Plitidepsin inhibits autophagy, the main mechanism of acquired resistance to bortezomib

Abstract # B057

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Summary

Plitidepsin (PLD), an antitumor agent isolated from the sea squirt *Aplidium albicans*, has been tested with positive results in multiple myeloma (MM) patients in a phase III pivotal trial in combination with dexamethasone (clinicaltrials.gov identifier: NCT01102426) and in a phase I trial in combination with bortezomib (Bz) and dexamethasone (clinicaltrials.gov identifier: NCT02100657). PLD targets eEF1A2, one of two isoforms of the alpha subunit of the eEF1 complex. In mammals, eEF1A2 has a selective pattern of expression in those tissues that do not express the A1 isoform, namely brain and muscle. Nonetheless, eEF1A2 is aberrantly expressed in many cancers, including solid tumors (1-3) and MM (4), and has been shown to hold oncogenic properties (5). We have previously demonstrated the interaction of PLD with eEF1A2 and calculated a K_D of around 80 nM for this interaction (6). Here we explored whether eEF1A2 overexpression had any effect on the sensitivity of MM cells to PLD and Bz, to better understand the synergistic effect these two drugs yield when combined (7). eEF1A has been shown to regulate chaperone-mediated autophagy (8) and to trigger the aggresome formation after proteasome failure (9), essential for the autophagic degradation of unfolded, newly synthesized peptides. Since PLD targets eEF1A2, it seemed likely that it could interfere with autophagy. We first explored this possibility in HeLa wt and APL-R (PLD-resistant derivative) cells and, indeed, we observed that plitidepsin inhibited autophagy, since there was a clear reduction in lipidated LC3B protein, and it did it without inhibiting Class III PI3K. The basal level of autophagy was higher in HeLa wt than in the APL-R cells. Since the main difference between both cell lines was the expression of eEF1A2, lost in the resistant derivative, we analyzed the expression of the elongation factor in a small panel of MM cell lines to select two of them with very different level of expression. We picked MM1S, with low level, and OPM1, with high level of the elongation factor. We then checked whether inhibition of autophagy held for multiple myeloma cells and we confirmed that plitidepsin had a clear inhibitory effect on autophagy in MM1S and OPM1 cells. OPM1 cells showed a higher level of basal autophagy (as HeLa wt cells also did). We then checked their sensitivity to both, bortezomib and plitidepsin through dose-response experiments. Interestingly, eEF1A2 overexpression in OPM1 cells could be related to an increased resistance to bortezomib, probably due to an enhanced autophagy that compensates for proteasome inhibition. Finally, when given in combination (R 1:0.52), plitidepsin and bortezomib showed a synergistic effect in the OPM1 cell line, while this effect was less clear in MM1S cells. Thus plitidepsin treatment presumably counteracts autophagy the main mechanism of resistance to bortezomib.

(1)Sun et al, 2014, Biochem Biophys Res Commun 450:1-6
(2)Xu et al, 2013, Clin Exp Metastasis 30:933-44
(3)Anand et al, 2002, Nat Genet 31:301-5
(4)Li et al, 2010, PLOS One 5, e10755
(5)Lee and Surh, 2009, Ann N Y Acad Sci 1171:87-93
(6)Losada et al., 2004, Br J Cancer 91:1405-13
(7)Mitsiades et al, 2008, Cancer Res 68:5216-25
(8)Bandyopadhyay et al, 2010, Mol Cel 39:535-47
(9)Meriin et al, 2012, J Cell Sci 125:2665-74

Results

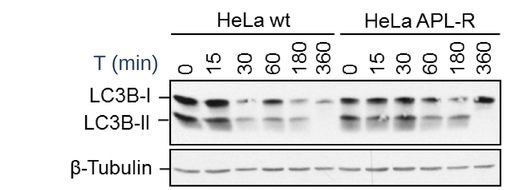


Fig 1: PLITIDEPSIN INHIBITS AUTOPHAGY IN HeLa CELLS
HeLa wt and APL-R cells were treated with 450 nM PLD for the indicated times. Treated cells were then lysed with lysis buffer (1% Triton-X100, 50mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, Complete™ and PhosStop™). From each sample, 15 µg of protein were subjected to PAGE and electroblotted onto a PVDF membrane. Finally the membranes were hybridized with the appropriate anti-LC3B and anti-tubulin primary antibodies and the appropriate HRP-labeled secondary antibodies.

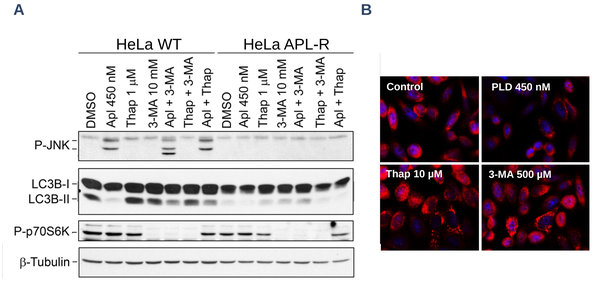


Fig 2: Plitidepsin inhibits autophagy in a VPS34 (Class III PI3K)-independent fashion.
(**A**) HeLa wt and APL-R cells were treated with DMSO (vehicle) or the indicated concentrations of PLD, Thapsigargin (Thap), 3-methyladenine (3-MA) or combination of them for 3 hours. Then cells were lysed with lysis buffer. From each sample, 15 µg of protein were subjected to PAGE and electroblotted onto a PVDF membrane. Finally the membranes were hybridized with the appropriate anti-phospho-JNK, anti-LC3B, anti-phospho-p70S6K and anti-tubulin primary antibodies and the appropriate HRP-labeled secondary antibodies.
(**B**) HeLa wt cells were seeded in 16 well slides, treated for 3 hours with the indicated concentrations of PLD, 3-MA or Thap. Then, cells were fixed with 4% paraformaldehyde for 10 min, blocked with 1% BSA in TBS-T, hybridized with anti-LC3B primary and Alexa-594 secondary antibodies. Nuclei were counterstained with Hoechst-33342 and preparations mounted with Mowiol. Pictures were taken with a Zeiss Axiovert 200M microscope equipped with Apotome structured illumination.

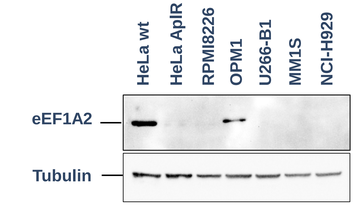


Fig 3: eEF1A2 EXPRESSION IN A PANEL OF MM CELL LINES.
A culture from each cell line in 3.5 cm plates was lysed with lysis buffer. From each sample, 10 µg of protein were analyzed by Western blot using the appropriate primary (Genetex GTX102326) and secondary antibodies to determine the expression of eEF1A2.

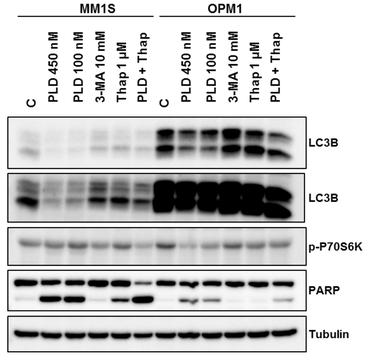


Fig 4: Plitidepsin inhibits autophagy in multiple myeloma cells.
MM1S or OPM1 cells were treated with the indicated concentrations of PLD, 3-MA, Thap or mixtures of them for 3 hours and then lysed with lysis buffer and 10 µg of protein extract subjected to PAGE and electroblotted onto a PVDF membrane. Finally the membranes were hybridized with the appropriate anti-LC3B, anti-phospho-p70S6K, anti-PARP and anti-tubulin primary antibodies and the appropriate HRP-labeled secondary antibodies

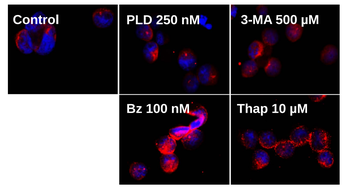


Fig 5: Plitidepsin inhibits while bortezomib enhances autophagosome formation in MM1S cells
MM1S cells were seeded in 16 well slides, treated for 3 hours with the indicated concentrations of PLD, 3-MA, Thap or Bz. Then, cells were fixed with 4% paraformaldehyde for 10 min, blocked with 1% BSA in TBS-T, hybridized with anti-LC3B primary and Alexa-594 secondary antibodies. Nuclei were counterstained with Hoechst-33342 and preparations mounted with Mowiol. Pictures were taken with a Zeiss Axiovert 200M microscope equipped with Apotome structured illumination

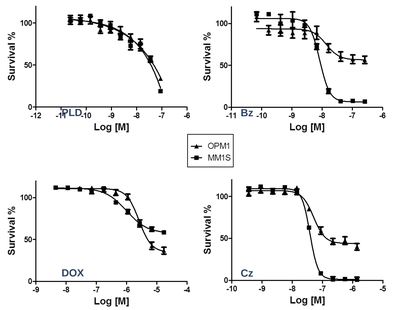
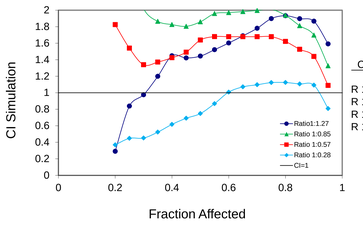
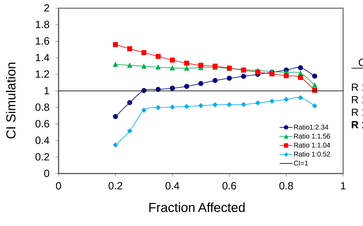


Fig 6: Dose-response curves for plitidepsin, bortezomib and carfilzomib in MM1S and OPM1 multiple myeloma cells.
1.5 x 10^4 cells per well were seeded in 96 well plates and treated with the appropriate concentrations of PLD, Bz, carfilzomib (Cz) or doxorubicin (DOX) (as a control). After 24 hours of treatment MTT was added to the samples and formazan crystals allowed to form for 8 hours. Finally the crystals were dissolved in DMSO and quantified by spectrophotometry. Experiments were performed 3 times in triplicate. Dose-response curves were then adjusted with the GraphPad Prism 5 software.



MM1S

CI Values at following EDs	ED20	ED50	ED80
R 1:1.27	0.29	1.44	1.93
R 1:0.85	2.57	1.86	1.94
R 1:0.57	1.82	1.64	1.62
R 1:0.28	0.37	0.75	1.12



OPM1

CI Values at following EDs	ED20	ED50	ED80
R 1:2.34	0.69	1.09	1.25
R 1:1.56	1.32	1.28	1.22
R 1:1.04	1.56	1.31	1.18
R 1:0.52	**0.34**	**0.82**	**0.9**

Fig 7: Fa vs CI plot for the combination of plitidepsin plus bortezomib in MM1S and OPM1 MM cell lines.
MM1S and OPM1 cells were treated with combinations of PLD and Bz at the indicated ratios and after 72 hours cell survival was measured with the MTT assay. Combination Index (CI) was then calculated by applying the Chou and Talalay method. Different degrees of synergism (CI < 1) were recorded when plitidepsin was combined with bortezomib, depending on the cell line tested. For MM1S cells the combination was not clearly synergistic at any ratio. For OPM1 cells the combination had a clear trend to moderate synergism at the ratio 1:0.52 (PLD:Bz)
Tables on the right summarize the different CI values at the effective doses ED20, ED50 and ED80.

Conclusions

- Plitidepsin inhibits autophagy in a Class III PI3K (VPS34) independent manner in HeLa cervix cancer cells and in MM1S and OPM1 multiple myeloma cells.
- OPM1 multiple myeloma cells that overexpress eEF1A2 are more resistant to bortezomib than MM1S cells that do not overexpress the elongation factor.
- While a moderate synergistic effect of the plitidepsin:bortezomib combination (R 1:0.52) can be seen in OPM1 cells, this effect is less clear in MM1S cells.