

HSP70 inhibition by 2-phenylethanesulfonamide induces lysosomal cathepsin D release and immunogenic cell death in primary effusion lymphoma

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Heat-shock protein (HSP) 70 is aberrantly expressed in different malignancies and has a cancer-specific cell-protective effect. As such, it has emerged as a promising target for anticancer therapy. In this study, the effect of the HSP70-specific inhibitor (PES), also Pifitrin- μ , on primary effusion lymphoma (PEL) cell viability was analyzed. PES treatment induced a dose- and time-dependent cytotoxic effect in BC3 and BCBL1 PEL cells by inducing lysosome membrane permeabilization, relocation of cathepsin D in the cytosol, Bid cleavage, mitochondrial depolarization with release and nuclear translocation of apoptosis-activating factor. The PES-induced cell death in PEL cells was characterized by the appearance of Annexin-V/propidium iodide double-positive cells from the early times of treatment, indicating the occurrence of an additional type of cell death other than apoptosis, which, accordingly, was not efficiently prevented by the pan-caspase inhibitor Z-VAD-fmk. Conversely, PES-induced cell death was robustly reduced by pepstatin A, which inhibits Bid and caspase 8 processing. In addition, PES was responsible for a block of the autophagic process in PEL cells. Finally, we found that PES-induced cell death has immunogenic potential being able to induce dendritic cell activation.

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Subject Category: Cancer

Heat-shock proteins (HSPs) are a family of proteins classified according to their molecular weight, with different intracellular locations and functions.¹ Cancer cells upregulate HSPs, in particular HSP90 and HSP70, to cope with their basal stress due to nutrient shortage, dysregulated growth and accumulating unfolded proteins.² These cells are highly dependent on HSPs to survive because of their role in preventing apoptosis, by helping the protein refolding and degradation. Particular emphasis has been given to HSP70,³ which comprises several isoforms, basally expressed or stress inducible.⁴ Elevated HSP70 expression in cancer cells of different origins seems to correlate with a faster disease progression⁵ and poor response to chemotherapy⁶ and its inhibition has been shown to be an effective strategy against cancer in animal models of colon cancer and melanoma.⁷ HSP70 depletion may reduce tumor growth *in vivo* also by activating the immune system.^{8,9} Recently, the small-molecule 2-phenylethanesulfonamide (PES), also called pifitrin- μ , has been identified as specific inhibitor of stress-inducible HSP70.¹⁰ PES induces caspase-independent cell death in cancer cells, by impairing lysosomal function and also by interfering with

cell survival pathways such as NF- κ B. Moreover, PES has been reported to impair all the main protein degradation pathways in cancer cells¹¹ and to be also effective in the killing of hematological cancer cells.¹² In some leukemia cell lines and leukemic blasts, it involves caspase activation while in others it acts in caspase-independent way, indicating that the mechanism underlying the PES-induced cell death is not unique and not completely defined yet. HSP70 is involved in maintaining the lysosome membrane integrity,^{13,14} frequently compromised in stress-induced cancer cell death.¹⁵ The treatment with recombinant HSP70 has been indeed shown to decrease the lysosome instability of cells derived from patients with lysosomal disorders such as Neiman Pick disease¹⁶ and in tumor cells, promoting cell survival.¹³ Lysosome destabilization can lead to necrotic or autophagic cell death and when the lysosomal disruption leads to a partial release of the lysosomal content in the cell cytoplasm, it can trigger apoptosis or necroptosis through the activation of mitochondrial pathway.¹⁷ The connection seems to be mediated by the processing of Bid to active tBid that, directly or indirectly, can cause mitochondrial permeabilization with

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Abbreviations: AIF, apoptosis-activating factor; AO, acridine orange; BORT, Bortezomib; DC, dendritic cell; HSP70, heat-shock protein 70; KSHV, Kaposi's sarcoma-associated herpes virus (KSHV/HHV-8); LC3, microtubule-associated protein 1 light chain 3; LMP, lysosome membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization; p62, sequestosome 1/SQSTM1; PCD, programmed cell death; PEL, primary effusion lymphoma; PES, 2-phenylethanesulfonamide or pifitrin- μ ; PI, propidium iodide; TMRE, tetramethyl-rhodamine-ethyl-ester; z-VAD, z-VAD.fmk

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release of molecules that in turn can mediate caspase-dependent¹⁸ or -independent cell death.¹⁹

Primary effusion lymphoma (PEL) is an aggressive B-cell lymphoma associated with Kaposi's sarcoma herpes virus (KSHV), characterized by poor prognosis.²⁰ We investigated the effect of HSP70 inhibition by PES in BC3 and BCBL1 PEL cell lines and found that PES induces lysosome membrane permeabilization (LMP), relocation of cathepsin D in the cytosol, Bid cleavage, mitochondrial depolarization with release and nuclear translocation of apoptosis-activating factor (AIF) that ended in programmed cell death (PCD). Cytosolic localization of cathepsin D has been shown to trigger mitochondrial outer-membrane permeabilization and caspase-dependent apoptosis when microinjected in fibroblasts.²¹ The lysosome permeabilization has been previously described for other drugs (granulysin) or for HSP70 inhibition with different strategies⁸ and to the best of our knowledge this is the first report describing such a mechanism for HSP70 inhibition by PES.

We then analyzed the impact of PES treatment on the autophagic process, which requires functional lysosomes for the degradation of the autophagic cargo.²² A basal autophagy occurs in PEL cells and it is a common feature of tumor cells,²³ which allows them to survive in stressful conditions. We found that PES treatment altered the expression of the LC3 (microtubule-associated protein 1 light chain 3) and p62 (sequestosome 1/SQSTM1) autophagic markers in PEL cells, indicating that it interferes with the autophagic process of these cells. Finally, we asked whether PES-induced cell death in PEL cells would have immunogenic potential and found that it was able to induce dendritic cells (DCs) activation.

Results

PES-induced loss of viability in PEL cells is dose and time dependent. BC3 and BCBL1 PEL cells were first exposed to different PES concentrations, ranging from 10 to 30 μ M, for 24 h. Cell viability assay indicated that PES induced a dose-dependent loss of viability in BC3 and BCBL1 PEL cell lines (Figure 1a). A time-kinetic investigation showed that PES treatment (20 μ M) increased cell death between 12 and 48 h in both cell lines (Figure 1b). BC3 cells showed a higher sensitivity to PES compared with BCBL1 cells and more importantly we found that PES treatment had much lower effect on human B cells isolated from healthy donors, even at the highest dose used (30 μ M; Figure 1c). The different sensitivity to the PES treatment may correlate with the level of HSP70 expression (Figure 1d), suggesting that HSP70 inhibition has specific pro-death effect on tumor cells, likely depending on their high HSP70 expression in comparison to normal B cells, previously reported also by other authors.⁴

Features of PES-induced cell death in PEL cells. We first evaluated the characteristics of PES-induced cell death by electron microscopic (EM) analysis. BC3 and BCBL1 cell lines displayed an incomplete chromatin condensation following treatment with the HSP70 inhibitor (20 μ M for 24 h), which was not visible in the control cells (Figure 2a).

To further investigate the mechanism of PES-induced cell death, we evaluated the AnnexinV- and propidium iodide (PI)-positive cells by flow cytometry. As shown in Figure 2b, PES induced the appearance of double stained AnnexinV/PI cells since the early time points (3 and 6 h), suggesting the occurrence of a type of cell death other than apoptosis, as in the latter AnnexinV-single-positive cells are observed at early times. According to that z-VAD.fmk (1 h-pretreatment at 100 μ M) was not able to prevent PES-induced cell death in BCBL1 and very slightly prevented it in BC3 cells (Figure 2b) in which a faint cleaved form of caspase 3, not detectable in BCBL1 cells, was observed (Figure 2c).

All together these results suggest that PES induces mainly a caspase-independent PCD in PEL cells characterized by cells displaying incomplete chromatin condensation and double AnnexinV/PI positivity at the early times of treatment, which resembles the previously described necroptotic¹⁹ or apoptosis-like cell death.²⁴

PES induced lysosome permeabilization and cathepsin D release from lysosomes. As HSP70 is known to be involved in the lysosome membrane stabilization,¹⁶ we aimed at investigating if HSP70 inhibition by PES would determine lysosome permeabilization in PEL cells and release of the lysosomal content in the cell cytosol. We first stained untreated or PES-treated PEL cells with acridine orange (AO), which accumulates in the lysosomes and stains red in the acid compartment of the intact lysosomes, while it gives a green staining of the other less acidic compartments, including lysosomes, when their pH increases, as for lysosomal membrane damage.¹⁶ As shown in Figure 3a, untreated BC3 PEL cells showed red fluorescence of the lysosomes, whereas PES treatment (20 μ M for 24 h) turned fluorescence completely green. Similar results were obtained for BCBL1 cells (data not shown). The staining with PE-conjugated antibody specific for cathepsin D, an aspartic lysosome protease with several functions,²⁵ showed that in control cells cathepsin D was normally localized into the lysosomes, where the immunofluorescence staining appeared punctate (Figure 3b-control); after PES treatment, the immunofluorescence became diffuse, suggesting that cathepsin D was relocated in the cytosol (Figure 3b-PES), in line with the concept of LMP. These results indicate that PES caused a lysosome membrane damage in PEL cells.

PES induces Bid cleavage, mitochondrial outer membrane permeabilization (MOMP) and AIF nuclear localization. We next investigated if lysosome permeabilization and cathepsin D release induced by PES would mediate Bid cleavage, as reported for lysosomal proteases relocated in the cytosol after Granulysin exposure¹⁹ and found that PES treatment caused Bid full-length decrease in BC3 cells and to a lesser extent in BCBL1 cells (Figure 4a). The cleaved products of Bid were undetectable probably due to its low concentration or to the Ab sensitivity, as reported by other authors.¹⁹ As Bid can also be cleaved by caspase 8, which in turn can be activated by the lysosomal proteases released in the cytosol,²⁶ we found that Bid cleavage was partially reverted by z-VAD.fmk treatment (Figure 4a). Cleaved Bid can, directly or indirectly, induce mitochondrial

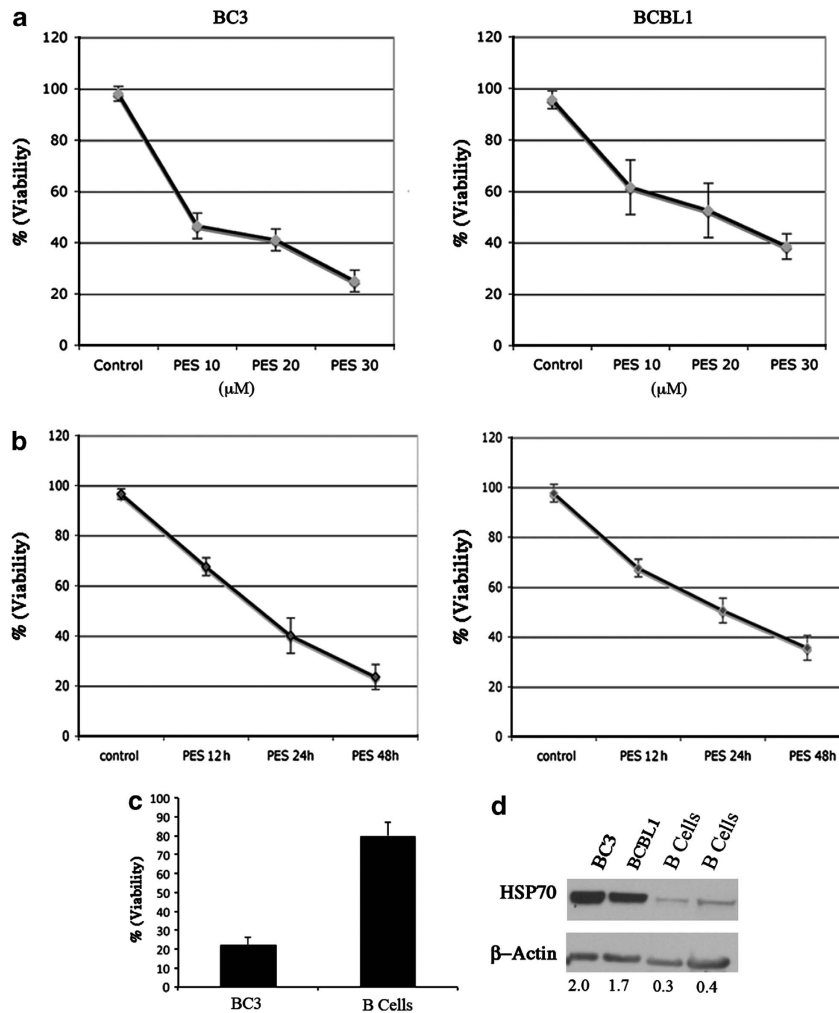


Figure 1 PES-induced loss of viability in PEL cell lines is dose and time dependent. (a) BC3 and BCBL1 were treated for 24 h with different PES concentrations (10, 20 and 30 μ M) or (b) for different times (12, 24 and 48 h) with 20 μ M of PES. Cells were counted by trypan-blue exclusion and mean \pm S.D. of three different experiments is reported. (c) Comparison of PES sensitivity between PEL cells (BC3) vs normal human B cells isolated from two different healthy donors. Cells were counted by trypan-blue exclusion and mean \pm S.D. of three different experiments is reported. (d) BC3, BCBL1 and normal B cell (from two different healthy donors) lysates were used to perform western blot analysis to evaluate the HSP70 protein expression. A β -actin antibody was used as protein-loading control. The numbers indicate the ratio HSP70/ β -actin

dysfunction^{27,28} and therefore we next investigated the mitochondrial involvement in PES-induced cell death by flow cytometric evaluation of tetramethyl-rhodamine-ethyl-ester (TMRE), a fluorescent probe that specifically responds to membrane potential. In both cell lines, a decrease of TMRE fluorescence occurred after PES treatment, indicating an outer mitochondrial depolarization (MOMP; Figure 4b). MOMP can cause a caspase-dependent¹⁸ or a caspase-independent cell death through AIF release and its nuclear translocation.¹⁹ As PES-induced cell death in PEL cells seems to be caspase-independent, according to the above-presented results, we finally investigated the subcellular localization of AIF. Western blot analysis on nuclear fraction of PEL cells, with a specific anti-AIF antibody, indicated that PES treatment induced AIF translocation into the nuclei (Figure 4c), suggesting that AIF can be responsible for the chromatin condensation observed by EM and the caspase-independent PCD. It has been reported that AIF binds to HSP70²⁹ and it can be released and nuclear-translocated also as consequence of HSP70 inhibition, mediated by PES.

Inhibition of cathepsin D counteracts PES-induced cell death. To evaluate the role played by the cathepsin D release in the cell death, we pretreated PEL cells with the aspartic protease inhibitor pepstatin A (20 μ M) for 30 min before PES exposure. We found that pepstatin A strongly reduced PES cytotoxic effect compared with the single PES treatment, whereas pepstatin A alone, at the same concentration, did not have any effect (Figure 5a). Concomitantly, we found that pepstatin A strongly reduced Bid processing in both PEL cell lines (Figure 5b). As cathepsin D can also mediate caspase 8 cleavage, which in turn can participate to the processing of Bid, we found that pepstatin A also reduced caspase 8 cleavage (Figure 5b). These results suggest that release of the lysosomal content in the cytoplasm and the direct or caspase 8-mediated Bid processing may be the main underlying mechanism of PES-induced PEL cell death.

PES sensitizes PEL cells to the bortezomib (BORT) killing. We have previously reported that treatment with the proteasome inhibitor BORT increases HSP70 expression in

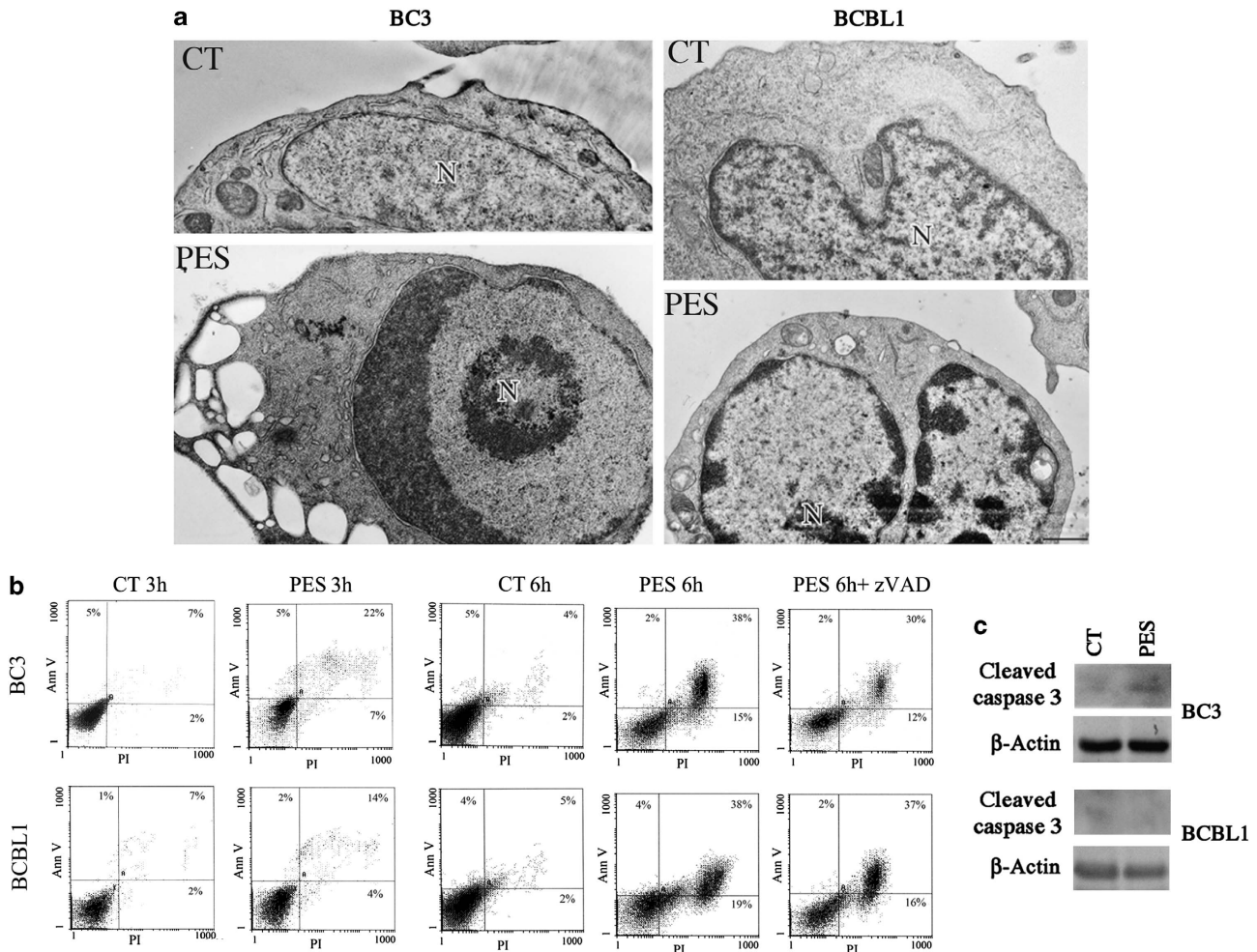


Figure 2 Features of PES-induced cell death. (a) BC3 and BCBL1 cells untreated or PES-treated ($20 \mu\text{M}$) were analyzed by electron microscopy after 24 h of treatment (Bar $1 \mu\text{m}$; N, nucleus). (b) PEL cells were double stained with AnnexinV/PI and analyzed by FACS analysis after 3 or 6 h of PES treatment with or without z-VAD.fmk pre-treatment. (c) Caspase 3 cleavage in untreated or PES-treated BC3 and BCBL1 cells. A representative experiment out of three is shown

BC3 and BCBL1 PEL cells.³⁰ High expression of HSP70 in cancer cells correlates with the sensitivity to its inhibition and based on this knowledge, we investigated if BORT could render PEL cells more sensitive to PES treatment. As reported in Figure 6 the combination of PES, used at suboptimal concentrations ($10 \mu\text{M}$ instead of $20 \mu\text{M}$), with BORT (10 nM) showed an increased cytotoxic effect against PEL cells. These results suggest that such a combination could be exploited for the therapy of this B-cell lymphoma and that PES could be probably combined also with other chemotherapeutic drugs that induce HSP70 upregulation, as a cell self-protective mechanism in response to stressful conditions.

An autophagic block occurred in PEL cells upon PES treatment. The lysosome membrane destabilization can lead to an increase of their pH and as a consequence to a block of autophagy.³¹ To find out the impact of PES treatment on PEL cell autophagy, we first analyzed by western blot the LC3 autophagic marker. An accumulation of the lipidated form of LC3 (LC3-II), which is formed and

degraded during the autophagic process, was observed in both PES-treated PEL cell lines (Figure 7). Hence, to clarify if the increased level of LC3-II was due to an increase of autophagy induction and/or to a block of the autophagic flux, we analyzed p62 protein, which is a read-out of a bona fide autophagic process. As shown in Figure 7, a accumulation of p62 was observed, suggesting that an autophagic block was likely occurring in these cells, upon PES treatment.

PES-induced cell death has immunogenic potential. The killing of tumor cells by chemotherapy can lead to a complete tumor eradication only if is able to activate also the immune system response. As HSP70 inhibition has been previously reported to activate the immune response *in vivo* in animal model,¹⁶ here we investigated whether PES-treated PEL cells would be able to induce DC activation. At this aim, we cocultured untreated or PES-treated BC3 and BCBL1 cells with immature DC and after 24 h light microscopy observations showed that DC cultured with PES-treated PEL cells appeared morphologically more differentiated (spindle-shaped cells) compared with the DC cocultured for the same

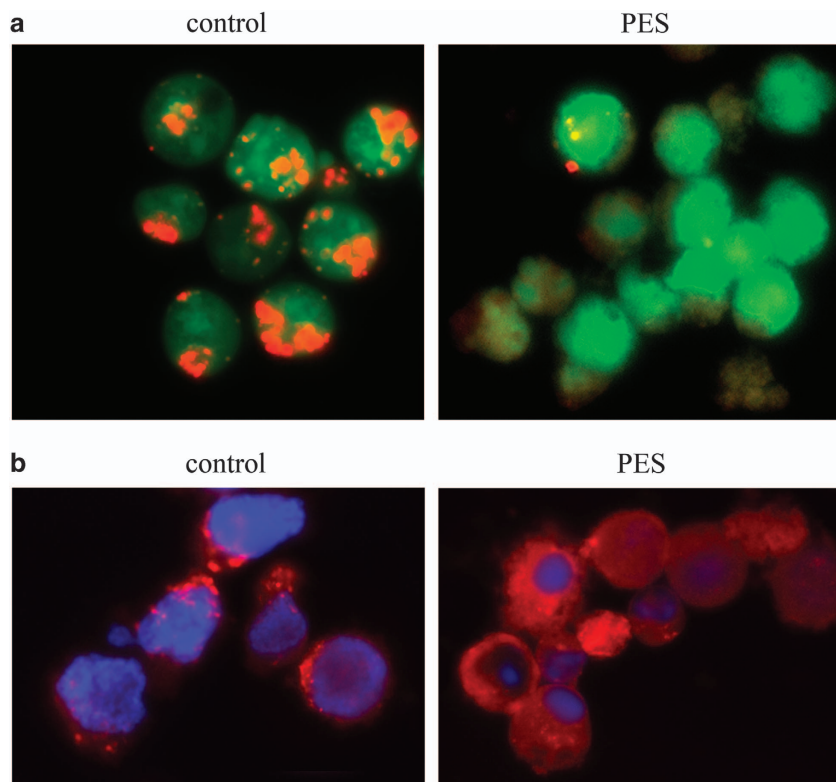


Figure 3 PES causes lysosome permeabilization in PEL cells. (a) BC3 cells untreated or PES-treated (20 μ M for 24 h) were stained with acridine orange (AO) or (b) with anti-cathepsin D monoclonal antibody. A representative experiment out of three is shown and similar results were obtained for BCBL1

time with mock-treated cells (Figure 8). According to that we found an upregulation of the Ag-presenting marker CD86 in the DC cocultured with PES-treated PEL cells, as indicated by FACS analysis (Figure 8). Altogether, these results indicate that HSP70 inhibition by PES induces an efficient cell death in PEL cells with immunogenic potential in terms of DC activation. This finding is particularly important because activated DC might, in turn, potentiate the killing tumor cells mediated by PES, by initiating a tumor-specific T-cell response, which can be investigated in further studies.

Discussion

In the search of new therapeutic approaches to selectively kill cancer cells, HSPs have emerged as suitable targets, being selectively expressed or upregulated in many tumor cells and allowing them to survive in stressful conditions.³² HSP high expression can be considered a negative prognostic marker, as it correlates with worse cancer prognosis, also by preventing chemotherapy-mediated apoptosis. PEL is a non-Hodgkin's lymphoma characterized by lymphomatous effusions of pleural, pericardial and abdominal cavities and by a poor response to conventional chemotherapy.²⁰ Given its association with KSHV and based on the evidence that an essential viral protein, LANA, is a client of HSP90, new therapeutic approaches have indicated that inhibiting this HSP can effectively suppress PEL survival.³³ The elevated HSP70 expression in BC3 and BCBL1 PEL cell lines also suggests that this molecule might be a suitable therapeutical

target. Hence, we found that HSP70 inhibition by the specific inhibitor PES, a recently identified¹⁰ molecule able to kill cancer cells, by impairing their protein clearance pathways,¹¹ induced an efficient cell death in PEL cells. Targeting HSP70 has been shown to affect tumor cell survival in several ways³⁴ because of its important role in protein refolding³⁵ and in the lysosomal membrane stabilization.¹⁶ HSP70 inhibition by adenovirus-mediated transfer of antisense cDNA⁸ can induce LMP. LMP, depending on the complete or partial release of the lysosomal content, can lead to different outcome in the modalities of cell death induction.¹⁹ Although PES has been shown to induce apoptosis in acute leukemia cells,¹² here we found that PES-induced cell death in PEL cells was slightly affected by caspase-inhibitor z-VAD.fmk. This suggests that cells were not undergoing apoptosis but rather a different kind of PCD, apoptosis-like or necroptosis, evidenced by the double AnnexinV/PI-stained cells at early times of treatment, observed by FACS analysis and by the incomplete chromatin condensation observed by EM analysis. The main underlying mechanism of PES-induced PEL cell death was a detrimental lysosome membrane damage, which leads to cathepsin D release into the cytosol that mediated Bid cleavage, mitochondrial depolarization and apoptosis-inducing factor (AIF) release. The most abundant lysosomal proteases released as consequence of LMP are cathepsins and some of them, including cathepsin D, B and L, remain active at neutral pH and can activate effectors that, in turn, mediate caspase-dependent or -independent cell death, involving the mitochondria.¹⁹ LMP induction is emerged as an alternative

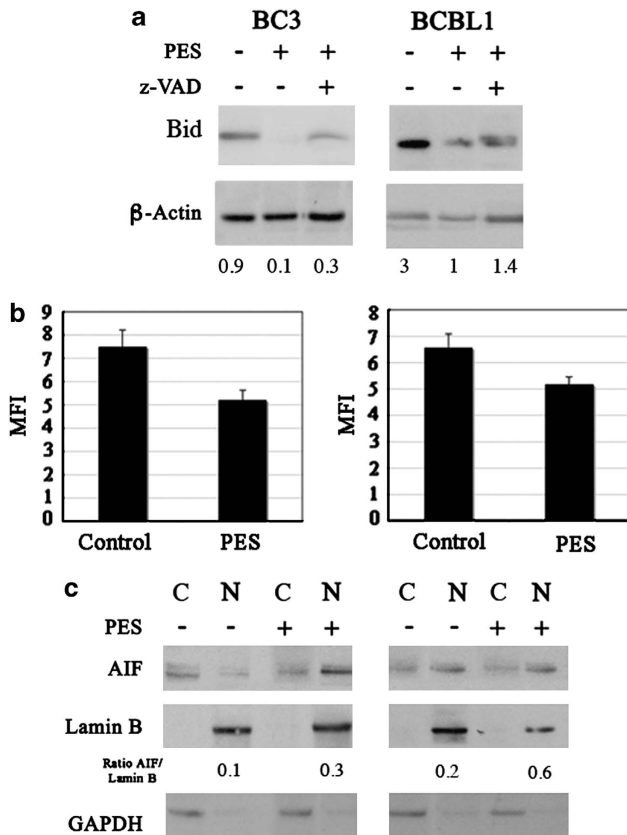


Figure 4 BID processing, mitochondrial depolarization and AIF nuclear translocation in PES-treated PEL cells. (a) BID and LAMP2 expression in BC3 and BCBL1 untreated or treated with PES was analyzed by western blot. β -Actin was used as loading control. (b) MOMP assay was performed by using 40 nM TMRE for 15 min at 37 °C on PEL cells untreated or treated with PES and mean \pm S.D. is reported. (c) Western blot analysis for AIF, Lamin B and GAPDH was performed on cytosolic (C) and nuclear (N) fractions of control PEL cells or after treatment with PES. For all these experiments PES was used at 20 μ M for 24 h

mechanism of cell death, particularly interesting because it can affect also tumor cells bearing gene mutations that prevent the classic apoptotic cell death.¹⁷ Thus, although BC3 and BCBL1 PEL cell lines harbor, respectively, phosphatase and tensin homolog and monoallelic p53 mutation,³⁶ PES treatment was able to lead them to a PCD. The role played by cathepsin D relocation in the cytosol was highlighted by the observation that its inhibition with pepstatin A strongly reduced the PES cytotoxic effect and Bid cleavage. To the best of our knowledge, this is the first report showing that PES induces cancer cell death through this mechanism. More importantly the pro-death effect of PES was found to be more specific for the lymphoma cells compared with normal PBL, strongly encouraging the use of such molecule *in vivo*.

A block in the autophagic process, evidenced by the accumulation of LC3-I, LC3-II and p62, essential autophagy markers,³⁷ was induced by PES. Autophagy is a self-digestive process that ensures the lysosomal degradation of superfluous or damaged organelles and misfolded proteins and in cancer it may act as a pro-survival mechanism.²³ Therefore, the pharmacological and/or genetic inhibition of autophagy

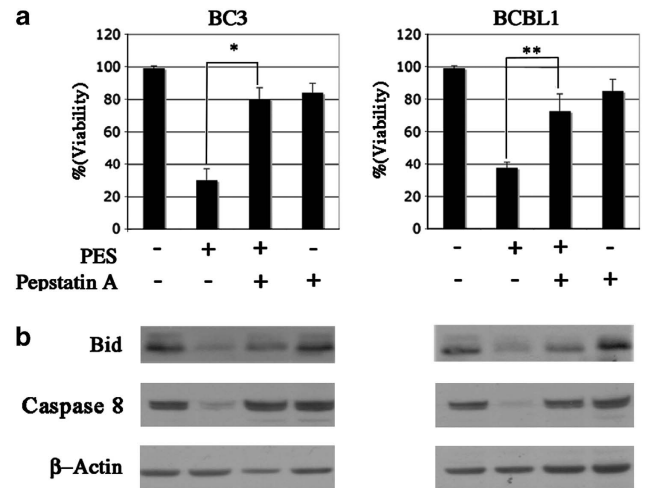


Figure 5 PES-induced cell death, BID processing and caspase 8 cleavage are reduced by pepstatin A. (a) BC3 and BCBL1 mock-treated or treated with PES (20 μ M for 24 h) in the presence or absence of pepstatin A (20 μ M). Cells were counted by trypan-blue exclusion and the mean \pm S.D. is reported **P*-value = 0.02, ***P*-value = 0.02. (b) Western blot was also performed concomitantly with the treatments reported above to analyze the processing of Bid and caspase 8. β -Actin was used as loading control and a representative experiment out of three is shown

was recently shown to sensitize cancer cells to the lethal effect of various cancer therapies, suggesting that the suppression of the autophagic pathways may represent a valuable sensitizing strategy for cancer therapy.²³ Hence, PES-induced autophagic block could contribute to the PEL cell death together with the effects of cathepsin D release in the cell cytoplasm. The PES-induced autophagic block is in line with the finding that the lysosomal membrane destabilization can lead to an increase of their pH and, as a consequence, to a block of the basal autophagy.³¹ We previously showed that HSP70 is upregulated in PEL cells, upon BORT treatment³⁰ and as previous studies reported that higher HSP70 expression renders cancer cells more sensitive to its inhibition, we found that the cytotoxic effect of PES in PEL cells was increased by its combination with BORT. Interestingly, we recently found that autophagy is activated by BORT in PEL cells as a survival mechanism (M.C. unpublished data) and here we show that PES is able to block the autophagic process in these cells. When autophagy is activated by a chemotherapeutic drug and helps the cells to survive, its blockage can increase its cytotoxic effect, and this could be another explanation for the synergistic effect of BORT and PES observed in PEL cells.

The killing of tumor cells can lead to a complete tumor eradication only if it is able to activate also the immune system and in particular DC response.³⁸ DC are powerful antigen-presenting cells and as such have a crucial role in initiating a specific immune response. DC, in their immature state, are highly specialized in antigen capture and processing and after maturation they also upregulate co-stimulatory molecules (ie, CD80 and CD86) and activate T lymphocytes. As molecules released in the tumor microenvironment can lead to DC dysfunction and tumor progression,^{39–44} it is important that anticancer drugs kill tumor cells through an immunogenic cell death^{30,38,45} able to counteract DC dysfunction.

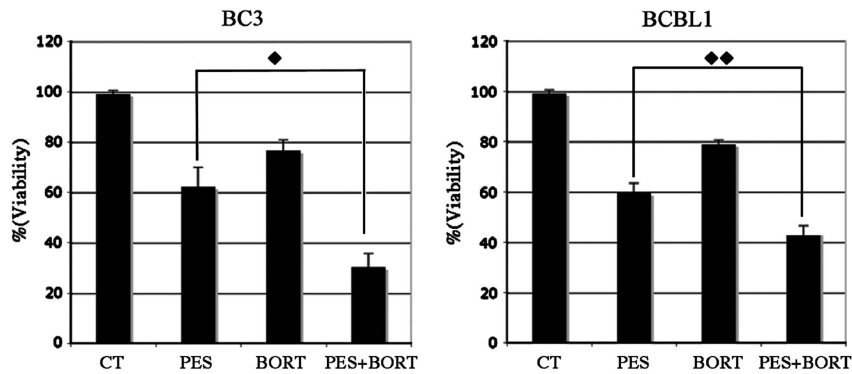


Figure 6 PES synergizes with bortezomib in the induction of PEL cell death. BC3 and BCBL1 were treated with bortezomib (10 nM), PES (10 μ M) or combination of both for 16 h and viability was assessed by trypan-blue exclusion. Mean \pm S.D. and *P*-value are also reported *P*-value = 0.01, *P*-value = 0.05

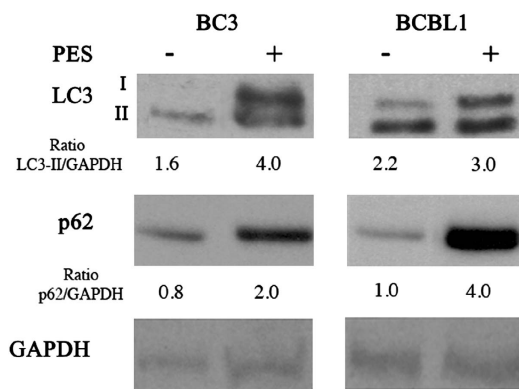


Figure 7 Autophagic process is blocked by PES treatment in PEL cells. BC3 and BCBL1 untreated or PES-treated were analyzed by western blot for the expression of LC3-I/II and p62. GAPDH was used as loading control and the ratios LC3-II/GAPDH and p62/GAPDH were also reported. One representative experiment out of three is shown

HSP70 inhibition has been shown in some studies to activate the immune system leading to *in vivo* tumor regression.⁹ In agreement, here we show that HSP70 inhibition by PES triggered PEL cell death that was able to activate DC, therefore, it was an immunogenic type of cell death that will be interesting to study further. In conclusion, our data underscore the use of HSP70 inhibitor PES as a new therapeutic strategy against PEL that could be exploited also because of its immunogenic potential.

Materials and Methods

Cells and reagents. The BC3 and BCBL1 PEL cell lines (ATCC) were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA; cat. no. R0883) 10% fetal calf serum (Euroclone, Milan, Italy; cat. no. ECLS0180L), glutamine (300 μ g/ml) and streptomycin (100 μ g/ml) and penicillin (100 U/ml; Gibco, Carlsbad, CA, USA; cat. no. 10378-016) in 5% CO₂ at 37 °C. B lymphocytes were isolated by Fycoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden;) from buffy coats and positively selected using anti-CD19 MAb-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA; cat. no. 130-050-301). The proteasome inhibitor BORT was purchased from Millennium Pharmaceutical Inc (Cambridge, MA, USA). The heat-shock protein (HSP)70 inhibitor PES (Calbiochem, San Diego, CA, USA; cat. no. 506155), the aspartic protease inhibitor pepstatin A (Santa Cruz Biotechnology, Heidelberg, Germany; cat. no. sc-45036) and the pan-caspase inhibitor z-VAD.fmk were purchased from Calbiochem (San Diego, CA, USA; cat. no. 219011).

Cell viability. BC3 and BCBL1 were plated in 12-well plates in complete medium at a density of 5×10^5 cells/ml and treated with PES (10–30 μ M; Calbiochem; cat. no. 506155), BORT (10 nM; Millennium Pharmaceutical), pepstatin A (20 μ M; Santa Cruz Biotechnology; cat. no. sc-45036) alone or in combination for the indicated time or with DMSO, as control. After treatment, cells were collected and counted by trypan-blue exclusion. Viability was assessed by calculating alive (trypan-blue excluding) cells as percentage of all cells. Each experiment was performed in triplicate.

EM analysis. Cells were fixed in 2% glutaraldehyde in PBS for 24 h at 4 °C, post-fixed in 1% OsO₄ for 2 h and stained for 1 h in 1% aqueous uranyl-acetate. The samples were then dehydrated with graded acetones and embedded in Epon-812 (Electron Microscopy Science, Società Italiana Chimici, Rome, Italy). One-micrometer thick sections were cut, stained with 1% methylene blue and viewed by light microscopy to select representative areas. Ultrathin sections were cut with a Reichert ultramicrotome, counterstained with uranyl-acetate and lead citrate, and examined with a Philips CM10 transmission electron microscope (FEI, Eindhoven, the Netherlands).

Cell death assay. The effect of PES after 3 or 6 h of treatment with or without z-VAD.fmk (Calbiochem, San Diego, CA, USA; cat. no. 219011) was examined. The cells were washed with ice-cold phosphate-buffered saline (PBS; Sigma; cat. no. D8537), resuspended in Annexin V-binding buffer, and subsequently stained with AnnexinV-fluorescein isothiocyanate and PI (BD Pharmingen, San Jose, CA, USA), according to the manufacturer's recommendation (Società Chimici Italiani; cat. no. IK-11120). The cells were analyzed in a cytofluorimeter (EPICS XL, Beckman Coulter, Brea, CA, USA). A minimum of 10 000 events were examined for each sample.

Determination of mitochondrial membrane integrity. TMRE (Life Technologies, Molecular Probes, Carlsbad, CA, USA; cat. no. T669) is a voltage-sensitive fluorescent indicator for mitochondrial transmembrane potential. At the end of the treatment period, unfixed cells were incubated with 40 nM TMRE in fresh medium for 15 min at 37 °C, in order to allow the dye to equilibrate between cytosolic and mitochondrial compartments, and then washed in fresh medium. The cells were analyzed in a cytofluorimeter (EPICS XL, Beckman Coulter). The gate used for treated cells was the same as in control cells. The red fluorescence intensity of the gated cells was analyzed on a log scale (FL2) and recorded as mean fluorescence intensity. A minimum of 10 000 events were examined for each sample.

Western blot analysis. Cells (5×10^5) were lysed in a modified RIPA buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 8), 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100, protease and phosphatase inhibitors. The lysates were prepared according to the manufacturer's instructions (Life Technologies), subjected to electrophoresis on 4–12% NuPage Bis-Tris gels (Life Technologies; cod. no. NO0322BOX) and transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA; cat. no. IPVH00010). The membranes were blocked for 1 h in a PBS (Sigma; cat. no. D8537), blocking solution, containing 3% bovine serum albumine (Sigma; cat. no. A4503), 0.1% Tween-20

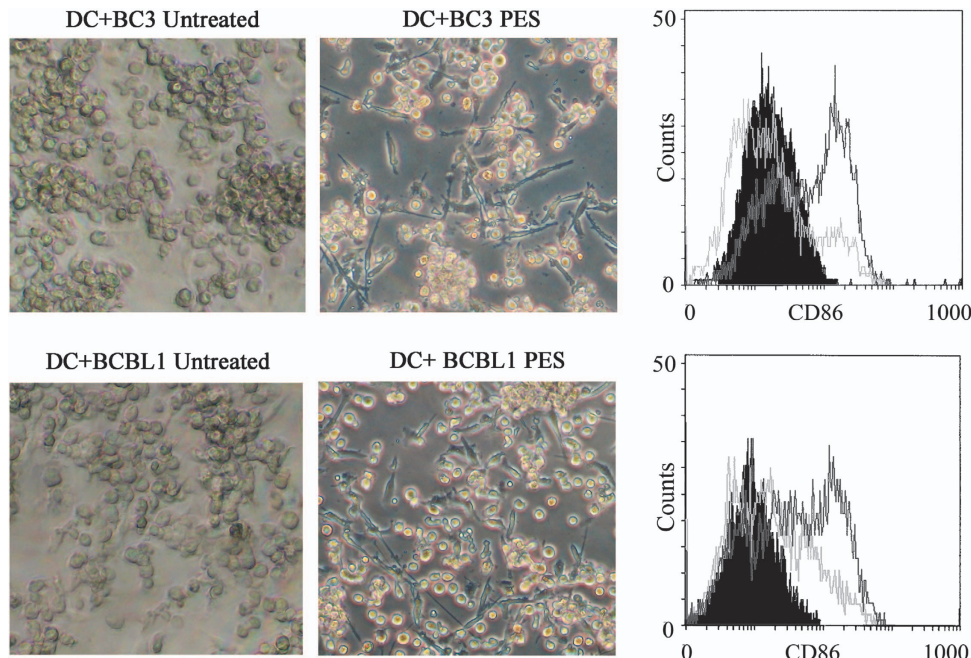


Figure 8 DC activation by PES-treated PEL cells. BC3 and BCBL1 cells mock treated or PES treated (20 μ M for 24 h) were cocultured with immature DC for 24 h. DC morphology and CD86 expression, analyzed by FACS analysis, is shown. Filled histograms represent the isotype control, gray empty histograms represent CD86 expression of DC cocultured with mock-treated PEL cells, whereas black empty histograms represent CD86 expression of DC cocultured with PES-treated PEL cells. A representative experiment out of three is reported

and then incubated with a primary antibody overnight at 4 °C. The membranes were washed 5 min for three times in the washing solution (PBS and 0.1% Tween 20) and incubated for 45 min with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology; cat. no. sc-2004, sc-2768, sc-2005). The membranes were washed as described before and the blots were developed using ECL Blotting Substrate (Thermo Scientific, Rockford, IL, USA; cat. no. 32209).

Antibodies. In this work, the following antibodies were used: rabbit polyclonal anti-LC3 (Novus Biologicals, Cambridge, UK; cat. no. NB100-2220SS), mouse monoclonal anti-p62 (BD Transduction Laboratories, San Jose, CA, USA; cat. no. 610833), goat polyclonal anti-lamin B (Santa Cruz Biotechnology, Europe; cat. no. sc-6216), mouse monoclonal anti-caspase8 (Cell Signaling, Boston, MA, USA; cat. no. 4927), goat polyclonal anti-caspase 3 (Santa Cruz Biotechnology; cat. no. sc-1225), mouse monoclonal anti-HSP70 (Santa Cruz Biotechnology; cat. no. sc-66049), rabbit polyclonal anti-BID (Cell Signaling; cat. no. 2002), rabbit polyclonal anti-AIF (Santa Cruz Biotechnology; cat. no. sc-5586), goat polyclonal anti-cathepsin D (Santa Cruz Biotechnology; cat. no. sc-6487), mouse monoclonal anti-CD86 (BD Pharmingen; cat. no. 558703), mouse monoclonal anti- β -actin (Sigma; cat. no. A2228) and mouse monoclonal anti-GAPDH (Santa Cruz Biotechnology; cat. no. sc-137179).

AO staining. Untreated or PES-treated PEL cells were stained with AO at 100 μ g/ml for 30 min at RT, as previously described.⁴⁶ Cells were analyzed using an Apotome Axio Observer Z1 inverted microscope (Zeiss, Oberkochen, Germany), equipped with an AxioCam MRM Rev.3 camera at 40 \times magnification.

Immunofluorescence. Untreated or treated PEL cells, as described above, were fixed with ice-cold methanol for 10 min, and to investigate cathepsin D localization, the cells were labeled with an anti-cathepsin D antibody (Santa Cruz Biotechnology; cat. no. sc-6487). Cells were analyzed using an Apotome Axio Observer Z1 inverted microscope (Zeiss), equipped with an AxioCam MRM Rev.3 at 40 \times magnification

Generation of monocyte-derived DC. To generate monocyte-derived DC, human peripheral blood mononuclear cells were isolated by Fycol-Paque

gradient centrifugation (Pharmacia) from buffy coats. CD14⁺ monocytes were positively selected using anti-CD14 MAb-conjugated magnetic microbeads (Miltenyi Biotec; cat. no. 130-050-201), as previously reported.⁴⁷ Purified monocytes were cultured at a density of 1×10^6 cells/3 ml in 12-well plates for 6 days in RPMI 1640 (Sigma; cat. no. R0883) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 mg/ml streptomycin, 50 mM 2-mercaptoethanol (Sigma; cat. no. M3148), and recombinant human granulocyte-macrophage colony-stimulating factor plus interleukin 4 (50 and 20 ng/ml, respectively; Miltenyi Biotec; cat. no. 130-095-372 and cat. no. 130-093-917). Cytokines were replenished every other day by adding 20% fresh medium to each well.

Analysis of DC phenotype after coculture with tumor cells.

Tumor cells (BC3 and BCBL1) untreated or PES-treated for 24 h were cocultured for an additional 24 h with immature CD1a-positive DC. Live cocultures were analyzed by light microscopy and the phenotype of DC was monitored by staining with fluorescein isothiocyanate-conjugated anti-CD86 (BD Pharmingen; cat. no. 558703) for 30 min at 4 °C and analyzed on a flow cytometer. DCs were gated according to their FSC and SSC properties. Appropriate isotype controls were included and 5 000 viable DCs were acquired for each sample.

Statistical analysis. All experiments unless differently indicated were performed at least three times. All experimental results, unless differently indicated, were expressed as the arithmetic mean and standard deviation (S.D.) of measurements. Student's *t*-test was used for statistical significance of the differences between treatment groups. Statistical analysis was performed using analysis of variance at 5% ($P < 0.05$) or 1% ($P < 0.01$).

Ethics statement. The study was approved by the ethical Committee of Policlinico Umberto I, Sapienza University, Rome, Italy.

Conflict of Interest

The authors declare no conflict of interest.

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