Anticancer Effects of Combination Therapy through Apoptosis and Autophagy
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Abstract
Recent studies suggest the significant role of epigenetics in carcinogenesis by silencing tumor suppressor genes. Epigenetic drugs such as AZA and histone deacetylase inhibitors (HDACi) re-express epigenetically silenced tumor suppressor genes, and are under clinical trials as anticancer agents. HDACi that cause increased acetylation of DNA have been shown to be effective in treating leukemia but not solid tumors. Clinical trials are under way to use combination therapies with HDACi and other cytotoxic agents. We hypothesized that the re-expression of tumor suppressor genes would make cancer cells more susceptible to the cytotoxic agents. In this work we sought to develop a combination therapy using HDACi and a calpain protease inhibitor, Calpeptin. We employed a suboptimal dose of each inhibitor. The combination therapy produced more than additive growth inhibition in different types of cancer cells including breast and ovarian cancer. These inhibitory effects were a result of cell cycle inhibition, induction of apoptosis and autophagy. Significantly, the combination therapy also proved effective with different cell lines exhibiting different characteristics, such as the triple negative breast cancer cell line, MDA231. Since this is not a molecule-targeted therapy, we propose that this combination treatment may prove more effective against a wider array of cancers than current treatments, including cancers that are resistant and refractory to other drugs.

Background
- Carcinogenesis involves activation of oncogenes and down regulation of tumor suppressor genes.
- Many tumor suppressor genes are silenced by epigenetic mechanisms, involving both histone modifications and methylation of CpG residues in the upstream regions of genes.
- Our previous work observed that histone deacetylase inhibitors (HDACi) demethylate many tumor suppressor genes leading to their re-expression in cancer cells. (Sarkar et al., Anticancer Research, 31, 2723-2732, 2011.)
- Treatment of breast cancer cells with HDACi and calpeptin allowed re-expression of epigenetically silenced tumor suppressor genes and the induction of apoptosis (Mataga et al., 2012).
- Apoptosis occurs in the cell through two pathways, intrinsic and extrinsic.
- The intrinsic pathway is mediated through the involvement of mitochondria. This activates the release of cytochrome c from the mitochondria and leads to the activation of a caspase cascade that will cause apoptosis.
- The extrinsic pathway involves signaling via death receptors. For example, a growth factor, Fas-Ligand, will bind to the Fas receptor and will initiate the caspase cascade that leads to apoptosis.
- Autophagy, or the degradation of cellular products, might also play a role in drug induced cell death in addition to apoptosis. Calpeptin is known to induce autophagy in cancer cells (Storr et al., 2011).

Objectives
- To determine the effects of combination therapy with HDACi and calpeptin on different breast and ovarian cancer cell lines.
- To investigate the mechanisms of apoptosis and autophagy after treatment.
- To quantitate the increased expression of pro-apoptotic markers.

Methods
- Breast and ovarian cancer cells were treated with HDACi and Calpeptin.
- The cells were observed for changes in cell morphology.
- Once the cells were harvested, antibody staining for the Cib autophagic protein were done on the cells treated with Calpeptin.
- The harvested cells were also used to perform a Western blot analysis to look at the protein levels of various tumor suppressor genes.
- qPCR analysis was also done to quantitate the upregulation of 11 pro-apoptotic markers. These results are still preliminary.

Results
- MDA MB 231 triple negative breast cancer cells were treated with 0.2mM sodium butyrate (SB), 7.5 uM SAHA, 7.5 ug/ml calpeptin or combination of SB and calpeptin, SAHA and calpeptin at the same concentrations.
- Live cell counts were taken on day 4 by trypan blue exclusion procedure and the untreated control cell survival is expressed as 100% cell growth.
- Combination treatments of SB-calpeptin and SAHA-calpeptin showed synergistic type growth inhibition.

Fig 1: Breast Cancer Growth Inhibition by HDACi and Calpeptin
- MDA MB 231 triple negative breast cancer cells were treated with HDACi and Calpeptin.

Fig 2: Morphology Change of Breast Cancer and Ovarian Cancer Cells
- Breast Cancer MCF-7 Cells
- Ovarian Cancer SKOV-3 Cells
- Cancer cells were treated with inhibitors, and photographs were taken after 4 days.

Fig 4 Induction of Autophagy by Calpeptin in Breast and Ovarian Cancer Cells
- MDA MB 231 breast cancer and SKOV-3 ovarian cancer cells were treated with 20ug/ml with calpeptin for 4 days. The cancer cells were stained with anti-C3 antibody followed by the secondary antibody conjugated to FITC, then developed.

Fig 3: Effects of HDACi and Calpeptin on ERK Phosphorylation (A), and BAX (B) and (C)
- CAOV-3 ovarian cancer cells were lysed after 48 hrs treatment.
- Equal amount proteins were run in an SDS-PAGE.
- Western analysis was performed for BAX and pERK. Blot was stripped. A western blot for Actin and ERK followed (A and B).
- Transcript upregulation of BAX was determined by qPCR of cDNA prepared from total RNA (C).

Fig 5: Model of HDACi and Calpeptin Combination Therapy

Analysis and Conclusions
- HDACi and calpeptin combination therapy produced synergistic type growth inhibition in breast and ovarian cancer cells. Varying concentrations of both inhibitors were necessary to induce similar growth inhibitions, considering differential characteristics of the types of cancer cells. (Fig. 1)
- Combination therapy induced morphological changes in both breast and ovarian cancer cells indicate cell death. (Fig. 2)
- ERK phosphorylation was differentially inhibited by inhibitors in ovarian cancer cells. (Fig. 3A)
- Expression of the pro-apoptotic gene BAX was upregulated. (Fig. 3B, C)
- Calpeptin treatment showed induction of autophagy in both ovarian and breast cancer cells. (Fig. 4)
- We hypothesize that during combination therapy, HDACi with calpeptin, cells are sensitized by the re-expression of tumor suppressor genes and subsequently killed by induction of apoptosis and autophagy. (Fig. 5)

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