Category: Biomedical and Health Sciences

Research Plan

Jason Linzer

Title: Examining P53 Mutant Triple Negative Breast Cancer Cell Viability and Sphingosine Kinase 1 in Response to CHK1 Inhibitor and Doxorubicin

3A. Rationale: Triple Negative Breast Cancer [TNBC] is a type of breast cancer that does not have abnormal expression of the progesterone, estrogen, or human epidermal growth factor 2 receptors. The lack of the abnormal expression of these hormone receptors means hormone therapy cannot be used to treat TNBC. In addition, 44% of TNBCs have a mutation in the gene for the p53 protein. Since p53 responds to DNA damage halting cell growth and inducing apoptosis, genotoxic agents, which cause DNA damage, are not as effective at stopping cell proliferation in p53 mutated cancers. As a result, there is a need for novel therapies for p53 mutated TNBCs as hormone therapy and genotoxic agents are minimally effective in treating TNBC.

3B. Hypothesis, Research Question, and Expected Outcomes:

(i) Research Question: Can the use of Check Kinase 1 Inhibitor in combination with a genotoxic agent reduce the viability of p53 mutated TNBC cells better than either substance on its own?

(ii) Hypothesis and Expected Outcomes: Check Kinase 1 [CHK1] is a protein that is activated in response to DNA damage and DNA repair. When activated, CHK1 halts cell cycle progression to provide the cell time to repair DNA. CHK1 halts cell cycle progression by blocking progression through S phase and late G2 phase checkpoints. In addition, p53 functions by blocking cell cycle progression at the G1 checkpoint. In p53 mutated cells with inhibited CHK1, the cell would not be able to respond to DNA damage at these three major checkpoints.

Therefore, it is expected that damaging the DNA in p53 mutated CHK1 inhibited cells would result in decrease viability of these cells because of the lack of DNA repair. It is then expected that that these treated cells would exhibit greater rates of death and lower rates of cell proliferation as compared to cells not treated with both the genotoxic agent and the CHK1 inhibitor. The cells are also expected to exhibit lower levels of the molecule sphingosine kinase 1 [SK1], which is associated with cellular proliferation, and less proliferation upon visual inspection.

3C. Procedures, Data Analysis and Risk & Safety:

This research will be conducted at Stony Brook Lipid Cancer Lab of Dr. Yusuf Hanan and Dr. Lina Obeid of Stony Brook, NY under the direct supervision and guidance of graduate student Joseph Bonica.

Procedures

(i) Cell Culture

- TNBC cell line MDA-MB-231 will be purchased from ATCC (Catalogue # HTB-26)
- Cells will be grown in Dulbecco's modified Eagle's medium (DMEM) with 10% Fetal Bovine Serum (FBS) in 37 °C incubator
- Cells will be grown in a flask and split into a new flask every 2 to 3 days
- 75,000 cells from the flask will be plated into 3 mL of DMEM medium with 10% FBS for experimentation. For LDH & MTT Assays, cells will be plated in 6 well 35 mm plates. For Western Blot Assays, cells will be plated in 60 mm plates

(ii) Drug Treatment

- DMEM with 10% FBS medium will be removed and replaced with serum-free medium

- 1 hour later, CHK1/2 inhibitor AZD7762 will be added to the plates at a concentration of 0.3 μM
- Another hour later, doxorubicin will be added to the plates at a concentration of 0.8 μM. For 24 hr LDH & MTT Assays and Western Blot & EVOS Imaging, cells will incubate in the drug treated medium for 24 hours. For 48 hr LDH & MTT Assays, cells will incubate in drug treated media for 48 hours

(iii) LDH & MTT Assays

- After drug treatment, 50 μL aliquots of each treatment group and 50 μL of lactate dehydrogenase [LDH] substrate will be added to a 96 well plate
- Two wells will be used for each treatment group
- Plate will then incubate at 37 °C in the dark for 30 min
- Wells will then be read by a spectrophotometer. Absorbance values will then be calculated by subtracting absorbance at 490 nm by the absorbance value at 680 nm. The two values for each treatment group will then be averaged for LDH values
- To start the MTT Assay, the remaining medium in the plate will be aspirated. 1 mL of fresh medium and 1 mL of thiazolyl blue tetrazolium bromide [MTT] will be added to the 6-well plate
- The 6-well plate will then at 37°C for 30 min in the dark.
- The MTT and medium mixture will then be removed. 2 mL of dimethyl sulfoxide will then be added to each treatment group and the plate will gently rock for 10 min
- Two 200 μL aliquots from each treatment group will the be placed in a 96-well plate
- The 96-well plate will then be read in a spectrophotometer at 570 nm. The average for the two absorbance values for each treatment group will then be the MTT value

(iv) Western Blot Analysis and EVOS Imaging

- Imaging will first be done on an EVOS microscope after the drug treatment
- RIPA buffer for cell lysis will then be added to each treatment group. Cell lysates will be extracted, sonicated, then undergo centrifugation at 14,500 g for 10 min at 4°C
- A BCA Protein Assay kit will then be used to create samples for each treatment group where the total protein concentration is 20 μg/ 20 μL
- Proteins will then be separated on an SDS/ PAGE (4% - 15% Tris/HCl) gel by molecular weight. Proteins will then be transferred to a nitrocellulose membrane
- Membrane will then be blocked 5% nonfat milk in phosphate buffered saline PBS with 0.1 % Tween-20 [PBS-T] for 1 hour. Membrane will be cut to allow for use of different proteins
- Primary antibody for SK1 (diluted 1:1000) and primary antibody for β-Actin (diluted 1:10000 will be used for immunoblotting. Membranes will then be incubated at 4°C in the primary antibodies overnight
- Primary antibodies will be removed next day, then washed with PBS, then secondary antibodies will be added (diluted 1:1000 for SK1 and 1:5000 for β-Actin) for 1 hour
- Membranes will then be washed again. Pierce ECL Western Blot Substrate will then be added, and membranes will be exposed to x-ray films. X-ray films will then be developed in dark

(v) Data Analysis

- Quantitative data from MTT & LDH Assays will be entered into Microsoft Excel and analysis using 1 Way-ANOVA
- Western Blots and EVOS images will be analyzed qualitatively

Risk and Safety

- All work will be done in the Stony Brook Lipid Cancer Lab, which is a BSL-2 Lab.
- Safety courses in handling biological materials and chemicals will be completed prior to entry into the lab
- While in the lab, gloves and a lab coat will be worn at all times
- The cell line that will be used, MDA-MB-231 from ATCC, is a BSL-1 cell line.
- Potentially hazardous substances that will be used are MTT, trypan blue (for cell counting), doxorubicin, 100% dimethyl sulfoxide, and 70% ethanol. Cell culturing and work with MTT, trypan blue and doxorubicin will be done in a bacteriological hood
- Biological waste will be disposed in biohazard disposals. Glass waste will be disposed in a sharp's container. These wastes will be collected and disposed by the Stony Brook University Health & Safety Department

3D. Bibliography

Adan, A., Kiraz Y., & Baran Y. (2016). Cell Proliferation and Cytotoxity Assays. *Current Pharmaceutical Biotechnology, 17*, 1213-1221.

Carroll, B.L., Bonica, J., Schamseddine, A.A., Hannun, Y.A., & Obeid, L.M. (2018). A role for caspase-2 in sphingosine kinase 1 proteolysis in response to doxorubicin in breast cancer cells – implications for the CHK1-supressed pathway. *FEBS Open Bio. 8*, 27-40.

Gorini, S., Angelis, A. D., Berrino, L., Malara, N., Rosano, G., & Ferraro E. (2018). Chemotherapeutic Drugs and Mitochondrial Dysfunction: Focus on Doxorubicin, Trastuzumab, and Sunitinib. *Oxidative Medicine and Cellular Longevity, 2018*, 1-15.

Hannun, Y.A. & Obeid, L.M. (2017). Sphingolipids and their metabolism in physiology and disease. *Nature Reviews Molecular Cell Biology, 19*, 175-191.

Heffernan-Stroud, L. A., Helke, K. L., Jenkins, R. W., De Costa, A., Hannun, Y. A., & Obeid, L. M. (2012). Defining a role for sphingosine kinase 1 in p53-dependent tumors. *Oncogene, 31*(9), 1166-1175.

Jeffries, K. A. & Krupenko, N. I. (2018). Ceramide Signaling and p53 Pathways. *Advances in Cancer Research, 140*, 191-215.

Ma, C. X., Cai, S., Li, S., Ryan, C. E., Guo, Z., Schaiff, W. T., et al. (2012). Targeting Chk1 in p53 deficient triple negative breast cancer is therapeutically beneficial in human-in-mouse tumor models. *Journal of Clinical Investigation, 122*(4), 1541-1552.

Michelena, J., Gatti, M., Teloni, F., Imhof, R., & Altmeyer, M., (2019). Basal CHK1 activity safeguards its stability to maintain intrinsic S-phase checkpoint functions. *Journal of Cell Biology, 218*(8), 2865.

Morad, S.A.F. & Cabot, M.C. (2013). Ceramide-orchestrated signaling in cancer cells. *Nature Reviews Cancer, 13*, 51-65.

Ogretmen, B. (2018). Sphingolipid metabolism in cancer signaling and therapy. *Nature Reviews Cancer, 9*, 139-150.

Patel, A. G. & Kaufmann, S. H. (2012). How does doxorubicin work?. *eLife, 1*.

Qiu, Z., Oleinick, N. L., & Zhang, J. (2018). ATR/CHK1 inhibitors and cancer therapy. *Radiotherapy Oncology, 126*(3), 250-464.

Taha, T.A., Osta, W., Kozhaya, L., Bielawski, J., Johnson, K.R., Gillanders, W.E., et al. (2004). Down-regulation of Sphingosine Kinase-1 by DNA Damage. *The Journal Of Biological Chemistry, 279*, 20546-20554.

NO ADDENDUM EXISTS