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Vincristine regulates the phosphorylation of the antiapoptotic protein HSP27 in breast cancer cells

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Abstract

Vincristine is an antitumor drug that inhibits microtubule polymerization, causes G2/M arrest and induces apoptosis. 2D-PAGE and MALDI-TOF-MS analysis of vincristine effects on MCF7 cells, revealed a vincristine upregulated form and a vincristine downregulated form of the antiapoptotic protein HSP27. These findings linked to the lack of vincristine effect over HSP27 mRNA, suggest a protein post-translational modification. Further assays indicated the presence of a phosphorylated peptide, containing serine 82, only in the vincristine upregulated form. Serine 82 phosphorylation was confirmed using specific antibodies. Thus, phosphorylation of HSP27 may play a role in the cellular response to vincristine.
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Keywords: 2D-PAGE; Breast cancer; HSP27; MALDI-TOF-MS; Phosphorylation; Vincristine

1. Introduction

Microtubule dynamics and stability have a relevant role in many cellular events, particularly in cell division where vivid changes in microtubule architecture occur. This cell division regulation makes microtubules an important target for cancer chemotherapy [1].

Vinca alkaloids are a group of antitumor drugs that includes natural compounds such as vincristine or vinblastine [2] and semisynthetic compounds such as vinorelvine [3] and vindesine [4]. This microtubule-interfering agents introduce a wedge at the interface of two tubulin molecules that prevents the addition of heterodimers onto growing microtubules [5]. As a consequence, the mitotic spindle of dividing cells cannot be formed, leading to c-Jun NH2-terminal Kinase (JNK) activation [6], Bcl-2 phosphorylation [7], G2/M arrest and apoptosis [6].

Resistance development to chemotherapy agents means a major clinical problem. Cancer cells turn resistant to vinca alkaloids by mutations in tubulin [8], expression of alternative tubulin classes [8,9] and other cytoskeletal alterations [10]. Another mechanism of resistance is the decrease of drug concentration inside the cell caused by overexpression

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of drug pumps like ABC transporters [11], decreased influx [12] or activation of detoxifying systems like cytochrome p450 [13]. Decreased apoptosis is also a highly relevant resistance mechanism.

HSP27 (HSPB1), a small heat shock protein, is an antiapoptotic molecule involved in survival of cells exposed to several stress types including anticancer drugs [14]. This chaperone inhibits apoptosome formation [15], regulates Bid distribution [16] and prevents FAS-mediated apoptosis [17]. This protein also stabilizes actin microfilaments [18], increases the anti-oxidant cell defences [19] and inhibits NF-κB signalling.

In mammalian cells, HSP27 is distributed from monomers or dimers to large oligomers of 600–800 kDa [20]. Monomers/dimers and oligomers have different role in apoptotic pathways. The oligomerization status of this protein is dependent on its phosphorylation, so that when the protein is phosphorylated large oligomers are depolymerised [21].

Using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), we have found that vincristine treatment induces a new spot identified as HSP27 by MALDI-TOF-MS. This induction is accompanied with simultaneous downregulation of another more basic spot, also identified as HSP27, whereas HSP27 mRNA levels are not affected. In this work, we demonstrated that the vincristine-induced form of HSP27 is phosphorylated at Serine 82.

2. Materials and methods

2.1. Cell culture and treatments

MCF7 cells were propagated in phenol red DMEM (Cambrex) containing 100 μg/mL gentamicine and 10% of heat inactivated fetal bovine serum (FBS) (Cambrex). Then cells were estrogen-depleted by transference to phenol-red free DMEM containing 0.5% of hormone-free human serum (Atom), 100 μg/mL gentamicine (Sigma) and 4 mM L-glutamine (Sigma). After three days cells were treated with vincristine (Sigma) dissolved in ethanol. The final concentration of ethanol was always 0.1%.

2.2. RNA extraction, cDNA synthesis and real-time PCR analysis

Total RNA was extracted using Tri® Reagent (Sigma) and the yield was measured spectrophotometrically. Quality and integrity of total RNA was assessed on 2% agarose gel. Single stranded cDNA was synthesized from 2 μg of total RNA using MMLV Reverse Transcriptase (Invitrogen) and oligo dT (Roche). All the procedures were performed following the manufacturer's instructions. PCR reactions were performed in an ABI PRISM® 7000 (Applied Biosystems) using SYBR® GREEN PCR Master Mix (Applied Biosystems) and 300 nM primer concentration. PCR conditions were an initial denaturation and DNApol activation step of 95 °C for 10 min followed by 40 cycles of denaturation for 15 s at 95 °C and annealing-extension for 1 min at 60 °C. All the samples were amplified in triplicate and PCRs were repeated two times. β-2-Microglobulin (B2M) gene was used as internal control. The following primers were designed with the Primer Express® software v2.0: HSP27 forward 5′-AGGAGC GGCAGGACGAGCAT-3′ and reverse 5′-GCGACTCG AAGGTGACTGGG-3′, and B2M forward 5′-TGAG TGCTGTCTCCATGTTTGA-3′ and reverse 5′-TCTG CTCCCCACCTCTAAGTTG-3′.

2.3. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cells were solubilized in UTATH buffer [7 M urea, 2 M thiourea, 1% Amidosulfobetaine-14, 50 mM 2-Hydroxyethyl disulfide (HED), 0.5% IPG buffer, pH 3–10 (Bio-Rad)]. The cell extract was desalted with a desalting column (Pearson) and 60 to 100 mg of protein loaded into a strip holder. First dimension was run in 7 cm Immobiline™ DryStrips, pH 3–11 (Amersham) for 12 h at 30 V, 250 Vh at 500 V, 500 Vh at 1000 V and 8000 Vh at 5000 V. For the second dimension, strips were equilibrated in equilibration buffer [6 M urea, 30% glycerol, 50 mM Tris (pH 6.8), 2% SDS, 0.002% bromophenol blue w/v] and run in 10% polyacrylamide gels supplemented with 50 mM HED and 6 M urea. Gels were fixed with fixing-solution (20% methanol v/v, 10% acetic acid v/v) for 24 h, stained with Coomassie-solution (0.25% brilliant blue R250 w/v, 45% methanol v/v, 10% acetic acid v/v) for 2 h and distained with fixing-solution for 24 h. Spot density was quantified using Scion Image software (Scion Image corporation).

2.4. Trypsin digestion, mass spectrometry analysis and informatic treatment of data

Gel spots were excised, distained with 25 mM ammonium bicarbonate:acetonitrile (70:30 v/v) and incubated with 12 μg/mL trypsin (Promega) in 25 mM ammonium bicarbonate at 60 °C for 1 h. 4 mL of 1% trifluoroacetic acid were added to each sample. Then, samples were sonicated for 10 min and desalted with a ZipTip (Millipore). Peptides were eluted with 2 μL of alpha cyano-4-hydroxycinnamic acid in acetonitrile:0.1% trifluoroacetic acid (50:50 v/v). MALDI-TOF-MS was performed on a Voyager-DE STR (Applied Biosystems). Data from 50 to 200 laser shots were collected to produce a mass spectrum

of 1000–2000 Da range. The mass spectrum data were analysed with Aldente software (http://www.expasy.org/tools/aldente) [22].

2.5. Western blot analysis

For mono-dimensional electrophoresis, cells extracts were obtained in Laemmli buffer, heat denatured and 30 to 60 mg of protein were electrophoresed on a 10% SDS–PAGE. For 2D-PAGE cell extracts were processed as described. After electrophoresis proteins were transferred to PVDF membranes. Membranes were blocked with TBS/Tween 20 supplemented with 5% w/v non-fat milk for 1 h at room temperature, then incubated with primary antibodies overnight at 4 °C, with secondary antibodies for 1 h at room temperature, and developed with enhanced chemiluminescence reagents (Amersham). Anti-phospho-HSP27 (Ser82) (Cell Signaling Cat. #2401), anti-HSP27 (Cell Signalling Cat. #2402) anti-rabbit peroxidase (Cell Signaling Cat. #7074) and anti-mouse peroxidase (Sigma Cat. A-9044) were used at 1:4000, 1:6000, 1:2000 and 1:10,000 dilution, respectively.

2.6. Flow cytometry

Cells were collected by trypsinization and incubated sequently in 300 μL of buffer A [0.5 mM Tris–HCl (pH 7.6), 0.1% Nonidet P-40 v/v, 3.4 mM trisodium citrate, 1.5 mM spermin, 30 μg/mL trypsin w/v (Sigma)] for 10 min, in 250 μL of buffer B [0.5 mM Tris–HCl (pH 7.6), 0.1% Nonidet P-40v/v, 3.4 mM trisodium citrate, 1.5 mM spermin, 500 μg/mL trypsin inhibitor (Sigma), 100 mg/mL RNase A (Sigma)] for 10 min and in 250 μL of buffer C [0.5 mM Tris–HCl (pH 7.6), 0.1% Nonidet P-40 v/v, 3.4 mM trisodium citrate, 4.83 mM spermin, 416 μg/mL propidium iodide] for 10 min. Cell cycle was analysed in a FACscan flux cytometer (Becton Dickinson) using ModFYT software.

3. Results

3.1. Cellular response to vincristine

Vinca alkaloids are chemotherapeutic agents that induce mitotic arrest and apoptosis [23]. We have recently described that estradiol enhances vincristine-induced apoptosis [24]. In order to abolish estrogen interferences, experiments were performed in hormone-depleted conditions. MCF7 cells were estrogen-depleted, treated with 1 μM vincristine for 48 h and processed for flow cytometry. As expected, treatment with vincristine significantly increased both the number of cells that entered apoptosis (Control: 3.47%; Vc treated: 17.07%) and the number of cells in G2/M phase (Control: 11.54%; Vc treated: 17.74%) (Fig. 1).

3.2. Proteomic analysis of vincristine effects

To investigate which proteins might be altered upon vincristine treatment, cells were treated with either vehicle or 1 μM vincristine for 24 h. Protein expression after drug treatment was analysed by 2D-PAGE. Two vincristine-regulated spots were found (Fig. 2b). In vincristine treated cells (Fig. 2c, down panel) a spot appeared, labelled as A, which was upregulated as compared with control cells (Fig. 2c, upper panel). On the other hand another spot, labelled as B, was downregulated in vincristine treated cells. These data were confirmed by densitometry analysis with Scion Image software (Fig. 2d), in which spot labelled as C was used as endogenous control. Thus, spot A increased from 0.25 to 0.91 normalized units while

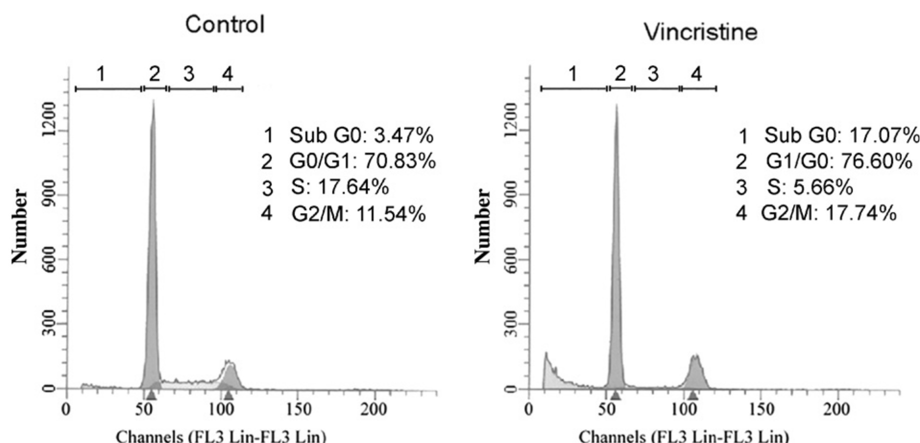


Fig. 1. Flow cytometry analysis of vincristine effects. Estrogen-depleted MCF7 cells were treated with vehicle (control) or 1 μM vincristine for 48 h. Cells were processed for propidium iodide staining and flow cytometry.

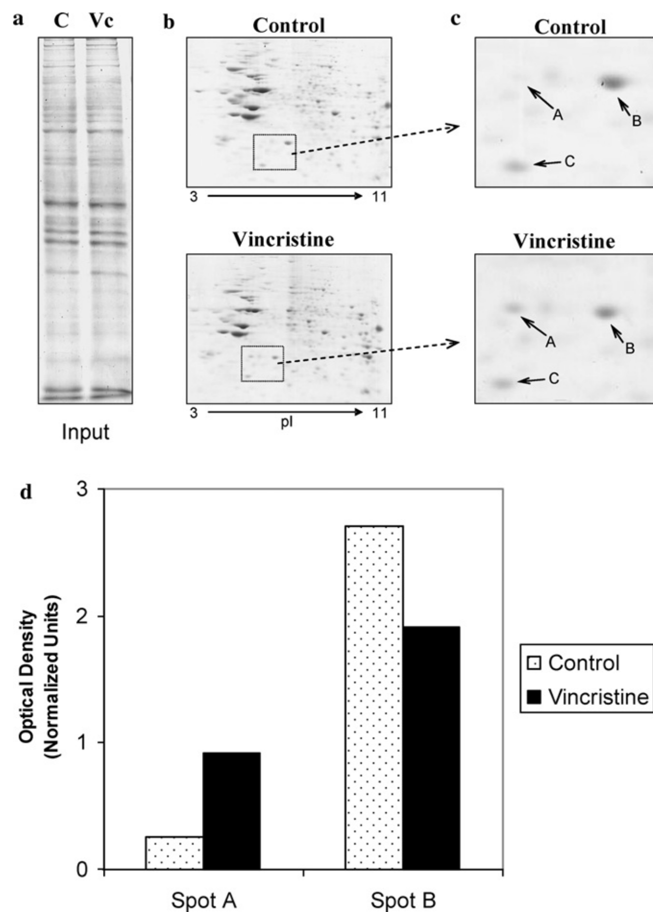


Fig. 2. Analysis, by 2D-PAGE, of vincristine effects. Estrogen-depleted MCF7 cells were treated with vehicle (control) or 1 μM vincristine for 24 h. (a) Coomassie staining of a mono-dimensional SDS–PAGE used as input control, (b) Coomassie staining of a 2D-PAGE, (c) magnification of the marked zone, (d) spot densitometry analysis.

spot B decreased from 2.71 to 1.91 normalized units. A mono-dimensional SDS–PAGE was run to demonstrate that identical amounts of total protein were loaded in each 2D-PAGE (Fig. 2a).

3.3. MALDI-TOF-MS identification

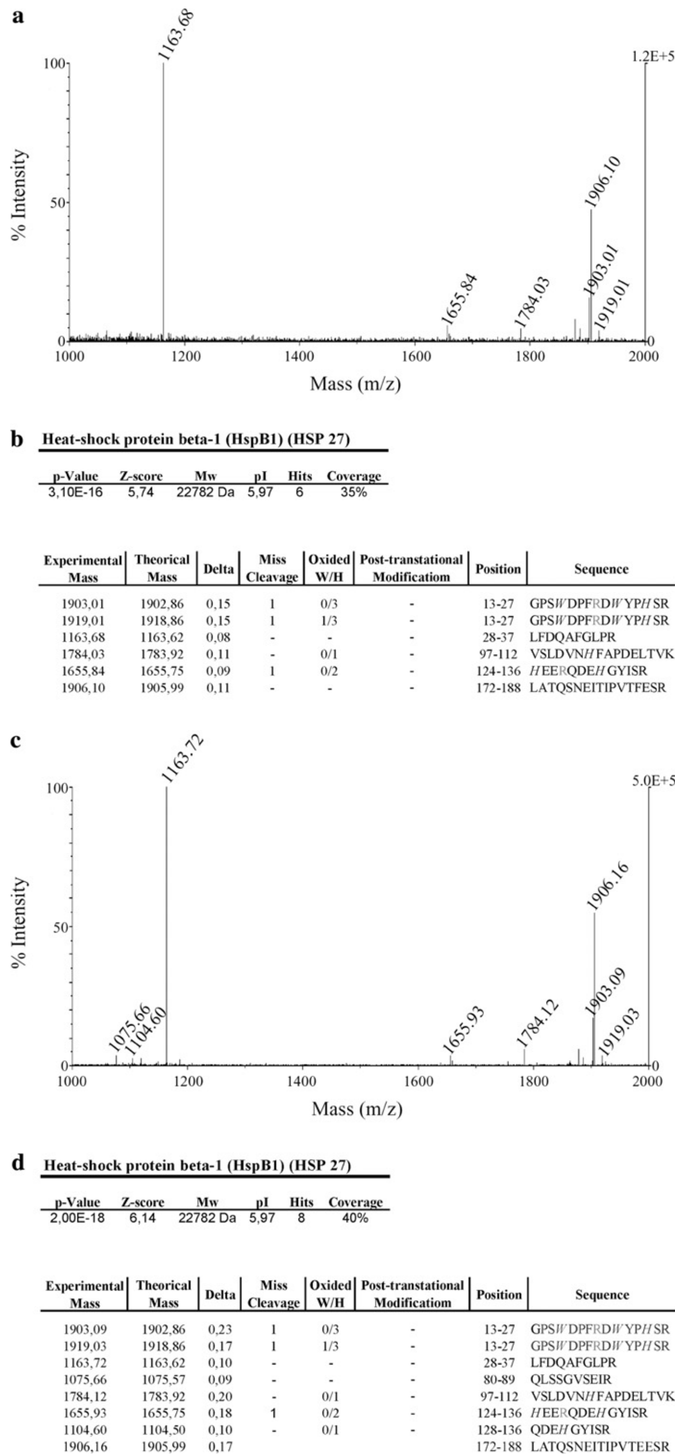
To identify the proteins of interest MALDI-TOF-MS in positive reflector mode was used. The two vincristine-regulated spots (A and B) were excised from the gel and digested with trypsin. The mass spectrometry data obtained for spot A (Fig. 3a) and spot B (Fig. 3c) were similar, this strongly suggested that the two spots were different forms of the same protein. Aldente software confirmed that both spots were HSP27 (HSPB1) with a p-value of 3.1×10^{-16} and 2.0×10^{-18}, respectively, (Fig. 3b and d).

3.4. mRNA expression analysis of HSP27

HSP27 is a heat shock protein that is regulated in response to stress; both increasing its expression and undergoing post-translational modifications [25]. Cells were treated with 1 μM vincristine (Vc) for 1 h, 3 h, 5 h, 10 h and 24 h. Then, the mRNA was purified, the cDNA was synthesized and the expression of HSP27 was determined by real-time PCR. Our results showed that there was no significant change in HSP27 mRNA expression at none of the times assayed (Fig. 4).

3.5. Identification of a post-translational modification in the Vc induced HSP27 form

Phosphorylation is the best characterised modification of HSP27. To investigate whether vincristine was regulating this type of modification, the A and B spots were subjected to MALDI-TOF-MS analysis in negative reflector mode. The fingerprints obtained for both spots revealed that in spot A appears a peptide of 1153.54 Da, absent in spot B (Fig. 5a and c). The informatic analysis of this mass spectrum revealed that the peptide corresponds to aminoacids 80–89 and that it is phosphorylated (Fig. 5b and d). Interestingly, the



b **Heat-shock protein beta-1 (HspB1) (HSP 27)**

p-Value	Z-score	Mw	pI	Hits	Coverage
3,10E-16	5,74	22782 Da	5,97	6	35%

Experimental Mass	Theorical Mass	Delta	Miss Cleavage	Oxided W/H	Post-transtational Modificatiom	Position	Sequence
1903,01	1902,86	0,15	1	0/3	-	13-27	GPS*W*DPFRD*W*YP*H*SR
1919,01	1918,86	0,15	1	1/3	-	13-27	GPS*W*DPFRD*W*YP*H*SR
1163,68	1163,62	0,08	-	-	-	28-37	LFDQAFGLPR
1784,03	1783,92	0,11	-	0/1	-	97-112	VSLDVN*H*FAPDELTVK
1655,84	1655,75	0,09	1	0/2	-	124-136	*H*EERQDE*H*GYISR
1906,10	1905,99	0,11	-	-	-	172-188	LATQSNEITIPVTFESR

d **Heat-shock protein beta-1 (HspB1) (HSP 27)**

p-Value	Z-score	Mw	pI	Hits	Coverage
2,00E-18	6,14	22782 Da	5,97	8	40%

Experimental Mass	Theorical Mass	Delta	Miss Cleavage	Oxided W/H	Post-transtational Modificatiom	Position	Sequence
1903,09	1902,86	0,23	1	0/3	-	13-27	GPS*W*DPFRD*W*YP*H*SR
1919,03	1918,86	0,17	1	1/3	-	13-27	GPS*W*DPFRD*W*YP*H*SR
1163,72	1163,62	0,10	-	-	-	28-37	LFDQAFGLPR
1075,66	1075,57	0,09	-	-	-	80-89	QLSSGVSEIR
1784,12	1783,92	0,20	-	0/1	-	97-112	VSLDVN*H*FAPDELTVK
1655,93	1655,75	0,18	1	0/2	-	124-136	*H*EERQDE*H*GYISR
1104,60	1104,50	0,10	-	0/1	-	128-136	QDE*H*GYISR
1906,16	1905,99	0,17				172-188	LATQSNEITIPVTEESR

Fig. 3. MALDI-TOF-MS identification of vincristine-regulated spots. Spots A and B were distained and trypsinized. The m/z of the obtained peptides was determined by MALDI-TOF-MS in positive reflector mode. (a) Mass spectrum for spot A; (b) result of Aldente analysis for spot A; (c) mass spectrum for spot B; (d) result of Aldente analysis for spot B. (Masses are given in Da).

analysis of spot B in positive reflector mode showed a 1075.66 Da peptide, also corresponding to aminoacids 80–89 which did not appear as a phosphorylated peptide (Fig. 3d). This non-phosphorylated peptide was absent in spot A when this analysis was carried out. (Fig. 3b)

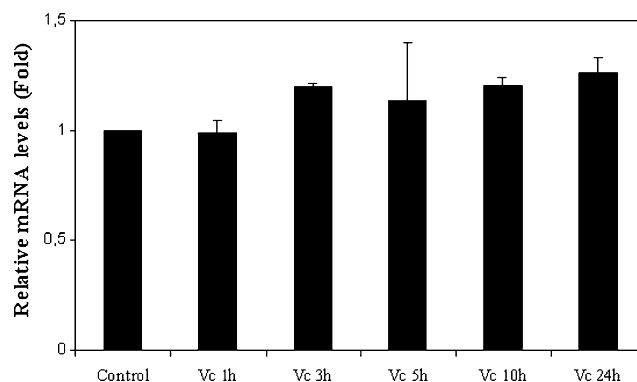


Fig. 4. Effect of vincristine on steady-state level of HSP27 mRNA. Estrogen-depleted MCF7 cells were treated with vehicle (control) or 1 μM vincristine (Vc) for the indicated times. Total RNA was isolated, the cDNA was synthesized and HSP27 mRNA expression was quantified by real-time PCR using B2M as internal control.

3.6. Immunoblot detection of HSP27 serine 82 phosphorylation

To prove that this HSP27 phosphorylation was located at a described serine [26], Western blot analysis was performed using antibodies that specifically bind to HSP27 only when it is phosphorylated at serine 82. Cells were treated with vehicle or 1 μM Vc for the indicated times. Then cells extracts were subjected to Western blot analysis (Fig. 6a, upper panel). The total amount of HSP27 was also determined (Fig. 6a, down panel). This study showed that Vincristine treatment induced a slight increase in HSP27 and, more importantly, that the ratio phosphorylated HSP27/total HSP27 increased near 3-fold. These data confirmed that HSP27 was phosphorylated at serine 82 (Fig. 6b). Western blot analysis of a 2D gel demonstrated that upon vincristine treatment only spot A was phosphorylated (Fig. 6c).

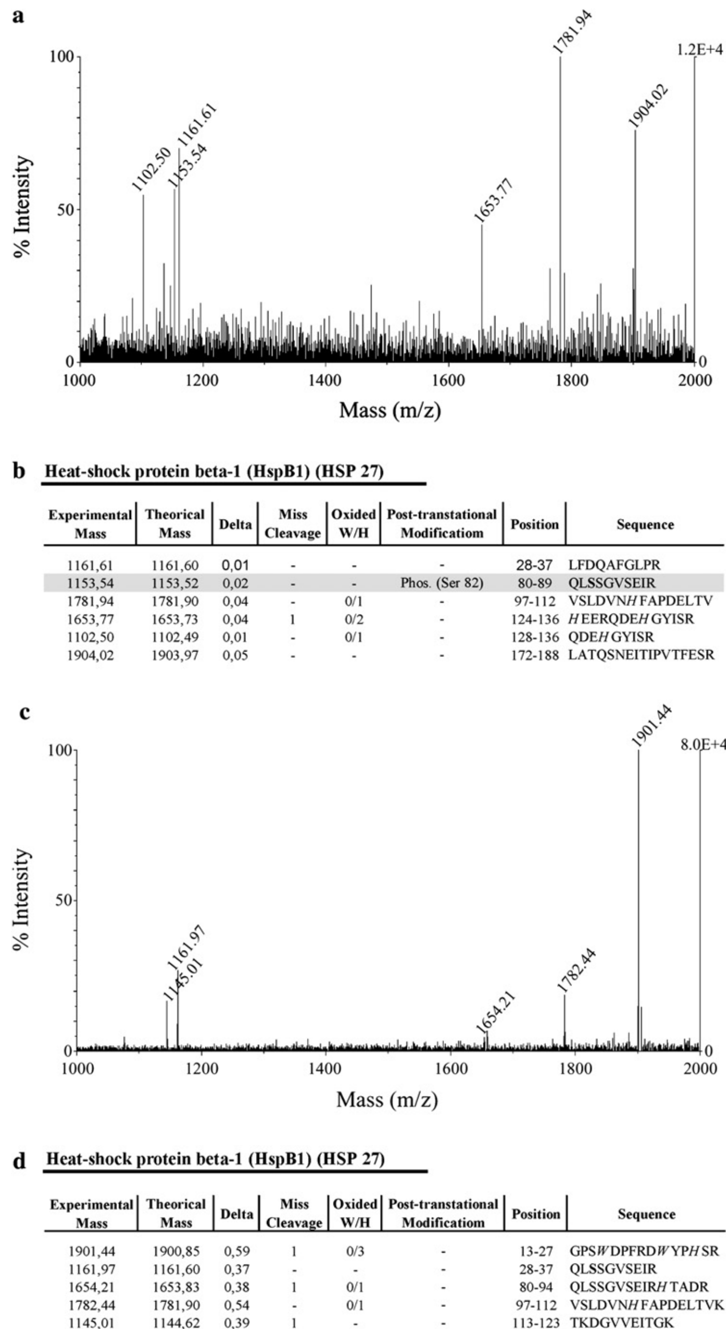
4. Discussion

Vinca alkaloids, such as vincristine, are drugs that block the formation of the mitotic spindle required for chromosome migration during mitosis [5]. These compounds also activate JNK [6], induce Bcl-2 phosphorylation [7], G2/M arrest and apoptosis [6]. However, the biochemical events occurring between vinca binding to tubulin and apoptosis are not well understood.

In the last few years, the development of proteomic techniques has made 2D-PAGE/MALDI-TOF-MS a powerful tool for analysis of complex protein mixtures. Using this methodology, it has been recently reported that in CCRF-CEM leukemia cells proteins such as FKBP59, TCTP, HSP90 or L-plastin are involved in cellular response to vincristine [10].

In this report, we have used MCF7 cells to study proteomic changes caused by vincristine treatment. This epithelial breast cancer cell line is characterised for its ability to process the estradiol signalling through its estrogen receptors. We have recently demonstrated that estradiol enhances vincristine-induced apoptosis [24]. Therefore, in order to avoid hormonal interferences, we performed our experiments in estrogen-depleted conditions.

After confirming that in this culture conditions vincristine still retained its capacity to induce G2/M arrest and apoptosis, we performed the 2D-PAGE analysis. This analysis showed two vincristine-regulated spots identified as HSP27 by positive reflector mode MALDI-TOF-MS, the more acid upregulated and the more basic down-regulated. These events linked to the lack of effect of vincristine on HSP27 mRNA levels, suggested that the vinca alkaloid was implicated in the regulation of a post-translational modification of the protein. We further explored this possibility using MALDI-TOF-MS in negative reflector mode, which facilitates the flight of negative charged peptides such as those that are phosphorylated. This analysis showed the presence of a 1153.54 Da peak only in the vincristine-induced spot. This peak corresponded to a phosphorylated peptide comprising aminoacids 80–89. This correlates with the presence, only in spot B, of a 1075.66 Da peak corresponding to a non-phosphorylated peptide also comprising aminoacids 80–89, when positive reflector mode mass spectrometry analysis was used. These data were also compatible with the fact that spot A is more acidic than spot B. Next, we determined the Phosphorylated/Total HSP27



b Heat-shock protein beta-1 (HspB1) (HSP 27)

Experimental Mass	Theorical Mass	Delta	Miss Cleavage	Oxided W/H	Post-transtational Modificatiom	Position	Sequence
1161,61	1161,60	0,01	-	-	-	28-37	LFDQAFGLPR
1153,54	1153,52	0,02	-	-	Phos. (Ser 82)	80-89	QLSSGVSEIR
1781,94	1781,90	0,04	-	0/1	-	97-112	VSLDVN*H*FAPDELTV
1653,77	1653,73	0,04	1	0/2	-	124-136	*H*EERQDE*H*GYISR
1102,50	1102,49	0,01	-	0/1	-	128-136	QDE*H*GYISR
1904,02	1903,97	0,05	-	-	-	172-188	LATQSNEITIPVTFESR

d Heat-shock protein beta-1 (HspB1) (HSP 27)

Experimental Mass	Theorical Mass	Delta	Miss Cleavage	Oxided W/H	Post-transtational Modificatiom	Position	Sequence
1901,44	1900,85	0,59	1	0/3	-	13-27	GPS*W*DPFRD*W*YP*H*SR
1161,97	1161,60	0,37	-	-	-	28-37	QLSSGVSEIR
1654,21	1653,83	0,38	1	0/1	-	80-94	QLSSGVSEIR*H*TADR
1782,44	1781,90	0,54	-	0/1	-	97-112	VSLDVN*H*FAPDELTVK
1145,01	1144,62	0,39	1	-	-	113-123	TKDGVVEITGK

Fig. 5. Identification of a phosphorylated peptide in the vincristine-induced spot. Spots A and B were distained and trypsinized. The masses of the obtained peptides were determined by MALDI-TOF-MS in negative reflector mode. (a) Mass spectrum for spot A; (b) result of Aldente analysis for spot A; (c) mass spectrum for spot B; (d) result of Aldente analysis for spot B. (Masses are given in Da.)

ratio using specific antibodies that recognized either serine 82 phosphorylated or total HSP27, respectively. The results showed that, despite the fact that a small increment of total HSP27 occurred during the first hour of vincristine treatment, the Phosphorylated/Total HSP27 ratio clearly increased after a vincristine treatment of 10 h or longer. Finally, we used a 2D-PAGE immunoblot to confirm that spot A was phosphorylated and spot B was not phosphorylated.

HSP27 is a small ATP independent heat shock protein that increases cell survival in stress

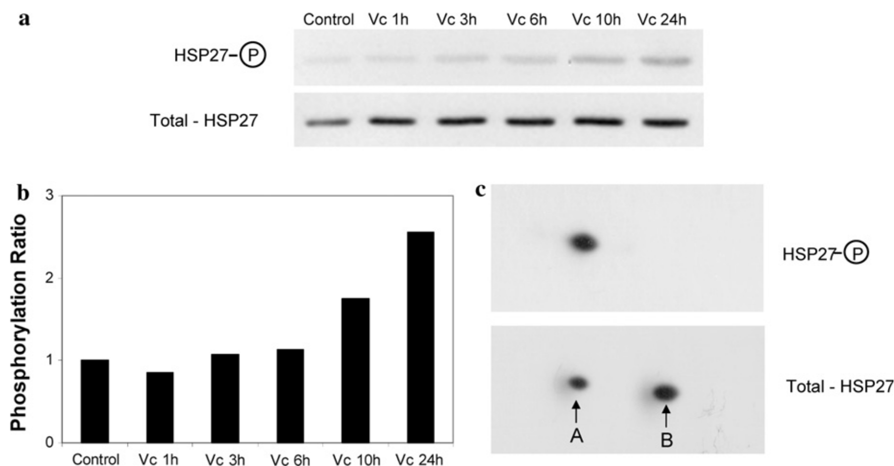


Fig. 6. Effect of vincristine on phosphorylation of HSP27 at serine 82. Estrogen-depleted MCF7 cells were treated with vehicle (Control) or 1 μM vincristine (Vc) for the indicated times. The relative amount of HSP27 phosphorylated at serine 82 present in whole cell extracts was analyzed by immunobloting with antibodies directed against HSP27 phosphorylated at serine 82 and total HSP27. (a) Immunoblot analysis, (b) band densitometry analysis of panel a, (c) 2D-PAGE immunoblot analysis of cells treated for 24 h with vincristine. Spots A and B are as in Fig. 2.

situations, such as those induced by treatment with anticancer drugs [14]. Epidemiological studies report that this apoptosis modulator is upregulated in breast cancer [27] and other tumour types [28]. This protein is also related with metastasis, poor prognosis and resistance to chemotherapy or radiation [29]. HSP27 forms large oligomers that are regulated by phosphorylation. When phosphorylated, oligomers switch to monomers or dimers, an event that implies a change in protein activity.

This chaperone regulates the function of several proteins involved in cell response to vincristine. Thus, non-phosphorylated oligomers of HSP27 bind to cytochrome C which blocks apoptosome formation[28], while phosphorylated monomers/dimers interact with DAXX and inhibit the apoptosis induced by Fas [17], a death receptor that can undergo ligand-independent activation by chemotherapeutic drugs such as vinblastine [30]. It has been demonstrated that phosphorylated HSP27 inhibits the TNF induced activation of NF-κB [31], a process that has been linked to vincristine-induced apoptosis [32]. This inhibition is caused by the chaperone binding to IKK-β that blocks IκB phosphorylation [31]. HSP27 has also been related with actin cytoskeleton modulation and Bid redistribution. However, there is little information about the HSP27 phosphorylation status in these events [16].

Recently it has also been shown that HSP27 is related to tumour cell migration. Thus, phosphorylation of HSP27 is implicated in TGF-β induced MMP2 activation [33] and in the invasive breast cancer cell line MDA-MB-231, the blocking of HSP27 phosphorylation results in tumour cell migration and invasion inhibition [34].

In summary, phosphorylation of HSP27 appears to play a significant role in cell survival and in metastasis formation. In this work we show that this modification is influenced by vincristine treatment, causing an increment of HSP27 phosphorylated at serine 82, an event that might be important in regulating cell migration and cell resistance to the drug.

Acknowledgments

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