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Investigation into the Combined Affects of Tumor Necrosis Factor-Alpha, Tamoxifen and 5-Azacytidine on HTB-26 Breast Cancer Cells

Gregory Roloff
SUNY Geneseo

Jessica Bosch
SUNY Geneseo

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Abstract

The primary goal of modern cancer therapeutic regiments is to develop treatments that are damaging to tumor cells but maintain the health of normal somatic cells. The "cocktails" of drugs used in current cancer treatment can interact in a mechanism that is not adequately understood. However, it is sometimes possible to use combinations of different chemicals to achieve the desired effect of killing the most cancer cells possible while preserving the patient's other rapidly dividing cells. Our work to this point has been focused on two chemicals, sulforaphane (SFN), a organosulfur compound found in cruciferous vegetables, and Tumor Necrosis Factor-α (TNF), a cytokine involved in regulation of the immune response. The scope of our research is centered around apoptosis, or programmed cell death. In this project, we tested the effects of combining SFN treatment with varying amounts of TNF-α based on prior research from the O'Donnell lab showing the combination of SFN and TNF-α together cause apoptosis despite the fact that TNF-α alone is an insufficient inducing agent. In our preliminary experiments using cytotoxicity assays, we did find increased killing when the drugs were used in combination. Experiments using caspase 3 apoptosis assays showed an increase in apoptosis with the combined treatment due to activation of the caspase 3 pathway. Moving forward, we have begun to investigate how altering gene regulation proteins might sensitize the cells to apoptosis induction via the use of 5-Azacytidine, a DNA methyltransferase inhibitor.

Introduction

The cell line we work with is HTB-26, derived from a 51 year old Caucasian female patient with breast cancer in 1979 (Cailleau). It can be described as an adenocarcinoma of the mammary gland that is derived from epithelial tissue. This cell line is tumorigenic in immunocompromised mice. One of the drugs we worked with is SFN, an isothiocyanate derived from broccoli and other cruciferous vegetables (Figure 1) (Jakubikova). Tumor necrosis factor α is a cytokine involved in inflammation. Recent research has shown that SFN and TNF-α may have synergistic effects when used together. Previous research has shown that TNF-α may activate NF-κB, an anti-apoptotic transcription factor (Figure 1).

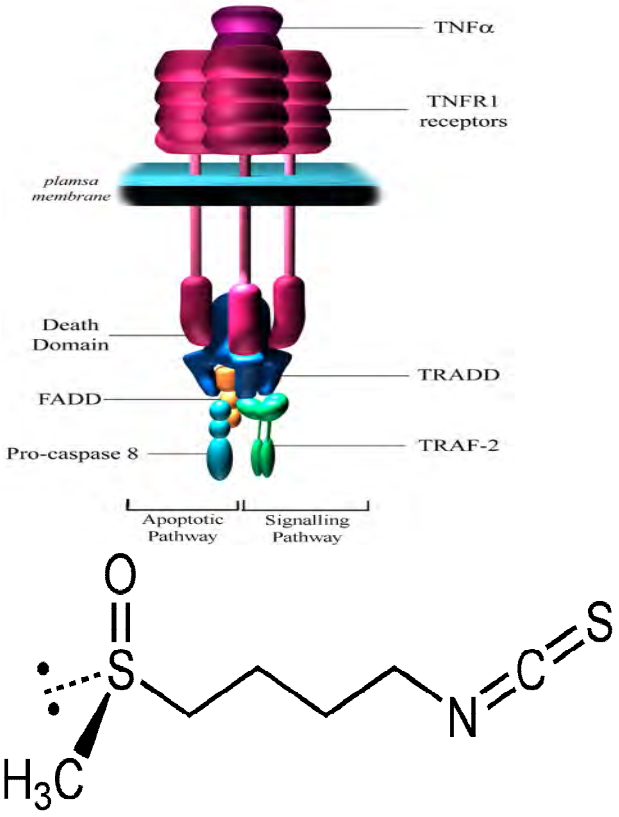

Figure 1. Membrane domain of TNF pathway and chemical structure of sulforaphane

Apoptosis is the process of programmed cell death and cancer cells often have mutations that prevent them from undergoing entering this process. TNF-α activates procaspase 8 which activates the executioner caspases, including caspase 3. Sulforaphane has also been shown to induce apoptosis through activation of reactive oxygen species-dependent caspase-3. This combination of drugs actually has a more lethal effect than either drug alone, leading to more apoptotic cells. Developing hypothesis look to test the levels of cell proliferation after first sensitizing the cells with 5-Azacytidine, a methyltransferase inhibitor (Figure 2.)

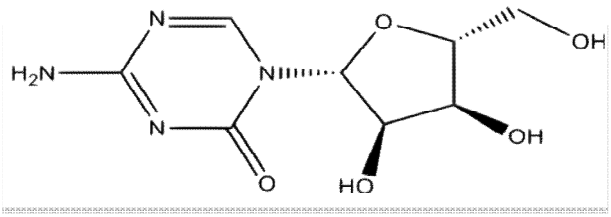

Figure 2. Chemical structure of 5-Azacytidine.

Materials

Dulbecco's PBS (Phosphate Buffered Saline), Trypsin, RPMI (Rochester Park Memorial Institute), L-15 Medium (Leibovitz), L-15, FBS (Fetal Bovine Serum), Glutamax, TNF-α, SFN, Annexin V Kit (10x Binding Buffer, PI, Annexin, Distilled H_20) , Caspase-3 Assay (Lysis Buffer, 2x Reaction Buffer 3, DTT, Caspase-3 calorimetric substrate), CyQuant Assay (CyQuant GR dye, Cell-lysis buffer, DNA standard). MTT, isopropanol, 1N HCl.

Methods

Cyquant Assay : A 96 well plate was set up after cells were cultured and incubated. Each well received 100µL of media. SFN was kept constant in all wells through column 10. The bottom four rows received varying amounts of TNF. The plate was incubated at 37^0 C for 72 hrs and excess fluids were removed by flicking and blotting. A CyQuant assay was prepared, and added to each well and the fluorescence was measured. These experiments allowed us to verify the dosages to use in future experiments.

Caspase 3 Assay: Cells were treated with nothing, SFN, tumor necrosis factor-α or a combination of drugs for 72 hours. Cells are collected by centrifugation at 250G for 10 minutes. The cells were lysed and then centrifuged. In a 96 well plate, 50 µL of each sample was added to each well along with 50 µL of 2x Reaction Buffer 3 and 5 µL of Caspase-3 colorimetric substrate . The plate was incubated for 1-2 hours at 37^0 C and absorbance was read at 405 nm.

MTT Assay: a 96 well plate was set up to measure cell proliferation. Cells from the breast cancer line HTB-26 were exposed to SFN alone, SFN and various amounts of TNF, or nothing. The cells were incubated for 72 hours. MTT was prepared and 20µL was added to each well and incubated for 5 hours. The supernatant was removed and 100µL of a mixture composed of 9.6 mL isopropanol and 0.4 mL HCl (1N) was added before the plate was read for fluorescence at 585 and 690 nm.

Results

Cytotoxicity experiments for HTB 26 cell lines revealed that the concentration of drug that showed the desired amount of killing was 0.035 mM, which ultimately came out to be 0.5mL of 0.28mM of SFN for every 4mLs of cells. The MTT cell proliferation assay detected an additional 7% killing in the presence of TNF, on top of the 57% caused by SFN alone. As the concentration of TNF increased, the amount of killing followed proportionally (figure 3). Results from the apoptosis assay, as depicted in figure 4, suggest that TNF and SFN may have a synergistic effect in inducing apoptosis, since the combination induced higher levels than the additive effects of the drugs alone. The CyQuant Assay illustrates that TNF alone is a poor killing agent, obtaining only a 6% increase from the cell control. However, TNF supplements the strong killing induced by SFN by an additional 6-7% (Figure 5).

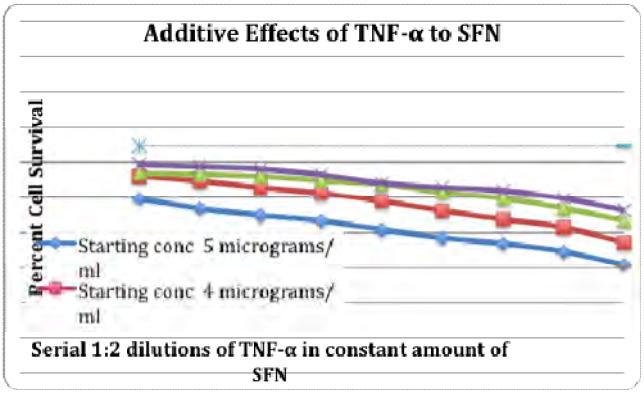

Figure 3. Graph depicting cell survival of HTB-26 cells when incubated in uniform amounts of sulforaphane, while varying the amounts of TNF in the experimental rows. Different doses of TNF are distinguished by separate lines

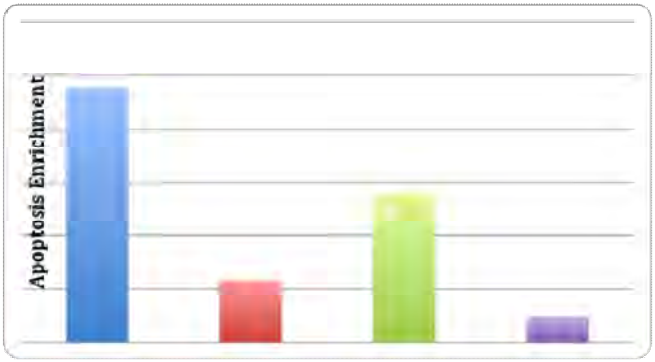

Figure 4. Apoptosis Enrichment of HTB-26 cells based on all experimental treatments of SFN, TNF and a combination of both vs. a cell control that received no drug.

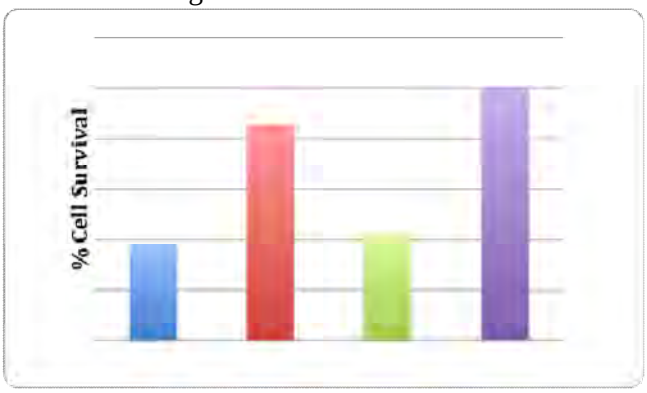

Figure 5. Results from CyQuant Assay for cell survival of each experimental group.

Discussion

Overall data provides support for the notion that SFN causes cytotoxicity in HTB-26 cells and that a combination of TNF-α and SFN has a greater apoptosis inducing effect. This can have great clinical implications if it is able to kill cancer cells more effectively in vitro. However, treatment would have to be relatively safe to the body's non-cancerous cells, which divide at various rates. The new direction we are following is to determine whether 5-Azacytidine treatment will first weaken cells so they will be more sensitive to apoptosis induction with Sulforaphane and Tumor Necrosis Factor-α . Our further studies will examine the influence these experiments will have on MRC-5 human fibroblasts (non-cancerous cells) and how 5-Azacytidine might enhance previously obtained results.

References

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