florence, AOU Careggi, Largo Brambilla 3, 50141 Florence, Italy. Tel.: +39 0557947296; fax: +39 0557947343.
E-mail address: santini@unifi.it (V. Santini).

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2.2. Protein extraction and 2D electrophoresis

Cells after 24 h incubation were washed twice in cold PBS plus 100 μM sodium orthovanadate and Protease Inhibitor Cocktail (Sigma). About 20×10^6 cells were lysed, and after ultracentrifugation (100,000 × *g*, 1 h, 4 °C), protein concentration determined by Bradford assay (Roche). Two-dimensional (2D) electrophoresis was performed as previously described [13].

2.3. Image analysis and 2D gel comparison

Two-dimensional gel images were acquired by a modified UMAX Utah.1100 Image Scanner (Amersham Biosciences, Uppsala, Sweden), at 1200 dpi of resolution. Gels were analyzed and compared using the software Image Master Platinum Ed. (Version 5.0, Amersham Biosciences, Uppsala, Sweden). Protein abundance was evaluated by comparing the Spot Staining Volume as calculated by Image Master Platinum ed. Gels were run in duplicate for each sample and scatter plot v/v for the matching spot pools were obtained to verify the degree of reproducibility among two replicates of each sample.

2.4. In gel digestion and mass spectrometry

Spots to be identified were excised from the gels, de-stained and subjected to tryptic in-gel digestion with Sequencing Grade Modified Trypsin (Promega, WI) at 37 °C for 2 h. The resulting peptides were treated by Zip-Tip C_{18} (Millipore), directly eluted by 2 μl of Matrix solution (5 g/l 2,5-dihydroxybenzoic acid in 50% ACN, 0.1%TFA), on 384 position-400 (m ⌀ Anchor Chip Target (Bruker Daltonics, Bremen, Germany). Mass spectra for Peptide Mass Fingerprinting (PMF) were acquired in reflectron positive ion mode on Ultraflex MALDI-TOF/TOF Mass Spectrometer (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 onto positions adjacent to the samples. Experimental Peptide Mass Fingerprinting in the range of 600–3500 Da was compared with NCBI protein database by the software MASCOT (www.matrixscience.org). Confirmatory fragmentation analyses (MS/MS) were performed when needed.

2.5. Western blotting

Kasumi-1 and human primary *t*(8; 21) AML cells were washed in cold PBS plus 100 μM sodium orthovanadate and Protease Inhibitors 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 assay (Pierce), 30 μg aliquot of each sample was separated by SDS-PAGE (9, 12.5 or 15% polyacrylamide gels) and then transferred onto PVDF membranes (Hybond-ECL; Amersham Biosciences) by electro blotting. Membranes were incubated with primary antibodies (anti-calreticulin, anti-alpha-Enolase, anti-hnRNP A2/B1, anti-HSP60, anti-HSP70, anti-HSP90 α/β, anti PRX I, anti TCP1β, by Santa Cruz Biotechnology; anti-cyclophilin A, anti-GAPDH, anti-PCNA, anti-α-tubulin, by Cell Signaling Technology, anti-β-tubulin by Upstate, 1:1000 16–18 h at 4 °C in PBS containing 0.1% Tween 20 and 5% BSA (T-PBS/5% BSA). To verify equal loading of sample per lane, membranes were incubated in stripping buffer (62.5 mM Tris–HCl pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol; 30 min at 50 °C) and extensively washed with T-PBS; then the same membranes were incubated in a 1:1000 dilution of anti-p38 (Cell Signaling Technology) in T-PBS/%5 BSA (16–18 h at 4 °C). Horseradish peroxidase conjugate secondary antibodies anti-rabbit IgG (Sigma) (1:5000 in T-PBS/2% BSA; 45 min at room temperature) were employed. Immunoblot were washed in T-PBS and antibody coated protein bands were visualized by ECL chemiluminescence detection (Amersham Biosciences).

2.6. Data analysis

Scanned images were analyzed by Image Master Platinum Ed. Version 5.0 (Amersham Biosciences). In order to analyze reproducibility, gel similarities or experimental variations, Scatter plots analysis was performed by software Image Master Platinum Ed. which calculates the linear dependence between spot values of one gel in comparison to another gel. For each protein we calculated ratio between spot volume in CTRL gel and spot volume in treated gel. After mass spectrometry analysis and identification by software MASCOT (www.matrixscience.org), proteins were classified by ExPASy Proteomic Server (http://expasy.org/) with UniProtKB database (http://www.uniprot.org). Functional categories were performed using David Bioinformatics Resources 6.7 database (http://david.abcc.ncifcrf.gov/) and cut off criteria used were a p-value of less than 0.001 using Benjamini–Hochberg correction.

Protein network analyses – The proteins identified by MS were included in a pathway analysis using the MetaCore™ network building (tool GeneGo Inc., St. Joseph, MI – http://www.genego.com/training.php). MetaCore™ is formed by an annotated database of protein interactions and metabolic reactions. Gene names of the all identified proteins and the corresponding mean fold-change values of such proteins were imported into MetaCore™ and processed using the network analysis algorithm. Like that, some networks were built. Pathways and direct and indirect associations among proteins were also investigated using String databases (Version 8.3, http://string-db.org/).

3. Results

3.1. Proteomic comparison after treatment with DNMT inhibitors

Protein gels were reproducible, as verified by scatter plot v/v among the replicates of each condition of culture (Fig. S1, a and b – Supplementary data). Changes of protein expression after 24 h azacitidine and decitabine treatment were considered significant when their quantity decreased or increased by at least 1.3-fold.

3.2. Treatment with 5-azacitidine

According to image analysis, 620 spots were constantly detected in control gel and 486 spots in treated cell gel and were quantified, normalized, inter gel matched, yielding 400 pairs of proteins. Sixty-four protein spots had significant volume differences occurring between untreated control and azacitidine treated, and we excised 51 protein spots from control gel and 13 spots from treated-cell gel. Mass spectrometry analysis by MALDI-TOF allowed us to successfully identify 43 protein spots whose levels changed (Fig. 1A, a and b) corresponding to 30 characteristic proteins, classified in functional categories by David Bioinformatics database. Results were reported in Table 1: Spot number obtained from Image Master correlates to corresponding spots in Fig. 2A.

As shown in Fig. 1B, nine proteins (30%) identified by MS included molecules functionally classified as Heat Shock Proteins/Chaperons, nine proteins (30%) were enzymes, most of which metabolic, one protein was involved in stress/redox homeostasis, five (17%) were structural proteins and six proteins (20%) were involved in signal transduction (Table 1 and Supplementary data). The majority of the detected proteins decreased after azacitidine treatment, as showed by the ratio of the volume of spots in treated and untreated cells (Table 1) and, after String analysis, we observed that all proteins were linked by different characteristics, and were involved in several neoplastic diseases (Fig. S2, a – Supplementary data).

Differential expression of PCNA, TCP1, hnRNP A2/B1 and Cyclophilin A was confirmed by western blot (Fig. 1C, a and b). Cyclophilin A, in particular, was identified by more than one spot in 2D maps, because of its different isoforms which could be presents, and its global expression in azacitidine treated cells was lower than that in untreated cells. Regarding biological variation of hnRNP A2/B1, western blot analysis showed an inhibition of expression of ribonucleoprotein B1 and an increase of A2, but global protein expression decreased (ratio of densitometry CTRL/AZA: 1.3, respect to loading control) (Fig. 1C, a and b).

We observed in 2D gels different spots of alpha-Tubulin, beta-Tubulin, alpha-Enolase, ACTB, HSP90, HSP70 and Calreticulin (Table 1 – marked with an asterisk), due to the presence of several isoforms. For this reason, global expression of these proteins was unmodified after azacitidine (Fig. 1C, c). Isoforms of these proteins, well separated by 2D electrophoresis, in 1D western blot clustered in the same band, precluding the validation of difference in expression.

The decrease of Cyclophilin A, PCNA and TCP1 beta was confirmed by western blot with specific antibodies also in primary cultures of 2 AML *t*(8; 21) cases after exposure of leukemic cells to azacitidine (Fig. 1D, a and b).

3.3. Treatment with decitabine

After image analysis, 576 spots were detected in control cells and 596 spots in treated cells; two gels were matched and 416 pairs

Table 1
Differentially expressed proteins identified by mass spectrometry after azacitidine treatment.

Spot CTRL	Spot AZA	Accession number[a]	Heat Shock Proteins/Chaperons	MW[b]	pI[c]	SCORE[d]	Vol. C/Vol. A[e]	Induced or overexpressed by AZA	Inhibited or reduced by AZA
C98	A69	P14625	Endoplasmin*	92,637	4.73	155	1.2		X
C96	A61	P14625	Endoplasmin*	90,351	4.73	32	0.4	X	
C9		P07900	Heat shock protein 90 kDa alpha, class B member 1*	83,638	4.94	75	CTRL		X
C121	A82	P07900	Heat shock protein 90 kDa alpha, class B member 1*	83,638	4.94	173	2.1		X
C139	A94	P07900	Heat shock protein 90 kDa alpha, class B member 1*	83,638	4.94	69	0.5	X	
C113	A83	P07900	Heat shock protein 90 kDa alpha (cytosolic), class B member 1*	83,638	4.94	46	0.5	X	
C181	A125	P11142	Heat shock 70 kDa protein 8*	71,138	5.37	186	2.5		X
C178	A123	P11142	Heat shock 70 kDa protein 8*	71,138	5.37	49	0.8	X	
C216	A151	P10809	Heat shock protein 60	61,388	5.24	219	1.0		
C249	A184	P78371	Chaperonin containing TCP1, subunit 2 (beta)	57,878	6.02	153	1.8		X
C173	A119	P38646	Heat shock 70 kDa protein 9	74,089	5.97	117	1.2		X
C862	A528	P62937	Cypa (Cyclophilin A)*	18,154	7.82	146	0.8	X	
C710	A524	P62937	Cypa (Cyclophilin A)*	18,154	7.82	98	1.4		X
C705	A523	P62937	Cypa (Cyclophilin A)*	18,154	7.82	103	1.2		X
C263	A173	P27797	Calreticulin*	48,325	4.28	75	2.2		X
	A306	P27797	Calreticulin*	48,325	4.28	46	AZA	X	
C235		P30101	Protien-disulfide isomerase A3	56,761	5.61	83	CTRL		X
Enzymes									
C295	A208	P06733	Alpha-Enolase*	47,538	6.99	61	0.4	X	
C292	A213	P06733	Alpha-Enolase*	47,538	6.99	94	1.5		X
C268	A199	P07237	Prolyl 4-hydroxylase beta-subunit	57,578	4.69	211	1.4		X
C236	A163	P12268	Inosine monophosphate dehydrogenase 2	56,338	6.44	115	1.5		X
C470	A355	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36,244	8.58	91	1.5		X
C546	A415	P60174	Chain B, Triosephosphate Isomerase (Tim)	26,877	6.51	191	1.2		X
C538	A406	P28066	Proteasome subunit, alpha type, 5	26,621	4.74	46	1.4		X
C815		P55072	Valosin-containing protein	71,660	5.14	49	CTRL		X
C665	A487	P15531	Non-metastatic cells 1, protein (NM23A) isoform a	19,925	5.84	64	2.7		X
C226	A157	P04040	Catalase	59,947	6.95	93	1.8		X
Cell redox homeostasis									
C596	A446	P09211	Glutathione S-transferase P	23,651	5.44	86	1.4		X
Structural proteins									
C253	A191	P68363	Alpha tubulin*	50,972	4.94	149	1.9		X
C260	A194	P68363	Alpha tubulin*	50,972	4.94	106	0.5	X	
C280	A204	P68363	Alpha tubulin*	50,972	4.94	60	0.9		
C445	A334	P07437	Beta tubulin*	48,233	4.78	182	1.6		X
C421		P07437	Beta tubulin*	48,233	4.78	127	CTRL		X
C284		P07437	Beta tubulin*	48,233	4.78	70	CTRL		X
C365	A274	P60709	ACTB protein (Beta-actin)*	40,620	5.29	142	1.8		X
C330	A251	P60709	ACTB protein (Beta-actin)*	40,620	5.29	48	0.8	X	
C413	A314	P52907	F-actin capping protein alpha 1	33,115	5.45	118	2.7		X
C475	A362	Q8TCG3	Tropomysin, TPMsk3	28,934	4.72	74	0.9		
DNA binding/signal transduction									
C278	A201	Q09028	Retinoblastoma binding protein 4 variant	47,910	4.74	67	1.1		
C400	A299	P06748	Nucleophosmin	32,653	4.64	76	1.3		X
C463	A350	P12004	Pcna	29,156	4.57	154	2.5		X
C424	A321	P22626	Heterogeneous nuclear ribonucleoproteins (hnRNP) A2/B1	37,464	8.97	95	2.2		X
	A541	P62158	Calmodulin	17,152	4.09	48	AZA	X	

[a] Accession number by UniProtKB.
[b] MW – theoretical molecular weigh obtained by ExPASy Proteomic Server.
[c] pI – theoretical isoelectric point obtained by ExPASy Proteomic Server.
[d] Score – index calculated by MASCOT-significant when greater than 56 ($p < 0.05$).
[e] Ratio between spot volume in CTRL and spot volume in treated gels.

Table 2
Differentially expressed proteins identified by mass spectrometry after decitabine treatment.

Spot CTRL	Spot DAC	Accession number[a]	Heat Shock Proteins/Chaperons	MW[b]	pI[c]	Score[d]	Vol. C/Vol. D[e]	Induced or overexpressed by DAC	Inhibited or reduced by DAC
C1591	D43	P07900	Heat shock protein 90 kDa alpha (cytosolic), class B member 1*	83,638	4.94	89	1.4		X
C1609	D66	P07900	Heat shock protein 90 kDa alpha (cytosolic), class B member 1*	83,638	4.94	85	2.3		X
C1614	D73	P07900	Heat shock protein 90 kDa alpha (cytosolic), class B member 1*	83,638	4.94	93	0.2	X	
C1689	D157	P10809	Heat shock protein 60	61,388	5.24	147	1.6		X
C1651	D119	P11142	Heat shock 70 kDa protein 8*	71,138	5.37	161	1.3		X
C2315	D71	P11142	Heat shock 70 kDa protein 8*	71,138	5.37	101	0.5	X	
C1674	D155	P27797	Calreticulin	48,283	4.28	90	4.6		X
C1713	D184	P30101	Protein-disulfide isomerase A3*	56,747	5.61	82	1.5		X
C1768	D251	P30101	Protein-disulfide isomerase A3*	57,146	5.61	72	2.2		X
C1752	D225	P30101	Protein-disulfide isomerase A3*	57,146	5.61	88	1.2		X
C2007	D660	P30040	Endoplasmic reticulum protein ERp29	29,032	6.08	70	1.8		X
C2119	D565	P23284	Cyclophilin B	22,785	9.25	122	1.3		X
C2178		P62937	Cyclophilin A*	18,229	7.82	61	CTRL		X
C2183	D606	P62937	Cyclophilin A*	18,229	7.82	75	0.4	X	
C2184	D612	P62937	Cyclophilin A*	18,229	7.82	91	1.4		X
C1732	D204	P78371	Chaperonin containing TCP1, subunit 2 (beta)	57,878	6.02	125	1.3		X
Enzymes									
C1824	D283	P00558	Phosphoglycerate kinase 1	44,985	8.30	100	3.9		X
C1875	D347	P06733	Alpha-Enolase*	47,481	6.99	121	2.4		X
C1778	D237	P06733	Alpha-Enolase*	47,481	6.99	195	2.1		X
	D256	P07954	Fumarate hydratase, mitochondrial	54,773	6.99	55	DAC	X	
C1707	D160	P04040	Catalase	59,947	6.95	62	0.8	X	
C1925	D403	Q15181	Inorganic pyrophosphatase	33,095	5.54	120	1.6		X
C1927	D402	Q15181	Inorganic pyrophosphatase	33,095	5.54	108	1.4		X
C1970	D424	P14207	Folate receptor beta	30,286	7.47	32	0.6	X	
C2142	D593	Q8TAE6	Protein phosphatase 1 regulatory subunit 14 C	17,946	5.09	31	0.8	X	
C1900	D363	P04406	Glyceraldehyde-3-phosphate dehydrogenase	36,201	8.58	59	1.5		X
C2037	D489	P60174	Chain B, Triosephosphate Isomerase (Tim)	26,877	6.51	155	1.2		
C1693	D137	Q02318	Cytocrome P450 27, mitochondrial	60,595	8.43	27	0.8	X	
Cell redox homeostasis									
C2060	D522	P09211	Glutathione S-transferase P	23,569	5.44	58	1.4		X
C2072	D531	Q06830	Peroxiredoxin-1	22,324	8.27	81	2.3		X
C1848	D331	O76003	Glutaredoxin-3	37,693	5.31	83	2.0		X
Structural proteins									
C2323	D304	P60709	ACTB protein (Beta-actin)	40,620	5.29	163	1.9		X
C1906	D375	P07437	Beta tubulin*	48,233	4.78	154	1.7		X
C1915	D391	P07437	Beta tubulin*	48,233	4.78	58	1.1		X
C2269	D205	P68363	Alpha tubulin	50,972	4.94	89	0.3	X	
DNA binding/signal trasduction									
C1995	D459	P35232	Prohibitin	29,843	5.57	142	1.6		X
C1933	D412	P12004	Pcna	29,156	4.57	127	0.8	X	
C1888	D345	Q13347	Eukaryotic translation initiation factor 3 subunit I	36,878	5.38	92	1.8		X
C1926	D394	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	37,464	8.97	112	1.4		X
C1940	D388	P09651	Heterogeneous nuclear ribonucleoprotein A1	38,936	9.17	132	1.4		X
C1834	D307	P29992	Guanine nucleotide-binding protein subunit alpha-11	42,382	5.51	54	2.0		X
C2035	D491	P52565	Rho GDP-dissociation inhibitor 1	23,250	5.03	80	1.4		X
C1862	D336	P06748	Nucleophosmin	32,653	4.64	64	0.9	X	
C1841	D320	Q9Y3F4	Serine-threonine kinase receptor-associated protein	38,756	4.98	51	0.6	X	
C2160		P62158	Calmodulin	17,152	4.09	49	CTRL		X

[a] Accession number by UniProtKB.
[b] MW – theoretical molecular weigh obtained by ExPASy Proteomic Server.
[c] pI – theoretical isoelectric point obtained by ExPASy Proteomic Server.
[d] Score – index calculated by MASCOT-significant when greater than 56 ($p < 0.05$).
[e] Ratio between spot volume in CTRL and spot volume in treated gels.

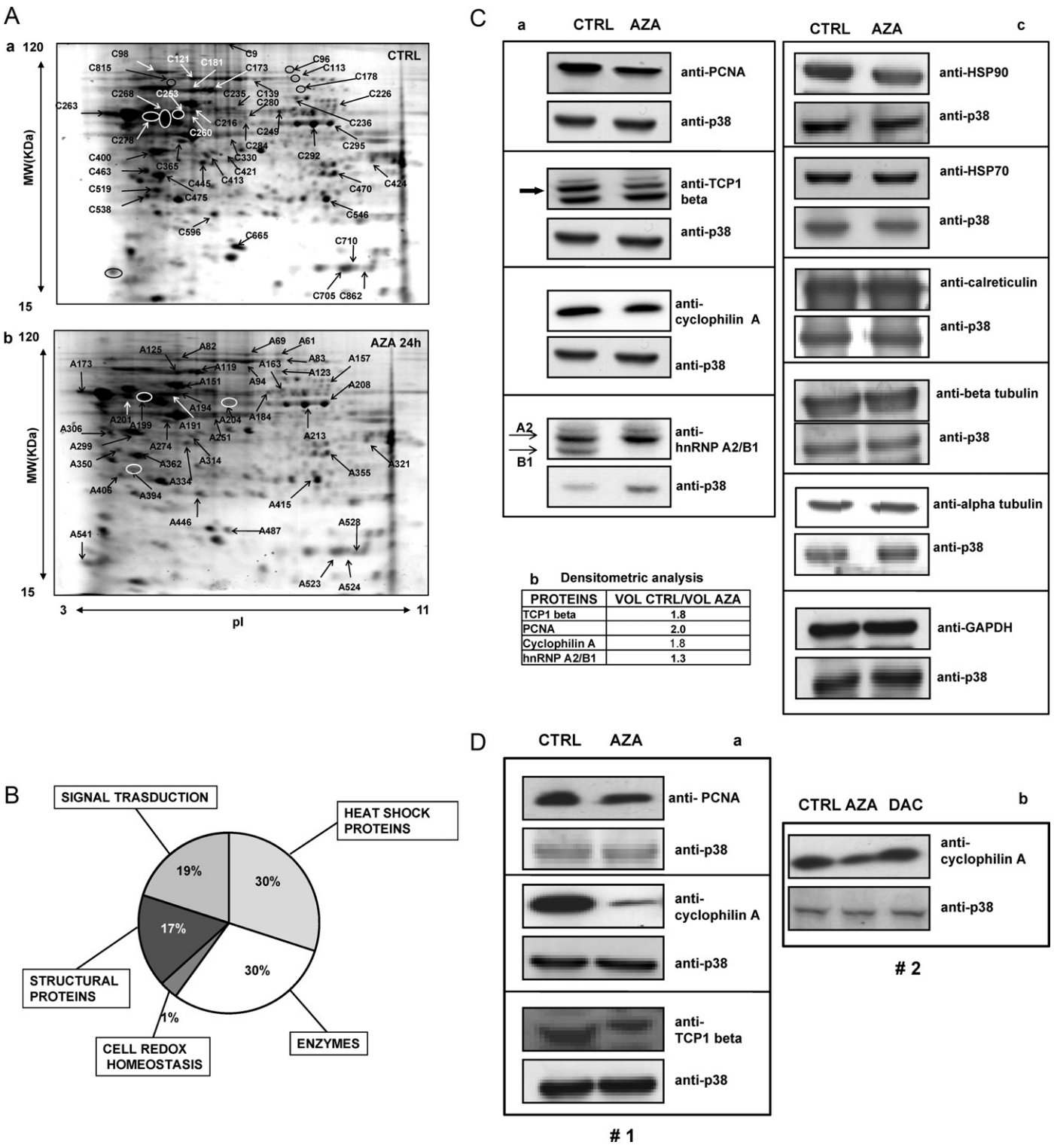
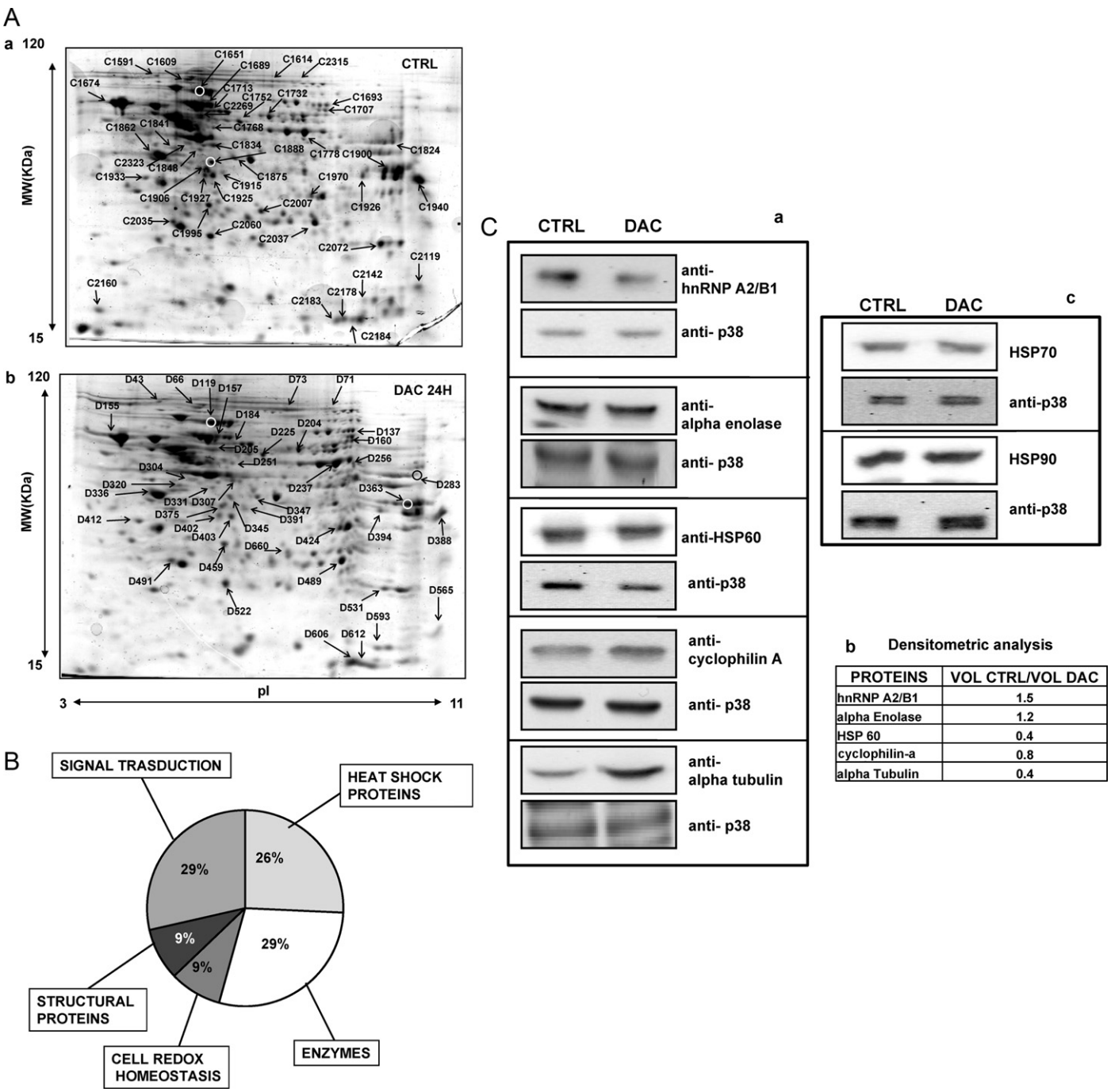


Fig. 1. Effect of azacitidine 24 h on proteome of Kasumi-1 cells. (A) Two-dimensional gels before (a) and after (b) treatment were analyzed. Spots were then picked and identified by MS analysis. (B) Functional distribution of identified proteins. (C and D) Confirmatory Western blot; anti-p38 is protein loading control. CTRL, control untreated cells; AZA, azacitidine; DAC, decitabine.

were found; 50 protein spots had significant differences between untreated control and decitabine treated cells. We excised 36 protein spots from control gel and 14 spots from treated gel; mass spectrometry analysis MALDI-TOF allowed us to identify 45 spots corresponding to 35 characteristic proteins (Fig. 2A, a and b), classified in functional categories by David Bioinformatics database. Results were reported in Table 2 (spot number obtained from Image Master, correlate to corresponding spots in Fig. 2A).

As shown in Fig. 2B, nine proteins (26%) identified by MS included molecules functionally classified as Heat Shock Proteins/Chaperons, ten proteins (29%) were enzymes, most of which metabolic, three proteins (9%) were involved in stress/redox



PROTEINS	VOL CTRL/VOL DAC
hnRNP A2/B1	1.5
alpha Enolase	1.2
HSP 60	0.4
cyclophilin-a	0.8
alpha Tubulin	0.4

Fig. 2. Effect of decitabine 24 h on proteome of Kasumi-1 cells. (A) Two-dimensional gels before (a) and after (b) treatment were analyzed. Spots were picked and identified by MS analysis. (B) Functional distribution of identified proteins. (C) Confirmatory Western blot; anti-p38 is shown as protein loading control. CTRL, control untreated cells; DAC, decitabine.

homeostasis, three (9%) were structural proteins and ten proteins (29%) were involved in signal transduction (Table 1 and Supplementary data). After decitabine treatment, the majority of identified proteins (those selected on the basis of a significant modulation in expression) were down-regulated, although protein spot number did not decrease (Table 2).

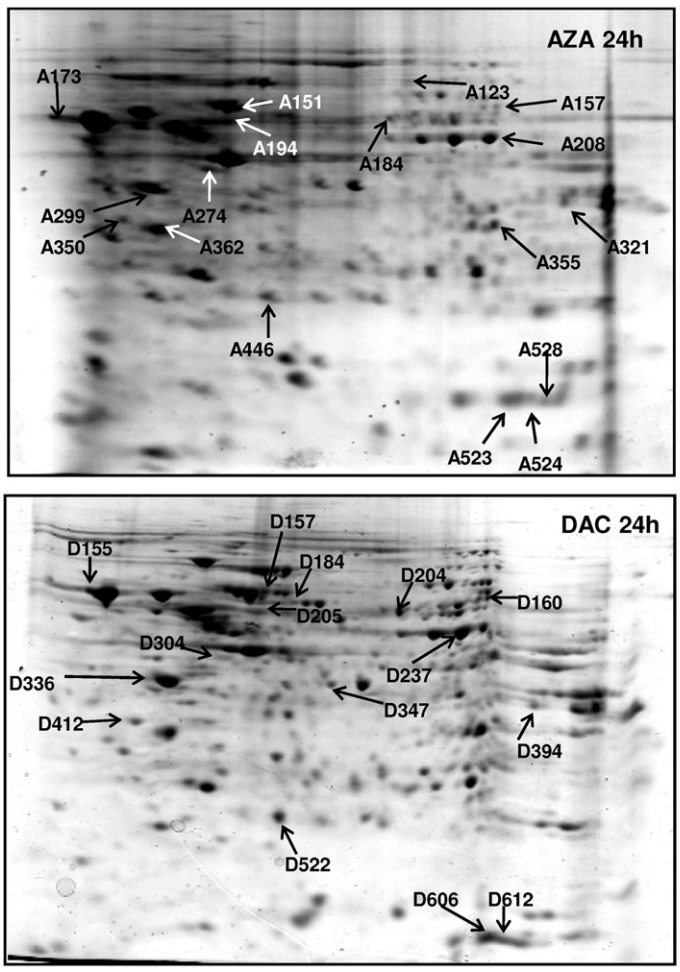
String analysis showed that the majority of modulated proteins were correlated (Fig S2, b – Supplementary data). Decrease of hnRNP A2/B1, alpha-Enolase and the increase of Cyclophilin A, HSP60 (ratio of densitometry CTRL/AZA: 0.4, respect to control loading p38) and alpha-Tubulin were confirmed (Fig. 2C, a and b). Several proteins (HSP90, HSP70, PDIA3, Cyclophilin A, beta-Tubulin, alpha-Enolase) had different migration sites into both 2D gels (Table 2 – marked with an asterisk), because of their isoforms (see above), so some of these did not change the global expression (Fig. 2C, c).

Cyclophilin A expression was increased in primary *t*(8; 21) AML cells after 24 h exposure to decitabine *in vitro* (Fig. 1D, b).

3.4. Comparison between AZA and DAC treatments

We matched gels after treatment with azacitidine and decitabine, in order to identify commonly modulated proteins and to evaluate possible different effects of the two agents on

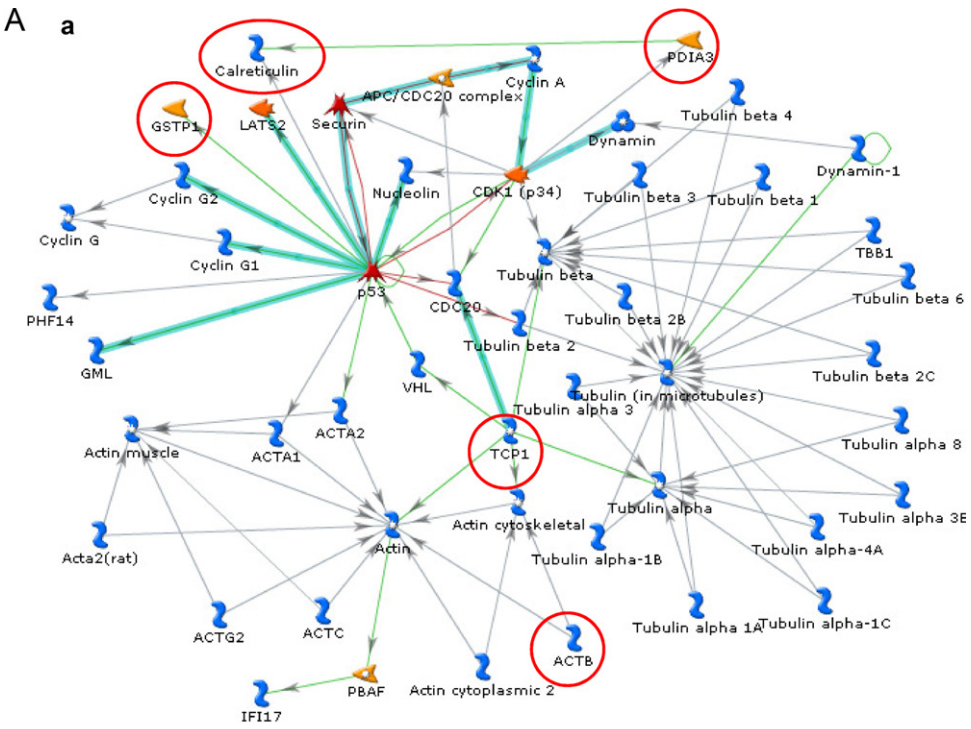
A



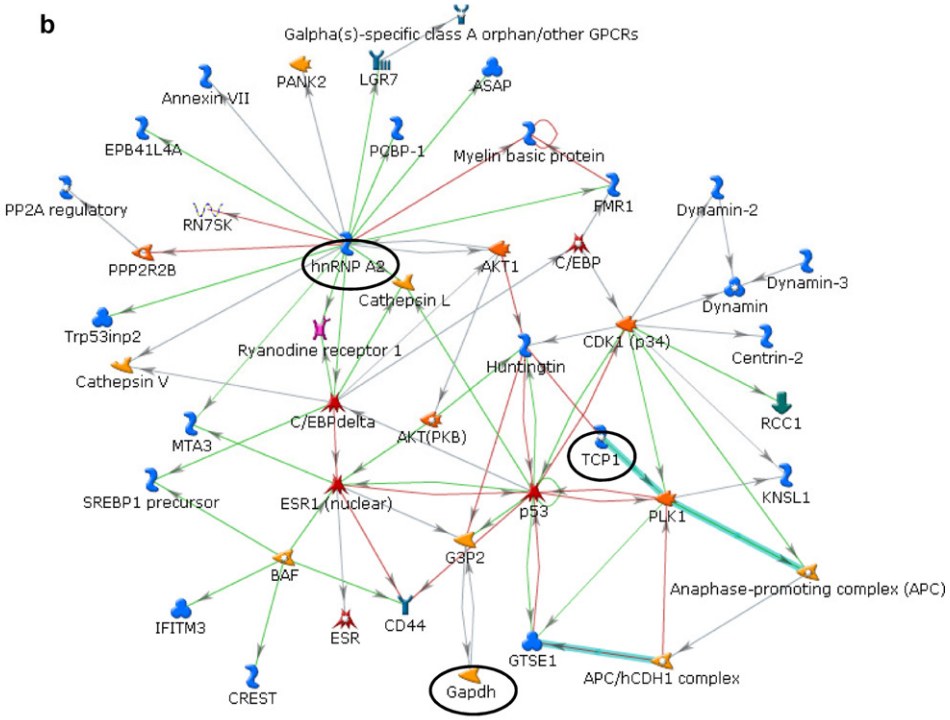
B

SPOT AZA	SPOT DAC	PROTEIN NAME	AFTER AZA	AFTER DAC
		HEAT SHOCK PROTEINS/CHAPERONS		
A151	D157	Heat shock protein 60	NO CHANGE	DECREASE
A184	D204	Chaperonin containing TCP1, subunit 2 (beta)	DECREASE	DECREASE
A528				
A524	D612	Cypa (Cyclophilin A)	DECREASE	INCREASE
A523	D606			
	D184	PDIA3	DECREASE	DECREASE
A173	D155	Calreticulin	DECREASE	DECREASE
		ENZYMES		
A208	D237	Alpha Enolase	NO CHANGE	DECREASE
	D347			
A355	D363	Glyceraldehyde-3-phosphate dehydrogenase	DECREASE	DECREASE
A157	D160	Catalase	DECREASE	NO CHANGE
		CELL REDOX HOMEOSTASIS		
A446	D522	Glutathione S-transferase P	DECREASE	DECREASE
		STRUCTURAL PROTEINS		
A194	D205	Alpha tubulin	NO CHANGE	INCREASE
A274	D304	ACTB protein (Beta-actin)	DECREASE	DECREASE
		DNA BINDING /SIGNAL TRANSDUCTION		
A299	A336	Nucleophosmin	DECREASE	NO CHANGE
A350	D412	Pcna	DECREASE	NO CHANGE
A321	D394	Heterogeneous nuclear ribonucleoproteins A2/B1	DECREASE	DECREASE

Fig. 3. Comparison between proteome of Kasumi-1 after azacitidine and decitabine treatments. (A) Two-dimensional Comassie stained gels after azacitidine (a) and decitabine (b) were compared and common proteins identified. (B) Table of common proteins. AZA, azacitidine; DAC, decitabine.



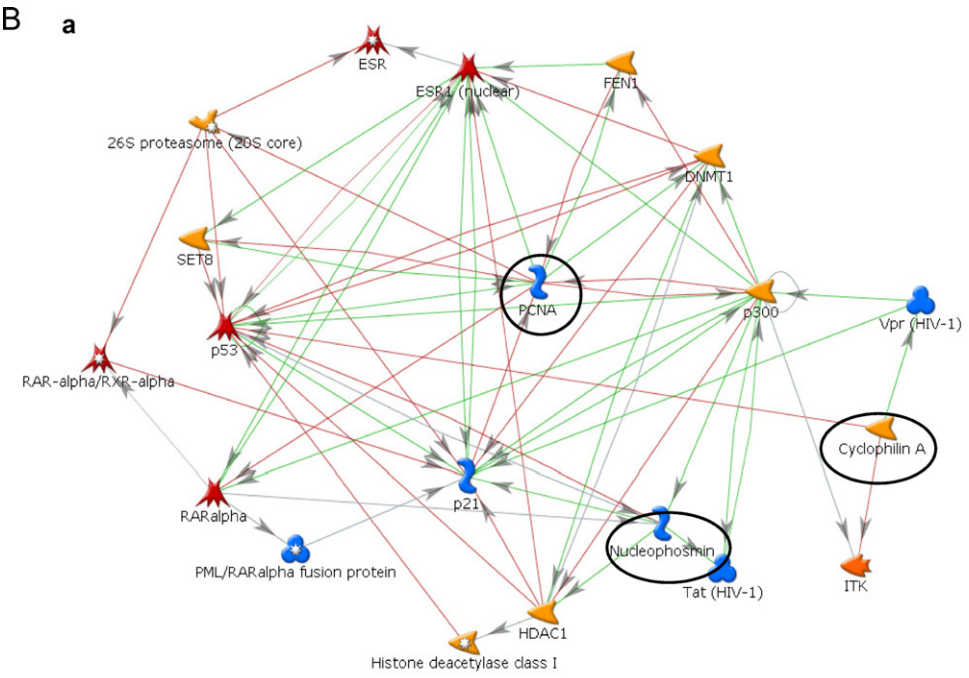
Key network objects	GO Processes	Total nodes	Seed nodes	Path ways	p-Value
TCP1, PBAF, Calreticulin, PDIA3, GSTP1	'de novo' posttranslational protein folding (40.0%), 'de novo' protein folding (40.0%), protein polymerization (36.0%), protein folding (46.0%), microtubule-based movement (36.0%)	50	6	6	1.38e-16



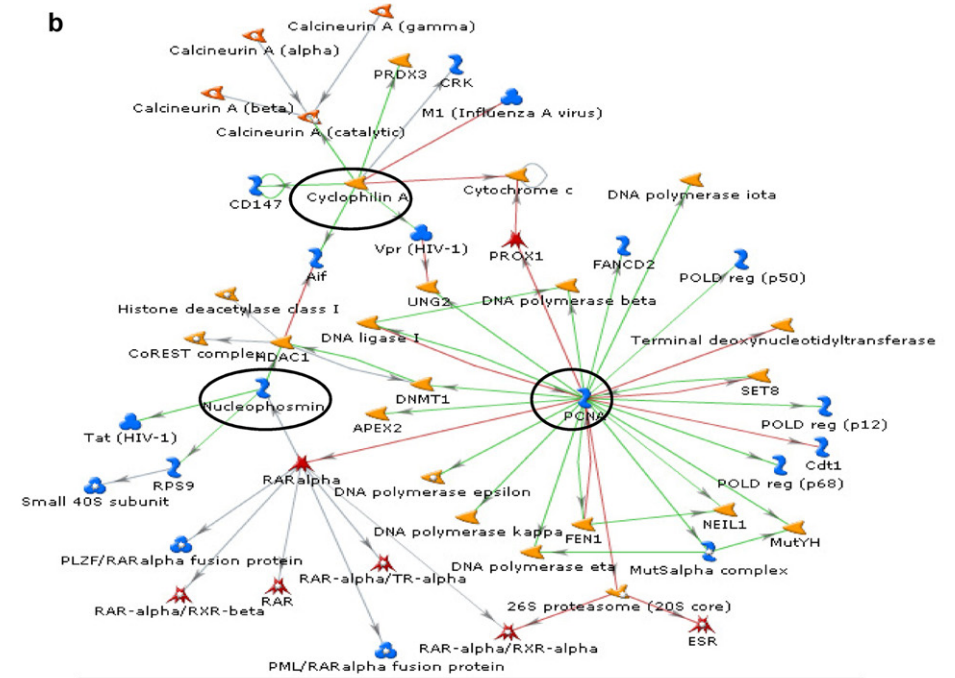
Key network objects	GO Processes	Total nodes	Seed nodes	Path ways	p-Value
hnRNP A2, BAF, G3P2, TCP1, p53	regulation of cell cycle process (26.7%), cell cycle process (35.6%), regulation of mitotic cell cycle (24.4%), mitotic cell cycle (31.1%), cell cycle (37.8%)	45	4	1	1.04e-10

Fig. 4. Proteome networks by Metacore analysis. Metacore analysis was performed to compare common proteins identified after azacitidine and decitabine treatments. (A) Proteins equally modulated by either drug: (a) protein folding network, (b) cell cycle network. (B) Proteins differently modulated by two drugs: (a) gene-specific transcription network, (b) response to stress-network. (C) Cyclophilin A pathway network.

B



Key network objects	GO Processes	Total nodes	Seed nodes	Path ways	p-Value
PCNA, Nucleophosmin, Cyclophilin A, p300, p53	regulation of gene-specific transcription from RNA polymerase II promoter (55.6%), regulation of cell cycle (61.1%), regulation of gene-specific transcription (55.6%), cellular response to estrogen stimulus (27.8%), regulation of transcription from RNA polymerase II promoter (66.7%)	20	3	0	1.40e-10



Key network objects	GO Processes	Total nodes	Seed nodes	Path ways	p-Value
PCNA, Cyclophilin A, Nucleophosmin, HDAC1, Cytochrome c	DNA metabolic process (55.3%), response to stress (83.0%), nucleic acid metabolic process (85.1%), cellular response to stress (59.6%), response to DNA damage stimulus (46.8%)	50	3	0	2.41e-09

Fig. 4. (*continued*)

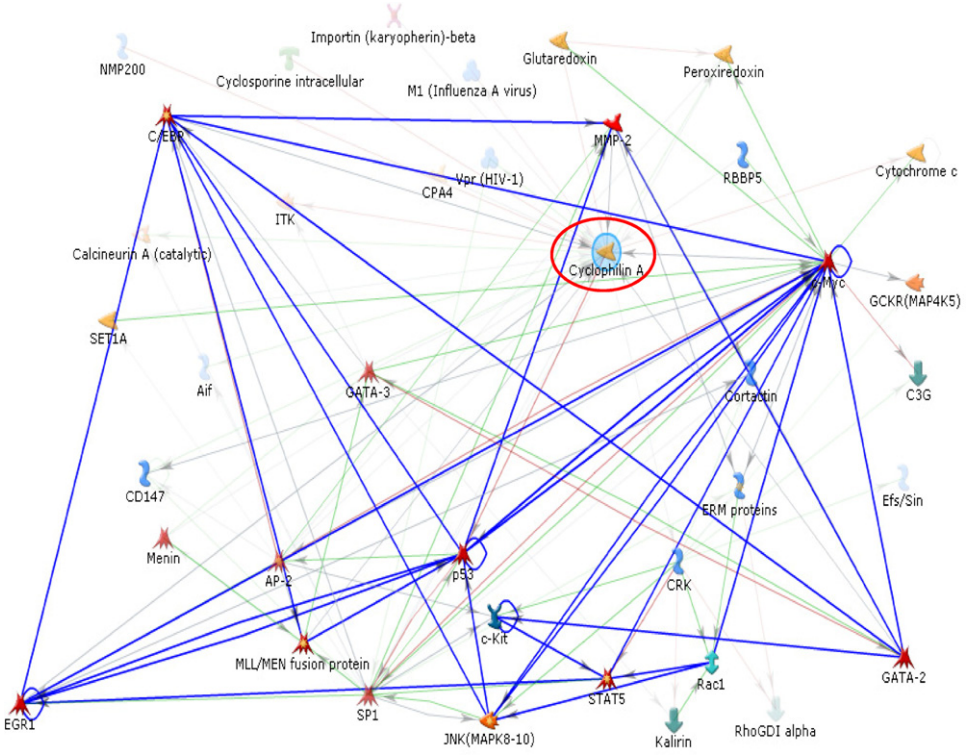


Fig. 4. (*continued*)

protein expression (Fig. 3A, a and b). Considering only proteins with a significant MASCOT score (Tables 1 and 2; score >56), and those proteins whose global expression (summing isoforms) was modulated, we found 14 proteins identified both in azacitidine and decitabine treated gels (Fig. 3B).

3.5. Protein network analysis

A pattern analysis was performed using the software MetaCore™ in order to evaluate the possible correlation between the 2D-electrophoresis/MS identified modulated proteins and possible cellular pathways affected by azacitidine and decitabine (Table 3). This analysis allowed also to relate the function of single proteins to the cellular context. All proteins analyzed were processed by the "network analysis" algorithm with fifty nodes, which generates sub-networks ranked by *p*-values.

First of all, we investigated the correlation between proteins that were equally modulated by either drug, particularly TCP1 beta, PDIA3, Calreticulin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Glutathione S-transferase P, ACTB and hnRNPA2/B1 (Fig. 3B). The analysis clustered proteins in two networks: the first, which include TCP1, Calreticulin, ACTB, PDIA3 and GSTP1 regulates protein folding (Fig. 4A, a) and the second, which includes GAPDH, Tcp1 and hnRNP A2/B1, modulates cell cycle (Fig. 4A, b).

Among proteins that had different modulation after two drugs treatments, we focused our analysis on proteins decreased only by azacitidine (Fig. 3B), particularly Cyclophilin A, Catalase, Nucleophosmin and PCNA. This analysis lead to cluster proteins in three network: two, including PCNA, Nucleophosmin and Cyclophilin A, are involved in gene-specific transcription and in response to stress (Fig. 4B, a and b), the third one is identified via Catalase presence, involved in triglyceride metabolism.

Since Cyclophilin A showed a decrease of expression after azacitidine and an increase after decitabine, we analyzed its pathway network as an interesting difference between the two agents. It seems particularly relevant that Cyclophilin A is involved in AML proliferation and maintenance (Fig. 4C – links marked in blue).

4. Discussion

Azacitidine and decitabine are used for treatment of MDS and AML. Although these two agents share a similar chemical structure and are often considered equivalent, they exert divergent biological effects and have some different clinical activity, as only azacitidine has been shown to significantly improve survival in MDS [14] while decitabine seems to have preferential activity on CMML [15] and AML [11]. Part of the differences may be due to prevalent incorporation into RNA of azacitidine and into DNA of decitabine, but it appears that other effects, beside hypomethylation, may explain their clinical activity [16]. Characterization of azacitidine and decitabine effects on protein expression, protein networks and pathways may help in understanding their molecular

Table 3
Summary of proteome modifications after azacitidine and decitabine treatment of AML1/ETO positive leukemic cells.

Drug	Number of protein spots	Proteins modulated			
AZA	Decrease	↓ 5 involved in protein folding ↓ 3 involved in cell cycle	↓ 3 involved in gene-specific transcription ↓ 3 involved in response to stress ↓ Catalase		
DAC	No change	↓ 5 involved in protein folding ↓3 involved in cell cycle		↑Cyclophilin A ↑ Catalase	↓ alpha-Enolase

mechanisms, whose discrepancies have started to be highlighted in scientific literature [3,5,6]. AML1/ETO positive leukemias have a particular good prognosis and are sensitive to chemotherapy [7–10]. Most interestingly, the AML1/ETO complex binding DNMTs and HDAC forms a putative ideal target for epigenetic drugs [7–10]. We employed a sensitive, 2D-electrophoresis approach followed by MALDI-TOF/MS to investigate proteins modifications after short term in vitro treatment with azacitidine and decitabine. This proteomic approach is increasingly used to implement studies of mechanisms of action of drugs [17,18].

We could demonstrate distinct expression pattern between protein extracts from untreated leukemic cells and extracts from azacitidine and decitabine treated cells. Specific proteins were significantly down-regulated, although not completely, by both drugs. We focused our attention both on the commonly repressed proteins, and on those decreased exclusively by one agent, to verify whether this difference could justify the diverse clinical effects of the two DNMT inhibitors. Quite differently from azacitidine, decitabine did not decrease the global number of protein spots, respect to untreated samples. This is consistent with the lack of inhibition of protein synthesis by decitabine, observed by other authors with different analytical methods [3].

The two published reports on gene expression modification by DNMT inhibitors [5,6] do not identify the same set of genes, and there is no significant overlap with our protein findings either.

Although the two agents are supposed to act and be efficient in clinics via hypomethylation of CpG islands of gene promoters, the majority of modulated proteins by in vitro DNMT inhibitors treatment were repressed, similarly to what observed in gene expression studies [6]. Moreover, we could not detect significant increase of tumor suppressor genes, indicating, again similarly to what already observed by other authors, that although the genes of these proteins may be up regulated via hypomethylation to levels detectable by PCR-based methods, they are not part of the general dominant expression pattern induced by these drugs, and proteomic methods are not sensitive enough to detect them [5].

In order to better understand the relationships among the proteins identified, we used String database, as well as MetaCore^{TM}. Most of the proteins whose expression was modified by DNMT inhibitors were inter-related in the pathway map, confirming the biological reliability of our analysis. In addition, the majority of modulated proteins were known to be involved in neoplastic progression, so that their decreased expression by DNMT inhibitors may help to understand the mechanisms by which these agents exert their anticancer activity.

In the MetaCore^{TM} produced network, the identified proteins clustered in two networks, one modulating protein folding and polymerization (TCP1, PBAF, Calreticulin, PDIA3, GSTP1) and the second involved in regulation of cell cycle and mitosis and RNA transport (hnRNPA2, BAF, G3P2, TCP1).

The Chaperonin Containing TCP1 in the eukaryotic cytosol ensures the correct folding and assembly of a variety of proteins. Neoplastic cells show an overexpression of TCP1 beta [19]. In our model, azacitidine and decitabine provoked decrease in TCP1 expression, and also a decrease in its best-characterized substrate beta-actin. We could indeed confirm this finding also in primary AML cells. Calreticulin is increased in several neoplastic diseases [20], and its expression decreased after both DNMTi treatments. The same inhibition is observed for Glutathione S-transferases (GSTs), which are a family of enzymes that catalyze the conjugation of glutathione (GSH) to various xenobiotics and participate in cellular protection against oxidative stress or resistance to drugs. These proteins exhibit genetic polymorphisms in their population distribution, and these variants are frequently expressed in AML patient and may influence their prognosis [21,22].

Heterogeneous ribonucleoproteins (hnRNPs) are complexes of RNA and protein present in the cell nucleus during gene transcription and subsequent post-transcriptional modification of the newly synthesized RNA (pre-mRNA). They are involved in splicing of pre-mRNA and in its transport out of the nucleus. Overexpression of hnRNP A2/B1 is correlated to cancer progression [23], particularly hnRNP B1 is over-expressed in MDS and AML-MDS [24] while hnRNP A1 is over expressed generally in solid [25] and hematological malignances [26]. From our analysis, it emerged that both agents, in our experimental conditions, have the same inhibiting activity on cell cycle/RNA metabolism and protein folding. Effects on gene expression and miRNA expression have been demonstrated to be time dependent, but we privileged the analysis of early modulation by these two drugs, to possibly examine non cytotoxic effects [5].

Regarding proteins selectively modulated by azacitidine, it looks quite important that one cluster of decreased proteins (PCNA, Cyclophilin A, NPM-1) is involved in response to stress, suggesting a role of azacitidine in impairing reaction of leukemic cells to DNA damage. The same proteins are regulating gene-specific transcription, and azacitidine could influence it, but not necessarily by CpG island hypomethylation. Besides, only azacitidine inhibits Catalase, which not only protects cells from the toxic effects of hydrogen peroxide, but also promotes growth of myeloid leukemia cells, thanks to ERK-1 activation [27]. In conclusions, azacitidine, but not decitabine, seemingly exerts an important down regulation of anti-stress proteins.

Cyclophilin A (CypA) modulation is quite peculiar, because its expression is reduced by azacitidine, but increased by decitabine, and these results were confirmed also in primary human *t*(8; 21) AML cells. Recent studies indicated different possible roles of CypA in tumor development, given that its expression resulted increased and induced by c-Myc and it has inhibitory activity on p53, as also shown by our analysis with MetaCore. This discrepancy after drug treatment could be consistent with a reported early and transient induction of pro-carcinogenic genes by decitabine [5]. Although a specific role in normal and malignant hemopoiesis has not been demonstrated for Cyclophilin A, it is possible that its effects on inflammation, neutrophil chemo-attraction and induction of reactive oxygen species may be critical for leukemic cells [28,29].Another interesting feature of our analysis is that decitabine is selectively able to down regulate alpha-Enolase. It could be that this occurs because of decitabine site-specific DNA incorporation. Certainly, by decreasing alpha-Enolase activity, decitabine could act reducing leukemic proliferation. Moreover, only treatment with decitabine down-regulates HSP60, which indeed plays a pivotal role in leukemogenesis [30].

Differences between the mechanisms of azacitidine and decitabine were observed in their activities on cell viability, protein synthesis, cell cycle, miRNA and gene expression [3,5,6]. The hypothesis that AZA and DAC have finely diverse biomolecular mechanisms is supported by the observation that these two agents have a specific and selective modulation of the target proteins which we identified. Their effects could be caused not exclusively by DNA hypomethylation. Our observations further indicate that azacitidine and decitabine are not interchangeable and that their alternate or combined use in clinics may be conceivable.

Conflict of interest

All authors have no conflict of interest to declare.

Acknowledgments

Contributions. V.S. designed the study and wrote the manuscript; F.B. performed experiments and wrote the results section; G.F.

performed mass spectrometry analysis; E.S. and E.M. performed Western blot experiments and A.S., A.G. and A.B. took part in designing the study and revised the manuscript. *Fundings*. Regione Toscana, Bando Salute 2009; Ente Cassa di Risparmio di Firenze (ECR); and Ministero per l'Istruzione, l'Università e la Ricerca (MIUR).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2011.11.024.

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