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Diseases & Research

Anti-Cancer Agents Work in Antagonism with Inhibitors of HSP 72

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Purpose: The heat shock genes are evidenced to be active in the migration and are major promoters of several human malignancies. The inclined expression of HSP 72 proteins play an inevitable role in protecting cancer cells from apoptosis, promoting cellular progression, which is one of the factors for reduced sensitivity towards conventional therapies. This *in-vitro* study used Acute Myeloid Leukaemia (AML) cell lines U937 and Chronic Myeloid Leukaemia (CML) cell line K562 to evaluate the effect of Pifithrin chloride (PES-Cl) - an inhibitor of HSP 72 protein activity as single agent and in combination with chemotherapeutic Bortezomib in order to elucidate the activity of these molecules in cellular apoptosis.
Methods: The cells were treated for initial response with PES-CL for 1h and a potential enhancement with another chemotherapeutic Bortezomib other over a 24 h time course and wise verse. The cytotoxic effects of the treatment were analysed by MTS assay and the combination index for the drug combinations tested were analysed for synergy, additive or antagonism using Compusyn software (Compusyn, Inc). Apoptosis was evaluated by Annexin V/PI assay. HSP 72 and BCL-2 expressions were analyzed by flow-cytometer.
Results: The MTS cell viability assay depicted no sign of enhancement of cytotoxicity by either of the PES-Cl/ Bortezomib drug combinations tested, but reflected antagonistic effects in U937 and K562 cell lines, exhibiting a CI of >1, explicitly indicating antagonism. Although both cell lines were responsive and showed antagonism, U937 were chosen for further investigations. The U937 cells showed apoptosis and no necrosis was found at any level of the investigation by Annexin V/PI assay. The cells exhibited HSP 72 inhibition for single and combination drug treatments. The results noted a high BCL-2 expression in U937 cells with the drug combinations of PES-Cl and Bortezomib disregard of the mode of administration followed. However, the highest expression was observed in cells exposed to PES-Cl initially and subsequently with Bortezomib treatment.
Conclusion: This present study is the first to investigate the potential activity of PES-Cl an HSP 72 inhibitor in combination with Bortezomib in leukemia cancers. And it is interesting to note the antagonism exhibited by the drug combinations. However, prospective studies are critical in elucidating explicitly the mode of action of these agents, to potentially overcome resistance to canonic chemotherapy and improve the therapeutic potential of leukemic treatments.
Keywords: Apoptosis; HSP 72; Necrosis; Pifithrin chloride; Bortezomib.

Introduction

Heat shock chaperone (HSP) play a crucial role in cell protection[1]. In case of cancers, the cells stressed by the pathophysiological and oxidative stress events results in incredibly high expression of heat shock proteins[2]. The expressive status of HSPs shows their profound roles in promoting cancer survival and metastasis through their diverse functions in translocation of proteins, regulation of cellular receptors and also in maintaining the protein conformational status[3]. Cancer cells rely on HSP's for their sustenance, as these chaperones are greatly involved in the repair and cellular homeostasis, reactivating the functional status of oncoproteins thus promoting cancer cell survival. HSP's 40, 60, 90 have been found to be associated with the control of proteasomal degradation, a normal intracellular mechanism involved in degradation of unwanted proteins[4]. In addition, these molecular chaperons safeguard the cells from extracellular stress responses resulting from oxygen depletion, fluctuation in temperature or pH[5]. However, HSP levels are highly elevated and also secreation is increased to the extracellular environment[6].

HSP 72 is found to mediate cancer progression through regulation of Her2-oncogene directed cellular senescence mechanism in breast cancers[7]. HSP 72 over expression reports to halt the apoptosis and senescence signalling pathways in cancer cells. The survival and metastases of several malignancies are highly associated with HSP protein expression profiles. HSP 90 proteins facilitates tremendous changes and initiate stabilization of mutated oncoproteins in mammary cancers, in turn trans-activating growth stimulating pathways, thereby permitting the proliferation of breast cancers[8]. The counter protective role of HSP's not only hinders programmed cell death process, but also creates resistance to chemotherapeutic treatments. One of the unique targets of pharmacological agents in humans is the G-coupled protein receptors, and interestingly, HSP 90 protein is closely linked with the regulation of these signalling molecules[9]. So, by inhibiting the heat shock proteins in certain cancers, the self-defence mech-

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anism of cancer cells will be blocked, making them more responsive towards treatments.

The stress proteins HSP 70 and HSP 60 have been found to be effective markers for cancer detection at preliminary stages due to their high expression observed in the intestinal, colorectal and prostate cancers, similarly, HSP 110 are highly linked with the migration of non-Hodgkin's lymphoma, tumours of the skin and colon[10–12]. Pharmacological agents such as Tanespimycin, Alvespimycin and Retaspimycin that actively inhibit HSP 90 have shown promising treatment outcomes in the treatment of metastatic skin cancer, renal, head & neck and peritoneal cancers[13]. HSP inhibitor compounds despite their similar mode of action, may reflect differential cytotoxic response in combination with well-known chemotherapeutics, an *in-vitro* investigation using N- terminal HSP 90 inhibitors shows enhanced cytotoxic response with HSP inhibitor compound 17-DMAG in contrast to other inhibitors such as NVP-AUY922 or NVP- HSP 990 in enhancing the sensitivity of chemo agents like 5-fluorouracil, Irinotecan and oxaliplatin *in-vitro* in colorectal cancers[14]. However, these cancer therapeutics that selectively target HSP's are under clinical trial phases[15].

Currently practising cancer treatment modalities such as chemo agents and ionizing radiation that harm the cancer cells seems to induce the production of heat shock proteins, in turn compromising treatment efficacy. Hence, to overcome the induced activity of HSPs, application of chemotherapy with membrane fluidizing modalities are proved to be effective *in-vitro* in reducing intracellular HSPs, and promoting apoptosis, resulting in improved drug sensitivity in chronic lymphocytic leukemic cancers[16]. Similarly, HSP 90 inhibitors ICPD47 and ICPD62 were reported to show strong synergistic activity in combination with chemotherapeutics including Gemcitabine, 5 fluorouracil and Doxorubicin *in-vitro* in pancreatic cancers[17]. However, great caution is required in selection of HSP inhibitors in combination with chemotherapeutics as striking antagonistic scenario was observed with HSP 90 inhibitors and chemotherapeutics which target the proteasome in prostate cancers[18].

Bortezomib is a well-known chemotherapeutic agent, which works efficiently as a single agent, especially for multiple myeloma patients not formerly treated[19]. One of the crucial targets of Bortezomib is the NF-KB signaling path focusing the proteasome. This drug takes up the space which is allocated for the entry of deteriorated proteins to the proteasome, in turn hindering their entrance, resulting in cellular stress, directing to apoptosis[20].

A report showed inclined expression of HSP 72 proteins in inducing resistance towards chemotherapeutic Imatinib in chronic myeloid leukaemia[21]. Other studies also highlight the contrasting role of HSP 72, showing treatment to be more responsive with down regulation of HSP 72[22]. Interestingly, HSP 72 has shown an eminent role in regulating BCR-ABL activity in CML cases and prone to have strong bond in activating mutant oncoproteins including c-Kit or FLT3 which are studied to be associated with poor prognosis in acute myeloid leukemic patients[23]. Therefore HSP 72 inhibitor compounds and their effectiveness in combination with conventional therapeutics may pave way for improving the therapeutic potential of anti-cancer agents. AML and CML patients are prone to developing resistance to chemo agents, therefore, novel therapeutic combinations of HSP and proteasomal inhibitors may in turn override the chemotherapeutic resistance and may potentially be first line therapeutic option in future. And this is the first study to investigate the potential activity of PES-Cl an HSP 72 inhibitor in combination with Bortezomib in leukaemia cancers.

Materials and Methods

The human leukaemia cancer cell lines U937 and K562 (85011440) were purchased from the European culture collections. Chemotherapeutic Pifithrin chloride (PES-Cl) was obtained from Calbiochem, UK; Bortezomib from Selleck Chemicals, Germany. MTS reagent (4, 5-dimethylthiazol-2yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium from Promega, UK. FITC Annexin V and PI from BD Biosciences, UK. The antibodies HSP 72 FITC – Stressmarq, UK and Anti-mouse\rat Bcl2 FITC from Invitrogen, UK.

Cell Treatment and evaluation of cell viability

Cancer cell lines U937 and K562 maintained under standard cell culture conditions were pre-seeded onto 96 well assay plates and subsequently exposed to treatments using with PES-Cl for 1 h, followed by treatment with Bortezomib for 24 h. Similarly, cells were treated with Bortezomib for 1 h followed by PES-Cl for 24 h. PES-Cl and Bortezomib controls were allowed for 24 h treatment. Live controls were placed as reference. Cells following subsequent treatments were incubated at 37 °C for 24 h. Following incubation, cells were assessed for cell viability, apoptosis or necrosis accordingly.

MTS assay

U937 and K562 cell lines following treatments were tested for cell viability by MTS assay. 20 µL of (333 µG/mL MTS working solution) was added to 100 µL of cells in 96 well assay plates. The plates were then incubated at 37 °C, with 5% CO2 for 2 h. The absorbance was measured at 490 nm. Untreated cells were placed as positive control. A dead cell control and blank were placed as negative controls. The cell viability was calculated as (mean of sample/mean of positive control x100).

Measurement of combination index (CI)

The results of MTS assay were used to analyse the combination index for the drug combinations tested, using Compusyn software (Combosyn, Inc). The results were interpreted by Chou-Talau equation, where CI < 1 = synergy, CI = 0 additive, CI >1 = antagonism.

Evaluation of apoptosis/necrosis

The cancer cell line U937 (1×10^6 cells/mL) following subsequent treatments were transferred onto V-shaped 96 well microtiter assay plates and centrifuged at 500 x g for 5 min and were resuspended in 100 µL cold Phosphate buffer saline (PBS) and centrifuged (This step was performed for 3 times). Following centrifugation, cells except controls were suspended in annexin V and PI reagents (2.5 µL each) pre-diluted in 50 µL of 1x binding buffer (1x). Annexin V and PI controls were also placed.

The cells were placed in a shaker for uniform mixing and incubated for 15 min in the dark. Following incubation, 200 µL of 1x binding buffer were added to the cells and measured for apoptosis/necrosis by annexin V and PI assay using flow cytometer.

Evaluation of HSP 72/BCL-2 protein expressions

The cancer cell line U937 following subsequent treatments were transferred onto V-shaped 96 well microtiter assay plates and centrifuged at 500 xg for 5 min and were resuspended in 100 µL cold Phosphate buffer saline (PBS) and centrifuged (This step was performed for 2 consecutive intervals). Following centrifugation, the cell pellets were suspended with 70 µL of cytofix\perm buffer and incubated at 4 °C for 20 min for allowing cell fixation and permeabilization. Following incubation, 70 µL of DPBS were added to the cells and centrifuged and resuspended in 100 µL of blocking agent (5% FBS in DPBS) and incubated for 5 min at room temperature. Following incubation, the cells were centrifuged and were resuspended with 50 µL of diluted antibody (HSP72/BCL-2 FITC labeled, 1:50 in blocking agent). The cells were then covered with foil incubated at 4 °C for 45 min, following this final incubation, 50 µL of blocking agent was added to the cells, centrifuged and resuspended in DPBS (100 µL). The cells were then analyzed for HSP 72/BCL-2 expression at 488 nm excitation and 525 nm emission using flow cytometer.

Data Analysis

Data were statistically analysed using Graph Pad Prism™ 6 version 6.05 (Graph Pad statistical Software, Inc, San Diego, CA, USA).

Results

The U937 cell lines were treated with PES-Cl at concentrations ranging 12.5 µM, 25 µM and 50 µM for every hour up to 6 h, to elucidate its response towards cell viability at the lowest tested dosage with limited time. It was interesting to note its minimal response at 1 h of minimal exposure with enhanced cytotoxicity at 6 h of treatment, killing more than 50% of U937 cell lines at the lowest dosage of 12.5 µM PES-Cl (Fig. 1) showcasing the initial HSP inhibition following short span of treatment. Reports in

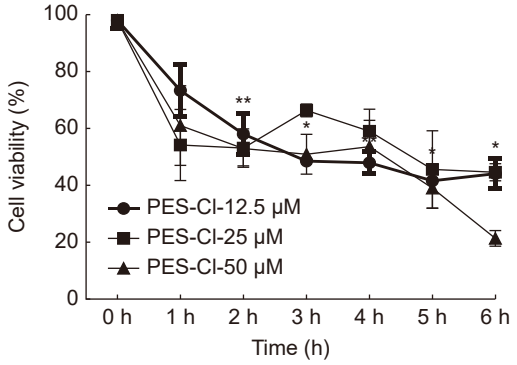

Fig. 1. Evaluation of cell viability by MTS assay on U937 cells following treatment with PES-Cl -12.5 µM, 25 µM and 50 µM for 1 to 6 h.
Data are presented as mean ± SD, n = 3 * (P < 0.05), ** (P < 0.01), using one-way ANOVA Dunnett's post hoc test. Lives cells were placed as controls.

pancreatic cell lines have showcased similar cytotoxicity at 5 h of treatment with HSP inhibitor Pifithrin-µ[21]. Although Pifithrin-µ is a different HSP inhibitor compound, the results strongly indicate the potential effect of PES-Cl and its effectiveness in arresting leukaemia cell lines.

Taking into account the treatment response of PES-Cl as single agent, a 24 h combination treatment intended to obtain an initial response with one agent for 1h and a potential enhancement with another chemotherapeutic Bortezomib other over a 24 h time course seemed a sensitive choice for investigation. Hence, U937 and K562 cell lines were subjected to PES-Cl (12.5 µM) treatment for 1h followed by Bortezomib at concentrations of 15.6 nM and at 31.2 nM. PES-Cl (12.5 µM) controls were allowed for 24 h treatment. Live controls are placed as reference. (Fig. 2A, B).

Cell death was further evaluated by MTS assay, the results of which showed that combination with Bortezomib was less effective at 15.6 nM and 31.2 nM dosages after 24 h exposure in U937 and K562 cell lines. Since, the drugs were found to more effective on U937 cells, than on K562 by MTS assay (Fig. 2), U937 cell lines were selected for further evaluation by Annexin V and PI assay, which reflected an apoptosis of 66.4 % and 66.2 % at 15 nM and 31.2 nM respectively in U937 (Fig. 3) and no necrosis was observed. The cells on the other hand expressed an incredible response upon 1h treatment with Bortezomib, followed by

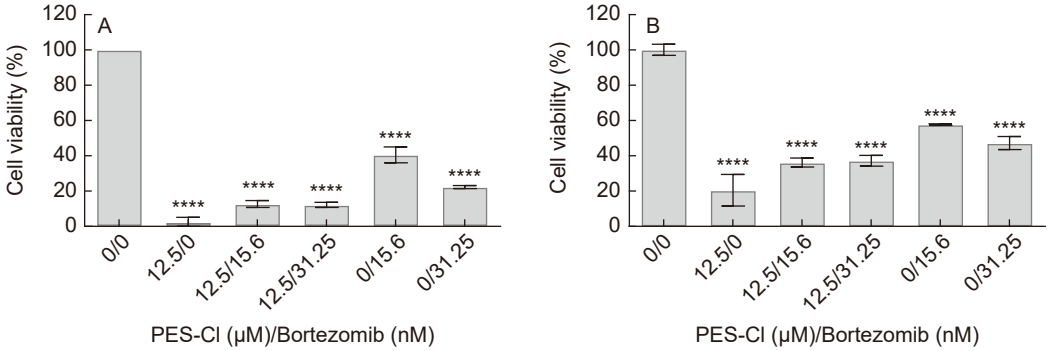

Fig. 2. Evaluation of cell viability assessment by MTS assay.
A. U937.
B. K562 cells.
1×10^6 cells\mL subjected to treatment with PES-Cl (12.5 µM) for 1 h and subsequent treatment with Bortezomib (15.6 nM, 31.25 nM) for 24 h. PES-Cl and Bortezomib controls were allowed for 24 h treatment. Live controls are placed as reference. Data are presented as mean ± SD, n = 3. **** (P < 0.0001) using one-way ANOVA Dunnett' post hoc test.

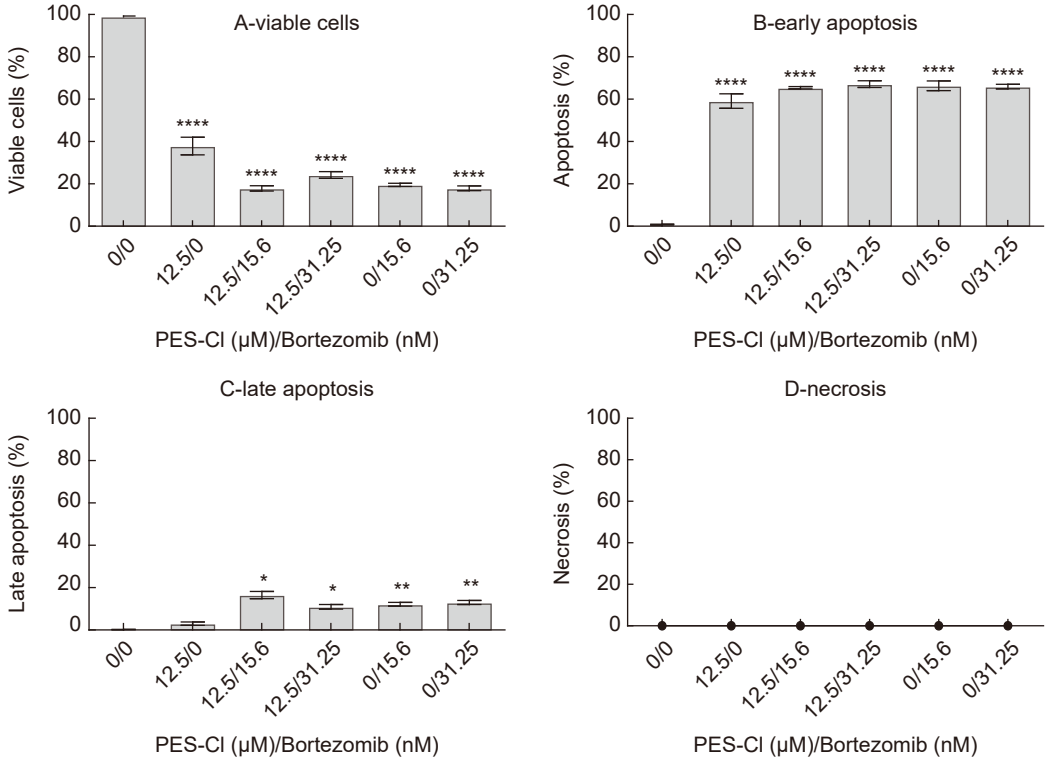

Fig. 3. Combined treatment effects of PES-Cl (12.5 μM) 1 h and Bortezomib (15.6 nM, 31.25 nM) for 24 h on U937 cells. U937 cells (1 × 10^6 cells\mL) following treatments were incubated for 24 h, PES-Cl (12.5 μM) and Bortezomib controls were allowed for 24 h treatment. Live controls are placed as reference. Cells were analysed by flow cytometer using Annexin V\PI assay.

A. Viable cells levels.

B. Early apoptosis levels.

C. Late apoptosis.

D. Necrosis levels.

Data are presented as mean ± SD, n = 3. * (P < 0.05), ** (P < 0.01), **** (P < 0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live control.

PES-Cl for 24 h, showing an increase in cell viability at 12.5/15.6 and 12.5/31.2 nM combination treatments (Fig. 4). The results of Bortezomib administration for 1hr, followed by administration with PES-Cl (12.5) for 24 h showed an apoptosis of 58% and 69.8% with 15.6 nM and 31.2 nM of Bortezomib treatment (Fig. 5). Intriguingly, these results indicated the antagonism between two mechanism of drug activity, and no sign of enhancement of cytotoxicity activity was noted by either of the drug treatment combinations.

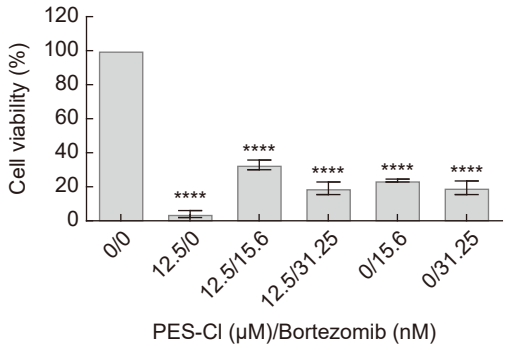

Fig. 4. Evaluation of cell viability by MTS assay.

U937 cells (1 × 10^6 cells\mL) following treatment with Bortezomib (15.6 nM, 31.25 nM) for 1 h and PES-Cl (12.5 μM) for 24 h, the cells following treatment were allowed for incubated for 24 h. PES-Cl (12.5 μM) and Bortezomib controls were allowed for 24 h treatment. Live controls are placed as reference. Data are presented as mean ± SD, n = 3. (P < 0.0001) **** using one-way ANOVA unnett's post hoc tests.

The MTS assay results were used to evaluate the combination index between PES-Cl and Bortezomib for synergistic, additive or antagonistic activity in U937 and K562 cell lines. The results exhibited antagonism, showing a CI of >1, explicitly indicating antagonism, see Table 1. All the drug combinations tested exhibited antagonistic activity regardless the order of administration. PES-Cl and Bortezomib as single agents and in proposed drug combinations exhibited significant inhibition of HSP 72 activity in U937 cell lines, the controls did not result in inhibition of HSP 72 activity (Fig. 6A, B). Taking into account the involvement of HSP 72 protein in the apoptotic mechanism and to further understand the signalling pathway directing towards apoptosis in U937 cells, the activity of BCL-2 was evaluated for the tested drug combinations (Fig. 7A, B). BCL-2 inhibition could determine the intrinsic/extrinsic apoptotic route. BCL-2 expression was noted to be 82.17 and 85.33% with Bortezomib as single agent at 15.6 and 31.2 nM. It was interesting to note the BCL-2 expression of 40.97% when Bortezomib 15.6 was administered initially, followed by PES-Cl (12.5) treatment for 6 h. BCL-2 expression at 31.2 nM Bortezomib, when administered as single agent showed 67.07%. BCL-2 expression was high as 91.06% when PES-Cl was administered as single agent. The results of this data strongly suggest that apoptosis is not mediated through the intrinsic pathway.

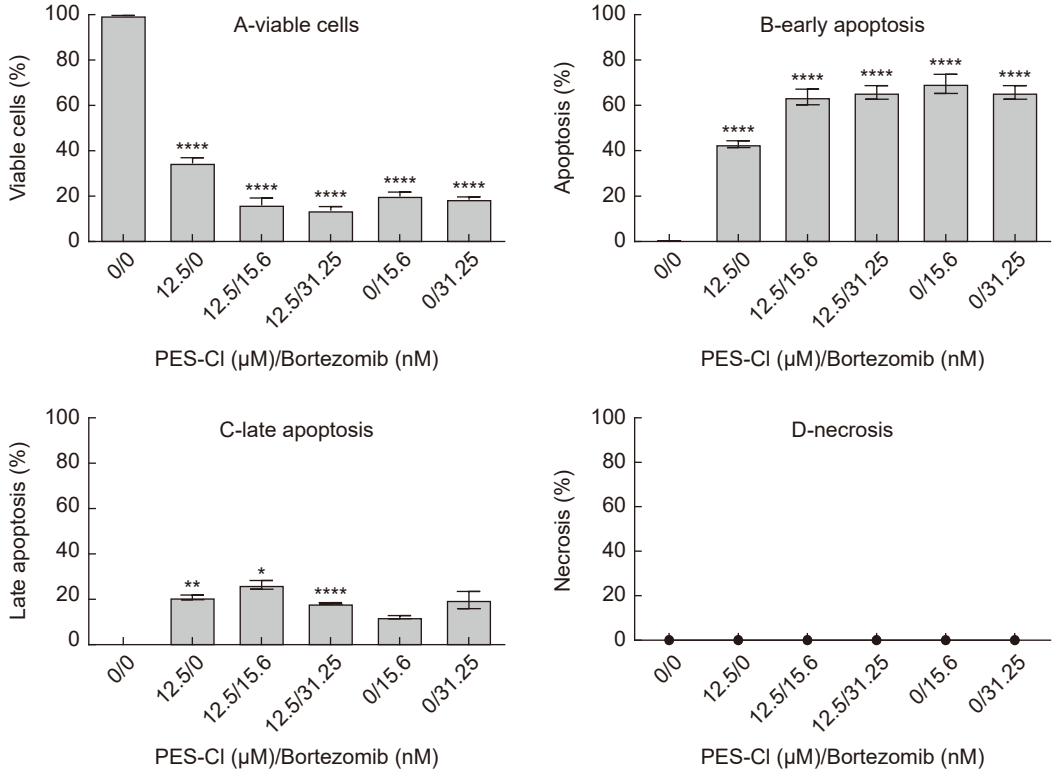

Fig. 5. Combined effect of Bortezomib (15.6 nM, 31.25 nM) treatment for 1 h and PES-Cl (12.5 μM) for 24 h on U937 cells (1 × 10^6 cells\mL). Cells following treatments were incubated for 24 h. PES-Cl (12.5 μM) and Bortezomib controls were allowed for 24 h treatment. Live controls are placed as reference. Cells were analysed by Annexin V and PI assay by flowcytometry.

A. Viable cells levels.

B. Early apoptosis levels.

C. Late apoptosis.

D. Necrosis levels.

Data are presented as mean ± SD, n = 3. * (P < 0.05), ** (P < 0.01), *** (P < 0.001), **** (P < 0.0001) using one-way ANOVA Dunnett's post hoc test. Data are all compared to live controls.

Table 1. Results of the combination index between PES-Cl and Bortezomib for synergistic, additive or antagonistic activity in U937 and K562 cell lines.

Cell Line	Combination Treatments	Combination Index (CI)	Results
K562	PES-Cl 1 h and Bortezomib 24 h	12.5/15.6 = 1.6912.5/3.12 = 1.99	Antagonism
U937	PES-Cl 1 h and Bortezomib 24 h	12.5/15.6 = 1.5212.5/3.12 = 1.81	Antagonism
U937	Bortezomib 1 h and PES-Cl 24 h	12.5/15.6 = 5.5712.5/3.12 = 2.14	Antagonism

Discussion and Conclusion

In leukaemia, HSP proteins especially HSP 72 and HSP 90 are over expressed and are related to poor prognosis[22]. Reports evidence the active role of HSP 72 inhibitor compound pifithrin-μ in enhancing the therapeutic potential in pancreatic cancers[24,25]. The proteasome inhibitor Bortezomib reported to induce heat shock proteins in human retinoblastoma cancer[26]. Abnormal proteasome activity was observed on acute myeloid leukaemia, multiple myeloma and in pancreatic cancers[27,28]. Besides the role of HSP's in cancer survival, the proteasome is an eminent target, considering its important function in cancer cell survival and resistance activity. Taking this into account, it seemed interesting to investigate a two-pronged approach, whether inhibition of HSP together with proteasome could potentially induce apoptosis and cell death in leukaemia cancer cells. Prior to combination response, the drugs were treated as single agents at different concentrations to evaluate the ideal concentration that could exhibit potential cytotoxicity. The results evidence severe cytotoxicity in all treated concentrations within 24 h treatment duration. Similarly, Bortezomib treatment evidence 15.6 and 31.2 nM as the lowest effective single agent doses. Bortezomib is widely administered for multiple myeloma cases[29] and recently considered effective for leukaemia cancers. Time dependent PES-Cl treatment showed minimal cytotoxicity at 1 h with 50% cytotoxicity in 6 h on U937cell lines, indicating an initial response to minimal doses, inhibiting HSP's within short period of treatment in U937.

A clinical trial study on prostate cancer case and on lymphoma case study[30,31] noted encouraging results on proteasome inhibition upon treatment with Bortezomib for minimal of 1 h. This study also observed an apoptosis rate of more than 60% on U937 cell lines, presenting a novel therapeutic option. PES-Cl at con-

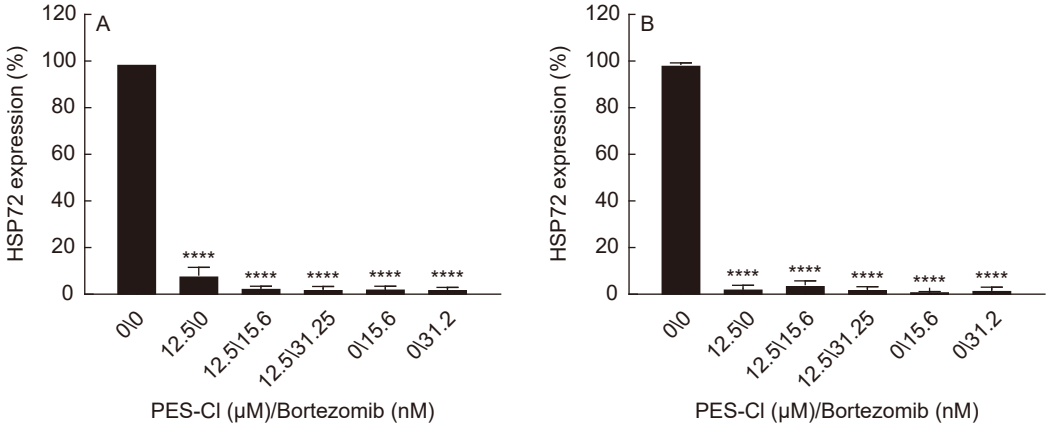

Fig. 6. HSP 72 protein expression in U937 cells following combined treatments.
A. 1 h PES-Cl (12.5 μM) and 6h Bortezomib (15.6 nM, 31.25 nM).
B. 1 h Bortezomib and 6 h PES-Cl combined treatment.
Cells following treatment course were investigated for HSP 72 expression. Data are presented as mean ± SD, n = 3. **** ($P < 0.0001$) using one way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated.

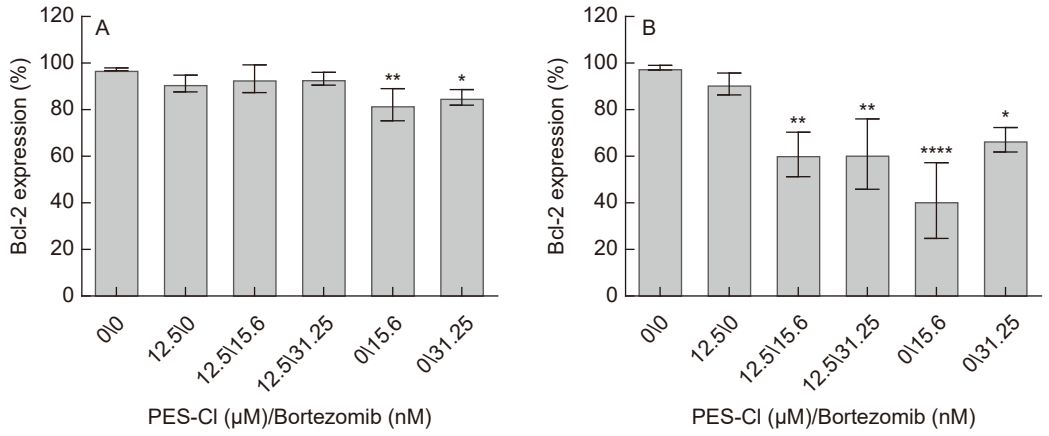

Fig. 7. BCL-2 protein expression in U937 cells following combined treatments.
A. 1 h PES-Cl (12.5 μM) and 6h Bortezomib (15.6 nM, 31.25 nM).
B. 1 h Bortezomib and 6 h PES-Cl combined treatment.
Cells following treatment course were investigated for BCL-2 protein expression. Data are presented as mean ± SD, n = 3. A. *($P < 0.05$), ** ($P < 0.01$). B. *($P < 0.05$), ($P < 0.01$), ($P < 0.0001$), using one-way ANOVA Dunnett's multiple comparisons test. Data are all compared to live cells control untreated.

centration of 12.5 μM was noted to be the minimal dose affecting cell viability at less a lesser tested duration of 1h. Similarly, Bortezomib at 15.6 nM and 31.2 nM doses were lowest doses chosen. Studies report similar cytotoxic effects with Pifithrin-α in combination with other chemo agents[32,33] explicitly indicating possible binding of HSP 72 to PES-Cl, possibly occupying the region of the proteasome, preventing the binding of Bortezomib, creating a competition between the two drugs. Previous studies in cancers of breast, lungs and lymphoma have shown the potential inhibitory role of HSP70 by Pifithrin-μ treatment[34,35]. A study on melanoma cell line highlights the potency of HSP 72 inhibitor Quercetin in increasing the activity of MG-132- a proteasomal inhibitor compound, suggesting a synergistic effect of HSP inhibitors and proteasome inhibitors[36]. Similar study reporting inhibition of heat shock factor -1 has improved treatment efficiency of Bortezomib in multiple myeloma[37]. Another report revealed that HSP 72 overexpression enhancing Bortezomib resistance in bladder cancers[38].

The results of this present study show the inhibition of HSP 72 and its profound role in apoptosis of U937 cell lines. Furthermore, this study reveals the antagonism between the two drugs, particularly upon initial administration of Bortezomib for 1h, followed by subsequent exposure to PES-Cl. This is the first report to evaluate the potential inhibitory role of HSP72 using PES-Cl as single agent and in combination with proteasome inhibitor Bortezomib in leukemic cell lines. Hence, the results outline the crucial activity of HSP 72 in inducing apoptosis. Moreover, taking into account the crucial role of BCL-2 in inhibiting apoptosis in cancers, the expression of BCL-2 upon treatments could reveal the apoptotic pathway involved in cell death is intrinsic/extrinsic or mitochondrial. The results noted a high BCL-2 expression in U937 cells with the drug combinations of PES-Cl and Bortezomib disregard of the mode of administration followed. However, the highest expression was observed in cells exposed to PES-Cl initially and subsequently with Bortezomib treatment. This suggests the possible activity of PES-Cl, affecting the proteasome inhibitor activity, halting the degradation of BCL-2, possibly by accumulation of HSP 72 near the proteasome. The re-

duced levels of BCL-2 were observed upon initial administration of Bortezomib, showing a partial inhibition. Our data confirms the complexity of the proteostasis network in cancer cells as suggested by Shkedi et al[18] To conclude, special attention is required in selection of HSP inhibitors in combination with chemotherapeutics.

Abbreviations

17-DMAG, 17-Dimethylaminoethylamino- 17-demethoxygeldanamycin; BCL-2, B-cell leukemia/lymphoma 2; BCR-ABL, BCR-ABL, Chimeric Gene of BCR and ABL; CML, Chronic Myeloid Leukemia; DPBS, Dulbecco's phosphate buffered saline; FITC, Fluorescein Isothiocyanate; FLT3, FMS - like tyrosine kinase 3; HSP 110, Heat shock protein 110; HSP 72, Heat shock protein 72; HSP 90, Heat shock protein 90; NVP- HSP 990, a novel HSP90 inhibitor; NVP-AUY922, a novel HSP90 inhibitor; PES-Cl, Pifithrin chloride; PI, Propidium iodide.

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Conflict of interests

The authors have indicated that they have no conflicts of interest regarding the content of this article.

Authors' contribution

MMS and PM have equal contribution in the investigation. JH-HW, MMS and PM designed the study. MMS and PM carried out the study and performed the analysis. MMS wrote the first draft and all authors contributed to the revision of the manuscript.

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