ERWINIA CHRYSANTEMI-DERIVED L-ASPARAGINASE STRONGLY ENHANCES PROTEASOME INHIBITORS ACTIVITY BY DEREGULATING METABOLIC CELL PROGRAM

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KEYWORDS: Multiple myeloma, L-asparaginase, Erwinia Chrysantemi, Glutamine, DNA-repair

INTRODUCTION AND AIMS: Some human tumor cells exhibit a high requirement for Glutamine (Gln) for metabolic processes and macromolecule synthesis, a condition that has been defined Gln-addiction. The potential role of Gln-dependence as therapeutic target in multiple myeloma (MM) has been recently investigated with a specific interest in inhibiting Gln uptake by acting on Gln transporters. Interestingly, MM cells lack significant levels of Gln synthetase, a condition that may explain the high sensitivity to Gln depletion.

It is known that clinically attainable doses of L-apsaraginase, the enzyme that catalyzes asparagine (Asn) hydrolysis is able to attain also Gln depletion due to a certain degree of glutaminase activity. L-Asparaginase from *E. chrysantemy* exerts a 10-folder higher glutaminase activity in comparison with the *Escherichia Coli*-derived formulation. I previously reported on the preliminary results of our study investigating the sensitivity of MM cells to pharmacological Gln depletion obtained by *Erwinia chrysantemi*-derived L-Asparaginase (L-ASP) and its synergistic effect in association with proteasome inhibitors (PIs).

The subsequent aim of my project was to disclose the biological mechanisms supporting the effectiveness of such an innovative combination.

MATERIALS AND METHODS: The IC50 value of L-ASP was determined by using tetrazolium salt MTS based assay in a panel of human MM cell lines (HMCLs) harboring different genetic background. Culture medium RPMI 1640 plus 10% FBS with 4 mM Gln concentration was used. Primary human MM cells were obtained from bone marrow samples with high disease load and mononuclear cells were isolated by Ficoll-Hypaque gradient and processed by MiniMacs high-gradient magnetic separation column (MiltenyiBiotec, Bergisch-Gladbach, Germany) to obtain highly purified CD138+ cells. Cells were either used immediately for viability assays.

Cell death analysis was performed by Annexin V/Propidium Iodide (AV/PI) staining followed by flow-cytometric analysis. L-ASP treatment effect on cell viability and mitochondrial membrane potential was evaluated by FACS analysis following PI and TMRE staining, respectively.

To evaluate the content of intracellular ATP, cells were lysed in PCA and the neutralized extracts were analyzed by HPLC, then ATP values were normalized to protein concentrations.
Combination drugs screening with L-ASP plus PIs bortezomib and carfilzomib was investigated with an isobologram analysis (CalcuSyn software program).

Combination of L-ASP and proteasome inhibitors was tested in standard culture conditions and in presence of IL6 and IGF-1, to mimic bone-marrow stromal cells (BMSCs) milieu.

To investigate the contribution of cMYC downregulation in the observed synergism, since cMYC protein is expressed in all HMCLs except for U266, a stable cMYC overexpressing U266 line was generated by lentiviral transduction (U266-cMYC OE). Transduced U266 cells were selected by 72 hours of puromycin exposure.

Western blot (WB) analysis was used to fully elucidate biological features of our findings.

**RESULTS:** L-ASP treatment showed potent anti-tumor activity in all HMCLs tested with an IC50 value ranging from 0.03 U/mL to 0.15 U/mL. L-ASP effect was also observed in primary MM cells (median IC50 =0.06 U/mL). L-ASP treatment induced an early (24 h) but irreversible “metabolic shutdown” in MM cells confirmed by cell cycle arrest in G1 phase and protein synthesis blockade as shown by increased non-phospho4EBP1 and decreased pP70S6K in WB analysis. A time-course WB analysis confirmed cMYC downregulation as the main event impairing cell cycle progression by CDK4 and CDK6 downregulation and loss of inhibition of p21 transcription. Notably, U266 cell line showed the highest IC50 value and did not show any modification of cell cycle progression under L-ASP treatment.

In co-treated cells, WB analysis revealed massive c-MYC down regulation, however, the effectiveness of the combination resulted independent from the oncogene deregulation since it was confirmed highly effective in cMYC negative U266 HMCL. Caspase-3 and PARP cleavage in co-treated cells pointed the crucial role of apoptosis in the observed synergism. Intracellular ATP measurement showed a significant NAD and ATP shortage in co-treated cells compared to single drugs exposure and a considerable accumulation of misfolded proteins, detected as a smear of high-molecular weight adducts in response to L-ASP and proteasome inhibitor co-treatment.

Further mechanistic studies highlighted induction of genomic instability in co-treated cells, due to an impairment of DNA-damage repair mechanisms as documented by a reduction of RAD51 expression, and a consistent increase in pH2AX on Ser 139 by WB analysis.

To corroborate the contribution of different amino acids depletion, MM cells were treated with PIs in different culture medium conditions. Remarkably, Asn depletion alone did not significantly increase PI activity on MM cells viability, instead it strongly contributed to L-ASP induced toxicity in Gln-starved cells.

**DISCUSSION:** Our findings suggest that L-ASP induced metabolic deregulation in MM cells is able to significantly increase plasma cells sensitivity to proteasome inhibitors. This effect may be explained by a huge proteasome activity injury secondary to co-treatment. Interestingly, as already shown in solid tumors,
Gln deprivation may also affect DNA-repair mechanisms. It has been already shown that PIs are able to induce “BRCAness” sensitizing MM cells to PARP inhibitors. We are currently exploring a model of synthetic lethality driven by PI-induced BRCAness and PARP1 activity impairment due to L-ASP treatment.

**CONCLUSIONS:** Our data show that the combination of *E. chrysanthemi* derived L-ASP and PI exerts a potent anti-MM activity mainly through a huge metabolic program deregulation. Our findings also suggest an intriguing role of DNA-repair mechanisms impairment.
REFERENCES


