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Characterization of HSP27 phosphorylation induced by microtubule interfering agents: Implication of p38 signalling pathway

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Abstract

Vincristine and paclitaxel are widely used antitumoral drugs that interfere with microtubule dynamics. We have previously demonstrated that vincristine induces phosphorylation of HSP27 at serine 82 in MCF-7 cells. In this report, we show that vincristine also causes phosphorylation of serines 78 and 15. Moreover, we demonstrate that phosphorylation of this chaperone is induced by the p38 signalling pathway while the JNK pathway is not implicated. Differences between vincristine and paclitaxel treatments are also appreciated. Thus, while vincristine induces a strong phosphorylation of the three serines, paclitaxel induces a weak phosphorylation of serine 78 and has no effect over serines 82 and 15 phosphorylation. Interestingly, pre-treatment of cells with a ten-fold excess of paclitaxel abolishes vincristine-induced phosphorylation of HSP27.

Keywords: Vincristine; Paclitaxel; Breast cancer; HSP27; p38; 2D-PAGE; Phosphorylation

Microtubule interfering agents such as taxanes or Vinca alkaloids (VAs) are widely used anticancer drugs. These drugs block the mitotic spindle dynamics needed for chromosome migration during mitosis, an event that causes the death of dividing cells [1]. These drugs also induce JNK[3] activation [2], Bcl-2 phosphorylation [3] and G2/M cell cycle arrest [2]. At high concentrations, VAs inhibit tubulin polymerization, while high doses of taxanes block tubulin depolymerization [1].

Resistance development to chemotherapy agents means a mayor clinical problem. HSP27 (HSPB1) is a small heat shock protein involved in metastasis formation and resistance to chemotherapy and radiation [4]. This chaperone promotes cell survival by blocking the apoptosome formation [5], by preventing Fas mediated cell death [6] and by other molecular mechanisms [7]. It also facilitates cell migration [8] and is implicated in TGF-β induced MMP-2 activation [9].

In response to different types of stress stimuli, HSP27 undergoes phosphorylation at serines 15, 78 and 82. The best characterized signalling pathway that induces these changes involves the p38 kinases also known as SAPK2, these MAPKs activate the p38 activated kinases MK2, MK3 and MK5. MK2, MK5 and at least "*in vitro*" MK3 phosphorylates HSP27 [10–12]. These modifications induce the switch of HSP27 distribution from large oligomers to monomers or dimers. This variation in the oligomerization status also causes a change in protein functions.

We have previously described that vincristine induces the phosphorylation of HSP27 at serine 82 [13]. In this report we demonstrate that the phosphorylations induced by the Vinca alkaloid also include serines 15 and 78 and that these

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[3] *Abbreviations used:* JNK, c-Jun NH2-terminal kinase; MMP2, matrix metalloproteinase-2; HSP27, heat shock protein 27; MIAs, microtubule interfering agents; MAPK, mitogen-activated protein kinase.

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modifications occur in a p38 dependent way. We also show that inhibition of JNK does not have any effect over HSP27 phosphorylation. Finally, we observe that vincristine and paclitaxel induce a differential phosphorylation pattern on HSP27.

Materials and methods

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). To avoid estradiol interferences with vincristine induced cell death, cells were estrogen-depleted by transference to phenol-red free DMEM containing 0.5% of hormone-free human serum (Atom), 100 μg/mL gentamicine and 4 mM L-glutamine. After three days cells were treated with vincristine (Sigma), vinblastine (Sigma), paclitaxel (Sigma), nocodazole (Sigma), anisomycin (Sigma), SP600125 (Biomol) and SB203580 (Sigma). SP600125 and SB203580 were dissolved in DMSO, anisomycin was dissolved in water and the other compounds were dissolved in ethanol. The final concentration of ethanol and DMSO was 0.1%.

Western blot analysis

Cells extracts were obtained in Laemmli buffer, heat denatured and 30–60 μg of protein were electrophoresed on a 10% SDS–PAGE. 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 (GE-Healthcare). Anti-phospho-HSP27 (Ser82) (Cell Signaling Cat. #2401), Anti-phospho-HSP27 (Ser78) (Cell Signaling Cat. #2405), Anti-phospho-HSP27 (Ser15) (Cell Signaling Cat. #2404), anti-HSP27 (Cell Signalling Cat. #2402), Anti-phospho-p38 MAPK (Thr 180 and Tyr 182) (Cell Signaling Cat. #9211), anti-phospho-c-Jun (ser 63) (Cell Signalling Cat. #9261), anti-rabbit peroxidase (Cell Signaling Cat. #7074) and anti-mouse peroxidase (Sigma Cat. A-9044) were used at 1:4000, 1:2000, 1:2000, 1:6000, 1:1000, 1:1000, 1:2000 and 1:6000 dilution, respectively.

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)], desalted with a desalting spin column (Pierce) and 60–100 μg of protein loaded into a strip holder. First dimension was run in 7 cm Immobiline™ DryStrips, pH 3–11 (GE-Healthcare), 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. For Western analysis, proteins were transferred to PVDF membranes and processed as described previously. For Coomassie staining, 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.

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 ng/mL trypsin (Promega) in 25 mM ammonium bicarbonate at 60 °C for 1 h 4 μL 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/)[14].

Flow cytometry

Cells were collected by trypsinization and incubated sequentially 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 (Sigma)] for 10 min, in 250 μL of buffer B [0.5 mM Tris–HCl (pH 7.6), 0.1% Nonidet P-40 v/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 flow cytometer (Becton–Dickinson).

Results

Analysis of vincristine effect over HSP27 phosphorylation

To investigate which proteins might be altered upon vincristine treatment, we used MCF7 cells cultured in estrogen-depleted conditions in order to avoid estradiol interferences with vincristine-induced cell death. Thus, these estrogen-depleted cells were treated with vehicle or 1 μM vincristine for 24 h. Protein expression after drug treatment was analysed by 2D-PAGE. We observed a vincristine-induced spot, named as A, while a second spot B was down-regulated upon vincristine treatment, (Fig. 1a). Spot A was identified as HSP27 by MALDI-ToF-MS (Fig. 1b). We have recently described that this spot is phosphorylated at serine 82 [13]. To further study whether this vincristine upregulated form is phosphorylated at any other residue, 2D-PAGE followed by Western blot analysis was performed. Western blot analysis using antibodies against total HSP27 confirmed that both spots contained HSP27 and that spot A was upregulated and spot B was downregulated (Fig. 1c, bottom line panels). Further analysis using antibodies against specific phospho-serine residues showed that spot A contained HSP27 phosphorylated at serine 15 (Fig. 1c, top line panels), serine 78 (Fig. 1c, second line panels) and serine 82 (Fig. 1c, third line panels). Antibodies against phosphoserine 78 detected another, more acidic form of phospho-HSP27 named as C (Fig. 1c, second line panels). No phosphorylation was detected in the downregulated spot B, indicating that this was the unphosphorylated form of HSP27 (Fig. 1c).

Signalling pathways implicated in vincristine-induced phosphorylation of HSP27

It has been shown that vincristine induces the activation of the JNK signalling pathway [2]. We therefore decided to study the possible implication of this signalling pathway in the vincristine-induced phosphorylation of HSP27. For that purpose, the specific inhibitor of JNK SP600125 was

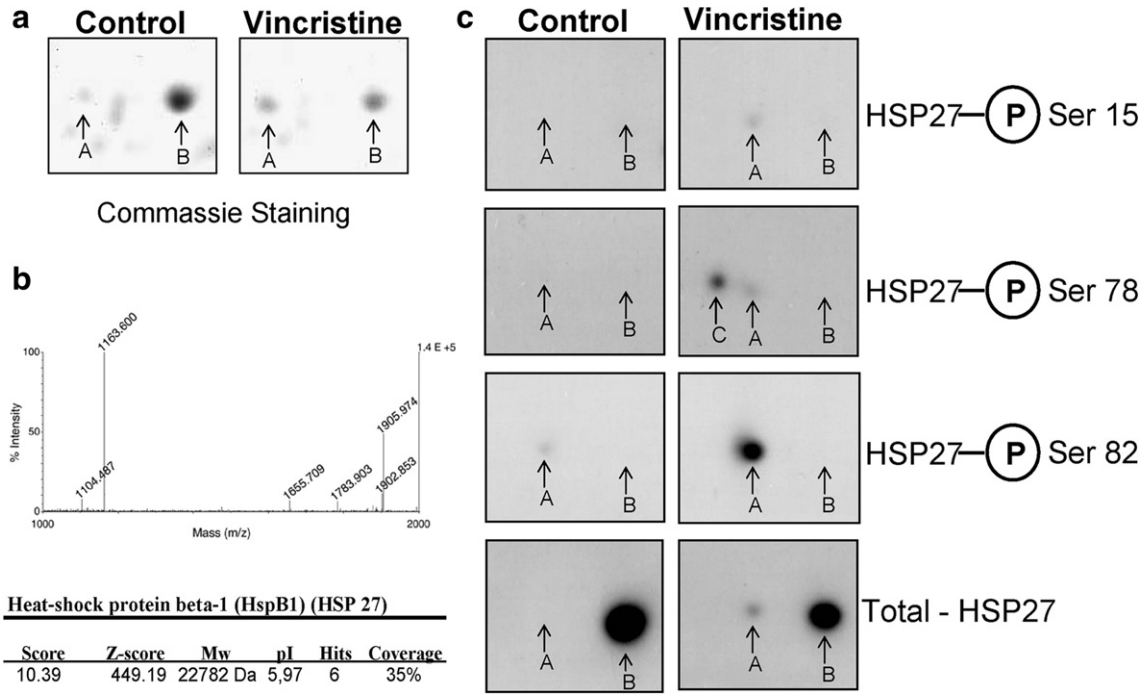

Fig. 1. Effect of vincristine on HSP27 phosphorylation. Estrogen-depleted MCF7 cells were treated with vehicle (Control) or 1 μM vincristine for 24 h. (a) Coomassie staining of a 2D-SDS–PAGE. (b) MALDI-ToF analysis of spot A. (c) Total and phosphorylated HSP27 at serines 15, 78 and 82 analyses by 2D-PAGE immunobloting. A, C: vincristine-induced spots, B: non-vincristine-induced spot.

used. Thus, estrogen-depleted MCF7 cells were treated with 1 μM vincristine for 24 h, 20 μM SP600125 or the combination of 20 μM SP600125 with 1 μM vincristine for 24 h. SP 600125 was added 30 min prior to vincristine treatment. After that, cell extracts were processed for Western blot. This analysis showed that vincristine induced the phosphorylation of both HSP27 and the JNK substrate c-Jun (Fig. 2, lane 2). Interestingly, SP600125 had no effect over the phosphorylation of HSP27 at serine 78, while clearly inhibited the phosphorylation of c-Jun (Fig. 2, lane 4). These results indicate that JNK is not implicated in the vincristine-induced phosphorylation of HSP27.

It is also known that HSP27 phosphorylation, involves the mitogen activated protein kinase (MAPK) named p38. To determine the implication of this signalling pathway in vincristine-induced phosphorylation of HSP27, the specific inhibitor of p38 kinases SB203580 was used. A treatment with anisomycin was also included as a positive control of p38 mediated phosphorylation of HSP27. Thus, estrogen depleted MCF7 cells were treated with vehicle, 1 μM vincristine for 24 h, 2 μg/mL anisomycin for 1 h, 10 μM SB203580, or the combination of 10 μM SB203580 with 1 μM vincristine for 24 h or with 2 μg/mL anisomycin for 1 h. SB203580 was added to cultures 30 min before the other compounds. After treatment, protein extracts were subjected to immunoblotting. As expected, vincristine and anisomycin induced the phosphorylation of HSP27 at serines 15, 78 and 82 (Fig. 3,

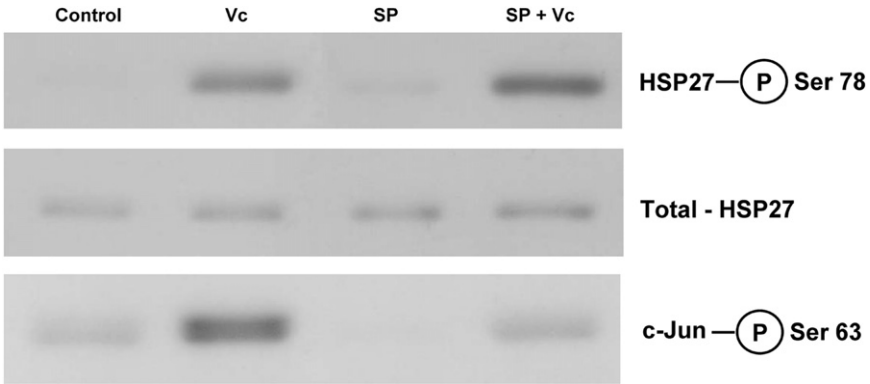

Fig. 2. Effect of SP600125 on vincristine-induced phosphorylation of HSP27. Estrogen depleted MCF7 cells were treated with vehicle (Control), 1 μM vincristine (Vc), 20 μM SP600125 (SP) or the combination of 20 μM SP600125 and 1 μM vincristine (SP+Vc). SP600125 was added 30 min before the treatment of 24 h with vincristine. Total HSP27 and HSP27 phosphorylated at serine 78 as well as phosphorylated c-Jun were detected by inmunobloting as described in Materials and methods.

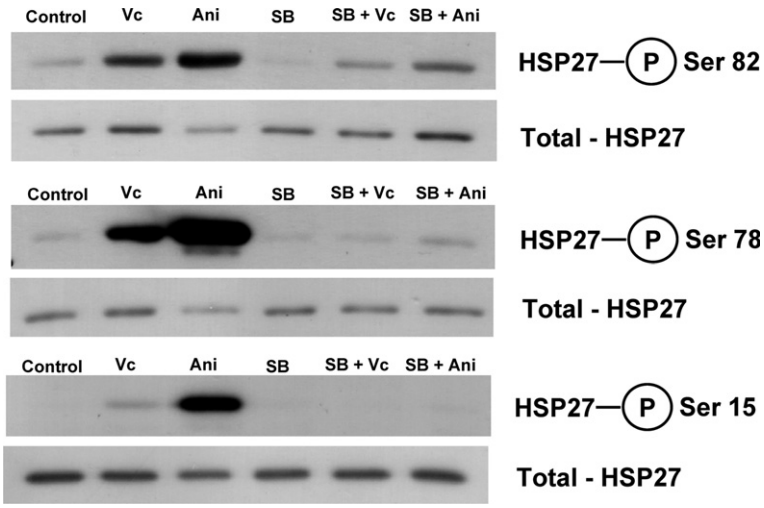

Fig. 3. Effect of SB203580 on vincristine and anisomycin-induced phosphorylation of HSP27. Estrogen-depleted MCF7 cells were treated with vehicle (Control), 1 μM vincristine (Vc), 2 μg/mL anisomycin (Ani), 10 μM SB203580 (SB) or the combination of 10 μM SB203580 with 1 μM vincristine or with 2 μg/mL anisomycin (SB+Vc or SB+Ani). SB203580 was added 30 min before the treatments of 24 h with vincristine or 1 h with anisomycin. Total HSP27 and HSP27 phosphorylated at serines 82, 78 and 15 levels of were detected by immunobloting.

lanes 2 and 3). Both vincristine and anisomycin induced phosphorylations were blocked by the p38 inhibitor SB203580 (Fig. 3, lanes 5 and 6).

To further support the hypothesis that p38 is implicated in the vincristine-induced phosphorylation of HSP27, we analysed the effect of vincristine over p38 phosphorylation. Thus, estrogen-depleted MCF7 cells were treated with vincristine or anisomycin as indicated above. Then cell lysates were processed for 2D-PAGE and analysed by Western blot using antibodies against the active form of p38 that is phosphorylated at threonine 180 and tyrosine 182. The results demonstrated that both vincristine and anisomycin dramatically increased the amount of phosphorylated p38 (Fig. 4).

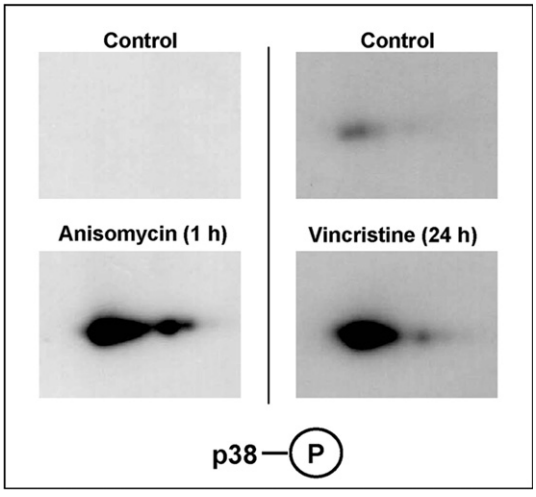

Fig. 4. Effect of vincristine and anisomycin on the levels of phosphorylated p38. Estrogen-depleted MCF7 cells were treated with vehicle (Control) and 1 μM vincristine for 24 h or 2 μg/mL anisomycin for 1 h as indicated. Cell extracts were subjected to 2D-PAGE and immunobloting, using specific antibodies against the phosphorylated forms of p38.

Distinctive effects of vincristine, paclitaxel and other MIAs over HSP27 phosphorylation

Together with vincristine, other compounds known collectively as MIAs (Microtubule-Interfering Agents) interact with microtubules dynamics. We decided to study whether some of these compounds could also induce the phosphorylation of HSP27. For this purpose estrogen-depleted MCF7 cells were treated with 1 μM of each vincristine, vinblastine, paclitaxel or nocodazole for 24 h or with 2 μg/mL anisomycin for 1 h. After treatment cell extracts were subjected to Western blot analysis. The results showed that phosphorylation of serine 15 and serine 82 only occurred upon vincristine or vinblastine treatment. Phosphorylation of serine 78 was activated by all reagents used, though vinca alkaloids were stronger inducers than the other MIAs (Fig. 5a).

To exclude the possibility that the moderate effect of paclitaxel over HSP27 phosphorylation could be due to the resistence of MCF7 cells to the drug, we compared the cell death induced by vincristine and paclitaxel using propidium iodide staining and flow cytometry analysis. Thus, estrogen-depleted cells were treated with 1 μM of each vincristine or paclitaxel for 24 h and after treatment, the cellular population was subjected to cell cycle analysis. The results showed that both vincristine and paclitaxel induced similar cell death rate (Fig. 5b). Therefore, the slight effect of paclitaxel over HSP27 phosphorylation cannot be attributed to a lack of effect of the drug on the cells, but rather to a genuine lack of activation of the pathways leading to the phosphorylation of HSP27.

It has been described that vincristine and paclitaxel have opposite effects over tubulin polymerization. To analyse whether paclitaxel can block vincristine effects over HSP27 phosphorylation, estrogen-depleted cells were treated with 0.1 or 1 μM vincristine, 1 μM paclitaxel

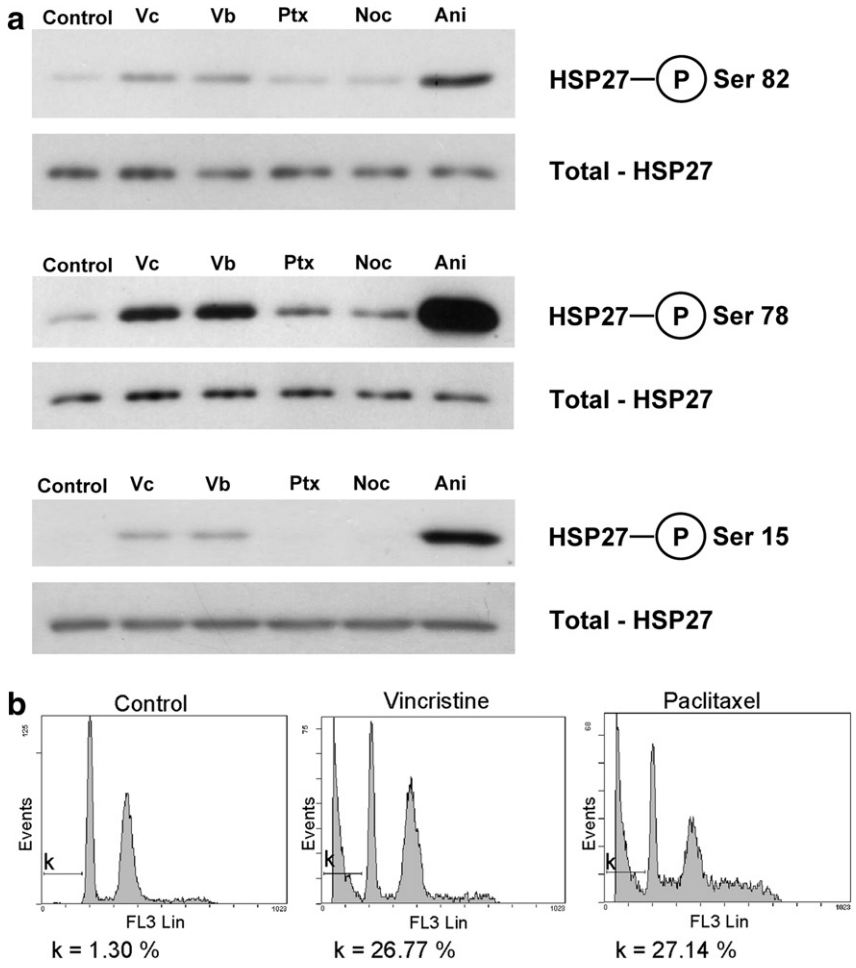

Fig. 5. Effect of MIAs on HSP27 phosphorylation and cell death. Estrogen-depleted MCF7 cells were treated with vehicle (Control), 1 μM vincristine (Vc), vinblastine (Vb), paclitaxel (Ptx), or nocodazole (Noc) for 24 h or 2 μg/mL anisomycin (Ani) for 1 h. (a) Cell extracts were subjected to Western blot analysis using specific antibodies against total HSP27 and HSP27 phosphorylated at serines 15, 78 and 82 as indicated. (b) Cells were processed for propidium iodide staining and flow cytometry analysis as described in Materials and methods. k indicates the percentage of dead cells.

and the combination of both. The cells were pre-cultured for 30 min with paclitaxel prior to vincristine treatment. Then, cell extracts were subjected to Western blot using specific antibodies against total HSP27 or HSP27 phosphorylated at serine 78. As expected, the treatment with either concentration of vincristine clearly induced the phosphorylation of HSP27 (Fig. 6, lanes 2 and 3) while paclitaxel treatment only had a slight effect (Fig. 6, lane 4). More interestingly, while no interference was observed when cells were treated with 1 μM of both vincristine and paclitaxel (Fig. 6, lane 5), their pre-treatment with a concentration of paclitaxel 10-fold higher than that of vincristine abolished the effect of the Vinca alkaloid on HSP27 phosphorylation (Fig. 6, lane 6).

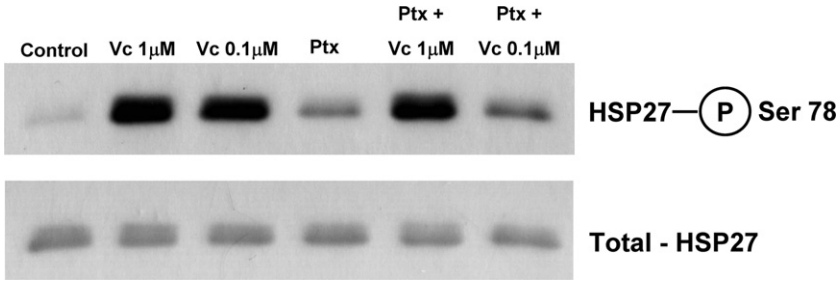

Fig. 6. Effect of paclitaxel pre-treatment on vincristine-induced phosphorylation of HSP27. Estrogen-depleted MCF7 cells were treated with vehicle (Control), 1 μM vincristine (Vc 1 μM), 0.1 μM vincristine (Vc 0.1 μM), 1 μM paclitaxel (Ptx), the combination of 1 μM paclitaxel and 1 μM vincristine (Ptx + Vc 1 μM) or the combination of 1 μM paclitaxel and 0.1 μM vincristine (Ptx + Vc 0.1 μM). Paclitaxel was added 30 min before vincristine and the treatment continued for 24 h. Total HSP27 and phosphorylated HSP27 were detected by immunobloting as described in Materials and methods.

Discussion

The use of compounds blocking microtubules dynamics is one of the most successful strategies for the treatment of cancer. These compounds, also known as MIAs, activate JNK [2], induce Bcl-2 phosphorylation [3], G2/M arrest and cell death [2]. However, the biochemical events occurring between the binding of the compound to tubulin and cell death are not well understood.

It is known that the small heat shock protein HSP27 increases cell survival in stress situations [15] by regulating the function of several proteins involved in the intrinsic [5] and the extrinsic [6] apoptotic pathways. Moreover, this chaperone has been also related to MMP2 activation [9] and tumour cell migration [8]. We have recently described that the MIA vincristine, induces the phosphorylation of the antiapoptotic protein HSP27 in the breast cancer cell line MCF7 [13]. In this work, we further study the effects of MIAs over HSP27 using this cell line as a model. We have previously demonstrated that estradiol enhances vincristine-induced apoptosis [16]. Therefore, in order to avoid hormonal interferences, we performed our experiments in estrogen-depleted conditions.

Thus, using 2D-PAGE immunoblotting we demonstrate that vincristine induces the phosphorylation of HSP27 at residues serine 15, serine 78 and serine 82. Furthermore, we determine that vincristine induced phosphorylation of HSP27 is inhibited by the p38 MAPK specific inhibitor SB203580 [17] which indicates that these phosphorylations are catalyzed by p38 or a downstream kinase. This is also supported by the fact that vincristine induces the phosphorylation of p38 MAPK. Moreover, our results show that vincristine-induced phosphorylation of HSP27 at serine 78 is not inhibited by the JNK inhibitor SP600125 [18] while this compound inhibits the vincristine-induced phosphorylation of the JNK substrate c-Jun. This strongly supports the idea that JNK is not implicated in vincristine-dependent HSP27 phosphorylation.

We have observed that of all MIAs tested, only the VAs vincristine and vinblastine induced the phosphorylation of HSP27 at serines 15 and 82. Serine 78, it is also phosphorylated by paclitaxel but to a lesser extent. This event can be explained by the fact that paclitaxel has been also described as a p38 activator [19]. We have determined that both types of compounds induced similar cell death ratios, suggesting that the low effect of paclitaxel over HSP27 phosphorylation cannot be attributed to a lack of activity of the drug, caused by ATP-dependent efflux pumps extrusion or any other mechanism of resistance.

We have also observed that a 10-fold higher concentration pretreatment with paclitaxel blocks vincristine-induced phosphorylation of HSP27 at serine 78. This inhibition clearly indicates that the vincristine-induced signalling pathway that causes HSP27 phosphorylation at serine 78 is not fully overlapping with the one induced by paclitaxel. Since both compounds have opposite effects over the tubulin polymerization status, it is also possible that the balance between polymerised and depolymerised tubulin could be implicated in the modulation of these signals.

In summary, we demonstrate that vincristine not only induces the phosphorylation of HSP27 at serine 82 but also regulates the phosphorylation of serines 78 and 15. These HSP27 phosphorylations are regulated by the p38 MAPK signalling pathway, while JNK appears not to be implicated in these modifications. Finally, we observe a differential regulation of HSP27 phosphorylation between vincristine and paclitaxel. Since HSP27 is implicated in cell survival and migration, these findings could be important for understanding the efficacy of chemotherapy treatments.

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