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Investigating Anti-Breast Cancer Properties of the Antibiotic Vancomycin-A Drug Repurposing study using *In Silico* Molecular Docking Techniques.

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Abstract:
The incidence of triple-negative breast cancer (TNBC) is approximately 10-15% of all breast cancer cases around the world. It is important to search for novel therapeutic agents against TNBC since this form of cancer is a leading cause of death in women worldwide. Various genetic mutations in TNBC patients have been identified. EZH2 is an oncoprotein involved in breast, prostate, colon, and other cancers. In silico drug design and bioinformatics studies helped to identify the FDA approved antibiotic, vancomycin as a potential inhibitor of the EZH2 oncoprotein. A drug repurposing study of vancomycin to investigate the potential anti TNBC properties was tested by doing cytotoxicity analysis with vancomycin in TNBC cell lines by WST-8 assay, spheroid forming assay and ROS generation experiments. The EZH2 inhibitor property of vancomycin was studied by RT-PCR and ELISA assay. Our initial results showed that Vancomycin effectively prevents cell proliferation, spheroid formation and generates ROS in the TNBC cell lines tested as well as inhibit the EZH2 gene expression.

Key Words: *Triple Negative Breast Cancer, Drug Repurposing, Epithelial Mesenchymal Transition, Molecular Docking.*

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I. Introduction:

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer that lacks estrogen receptors (ER), progesterone receptors (PR), and HER2 amplification. This makes it unresponsive to hormonal or HER2-targeted therapies, which are effective in other breast cancer subtypes. As a result, treatment and prognosis differ significantly, and research into effective therapies is ongoing (1-3).

Epigenetic regulators are naturally occurring compounds in the body that operate within the nucleus of the cell to turn “on” and “off” the expression of multiple genes involved in relevant cellular functions. EZH2 is an epigenetic regulator with an increased expression and activity in many cancer types, which, in general, potentiates cancer growth and expansion (4).When EZH2 regulates cell cycle progression, dysregulation of EZH2 accelerates cell proliferation, and prolongs cell survival which in turn leads to cancer development . Over expression of this oncogene can lead to uncontrolled cell growth because oncogenes can alter transcriptional activity and stability. In the past decade, numerous EZH2 inhibitors treatments have been explored for their anticancer properties (5-8).

A drug repurposing study (also known as drug repositioning or drug reprofiling) involves investigating existing drugs—often already approved for a particular disease—for new therapeutic purposes. This approach offers several advantages over traditional drug discovery, including reduced cost, shorter development timelines, and known safety profiles. Shorter path to market, Lower development costs, known pharmacokinetics and safety profiles and potential for therapeutic intervention for cancer, orphan diseases and rare conditions are advantages of drug repurposing studies(9-11).

Molecular docking is a key computational technique used in drug development to predict how a small molecule (a ligand, typically a drug candidate) binds to a target protein (usually an enzyme or receptor). Molecular docking simulates the interaction between a ligand and a target protein to predict the binding affinity

and binding mode (orientation and conformation). The goal is to find compounds that bind strongly and specifically to the target, potentially modulating its biological function (12-14).

Vancomycin is a tricyclic glycopeptide antibiotic originally derived from the organism Streptococcus orientalis. It is an antibiotic drug used to treat infections caused by bacteria, by killing bacteria or preventing their growth. This drug is in the cell wall synthesis inhibitor class of antimicrobial medications (15). Vancomycin is a medication used in the treatment of serious Gram-positive bacterial infections. Vancomycin can inhibit cell wall synthesis by binding to the D-Ala-D-Ala terminal of the growing peptide chain during cell wall synthesis, resulting in inhibition of the transpeptidase. Once bonded, it then prevents the cell wall from forming the cross-linking necessary cell wall integrity leading to bacterial cell death and inability to reproduce. When vancomycin inhibits the bacterial cell wall it then stops the bacteria growing and dividing properly (16).

In this study we did in -silico molecular docking software studies and discovered Vancomycin binds to the EZH2 oncoprotein, henceforth the objective of this study was to investigate the anti-breast cancer properties of vancomycin and its role in inhibiting EZH2 gene expression.

II. Materials And Methods

Molecular Docking Studies:

Generation of target structure and virtual screening:

Small molecules (SMs) from different libraries: Spectrum, Prestwick, Lopac, Tocriscreen, Selleckchem, SCREEN-WELL, NCI/DTP were used in the virtual screening. The in silico screening was done using AutoDock vina (21)

PDB ID 5HYN1 was used to model a full length EZH2 structure. The modelled systems after solvation in appropriate cubic boxes were electrically neutralized by adding NaCl ions. The ionized system was then minimized for 10,000 steps, equilibrated for 100 ps, and then simulated for 20 ns using the NAMD package.2 The structure after 20 ns of MD simulation was be used as targets for in silico screening. After in silico screening, the SMs were ranked based on their numerical docking affinity values, in a descending order of increasing numerical affinities values (decreasing actual affinity). The top 50 hits were then analyzed for proper 3-D conversion. We finally obtained 13 primary hits, including Vancomycin & Anidulafungin.

Cell Lines

MDA-MB-231 VIM RFP is a fibroblast-like reporter labeled cell line purchased from ATCC (USA) that was isolated from the pleural effusion of a 51-year-old, White, female with adenocarcinoma. This cell line can be used in drug development research. MDA-MB-231 VIM RFP reporter cell line provides a convenient and sensitive platform for research on the mechanisms of metastasis in vitro and the development of new anti-Epithelial Mesenchymal Transition drugs for metastatic breast cancer.

Cell Viability and Cytotoxicity Assay (WST-8 Assay)

MDA-MB-231 VIM RFP cell lines were tested with vancomycin for determining cytotoxic properties of vancomycin. The cancer cells were all plated in a 96-well plate and incubated for 48 hours in a three-trial study. Six wells were plated and each well contained 100µl of cancer cells and 200 µl of media specific to each cell line. After 24 hours of incubation each cell line was treated with vancomycin, and each reacted at a different concentration. After 48 hours of incubation each well is then hit with 10µl of the cytotoxicity dye solution. The plate is then incubated at room temperature for 1 hour. After an hour of incubation, the absorbance reading indicated that more cell death was occurring in the Vancomycin vs. the control.

Spheroid Forming Assay

MDA-MB-231 breast cells were grown to confluence on a Corning Ultra-Low Attachment surface six well plate. The ultralow attachment surface is a hydrophilic, neutrally charged coating covalently bound to a polystyrene vessel surface. The hydrogel inhibits specific and nonspecific immobilization which forces cells into a suspended state and enabling 3D spheroid formation. Vancomycin treated and untreated spheroid numbers were counted using a standard inverted microscope.

Reactive Oxygenated Species

ROS was developed in MDA cell line by plating the cells in two plates (one treated with a concentration of 200ng/mL of vancomycin and one control), After 24 hours of incubation the plates were exposed to dichlorodihydrofluorescein (DCF) to measure the ROS development in the vancomycin vs. control study in the cancer cells by a fluorescent microscope and photographed.

RNA Isolation/cDNA Synthesis

RNA Isolation and cDNA Synthesis are necessary for further investigation using techniques such as RT-PCR. The RNA Isolation was done using a kit (Lamda Biotech, Ballwin, MO, Catalog# D2312-100) via column isolation, and cDNA synthesis was completed using a kit from Lambda Biotech (Ballwin, MO, Catalog # G209) according to the manufacturer's protocols. After completing RNA isolation in the MDA cell line, each concentration was calculated using a spectrophotometer at a wavelength of 260 nm (SpectraMax QuickDrop, Molecular Devices, San Jose, CA) by using 2 µl of dH20 for control and 2µl of RNA for concentration analysis. RNA isolation was then validated by RNA gel electrophoresis. The gel was a ready-made, 1.25% agarose gel, specifically for RNA (.25-10kb) called Reliant Gel System (Lonzo Bioscience, Morrisville, NC, Catalog# 54922). The RNA loading dye was added to RNA samples and heated to 65ºC for five minutes to denature the RNA. Then, the RNA samples were loaded onto the gel was run in 1x MOPS buffer for approx. 20-30 mins. Lambda Biotech's 5X all-in-one master mix for cDNA synthesis contains reverse transcriptase, oligo, and DNase to remove any remaining genomic DNA. According to Lamba Biotech, 1 ng to 2 µg of RNA isolate is used for cDNA synthesis, and total volumes for this cDNA protocol are adjusted to 20µl total using dH2O.

Real-Time- Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed in triplicate using Lamda protocol and 2X Universal SYBR Green Mastermix from Lamda Biotech (Ballwin, MO, Catalog# qMX-Green). The wells were prepared according to the manufacturer's protocol with slight modifications in the cDNA amounts (2µl) placed into each well. However, all final template amounts for RT-PCR were adjusted to 20µl using dH2O. In addition, the SYBR green master mix and primers for β-actin [Housekeeping control] and EZH2 were used according to the protocol.

EZH2 - Enzyme-linked Immunosorbent Assay (ELISA)

To determine the effect of Vancomycin on EZH2 protein activity in MDA cell line investigated, a sandwich ELISA was performed. The ELISA was done using kits from Bio-source, San Diego, CA,(Catalog# MBS162978). The lysate of MDA cell line was added to each of the wells and "sandwiched" by a biotinylated antibody that binds to horseradish peroxidase. Colorimetric substrates were added that reacted with the horseradish peroxidase. The results of the ELISA were determined by absorbance by microplate reader at 450 nm. After analyzing the cell line, each reading subtracted the average reading of blank wells to normalize the results.

Statistics

Student t test and one way ANOVA was used for determining statistical significance of our data obtained.

III. Results

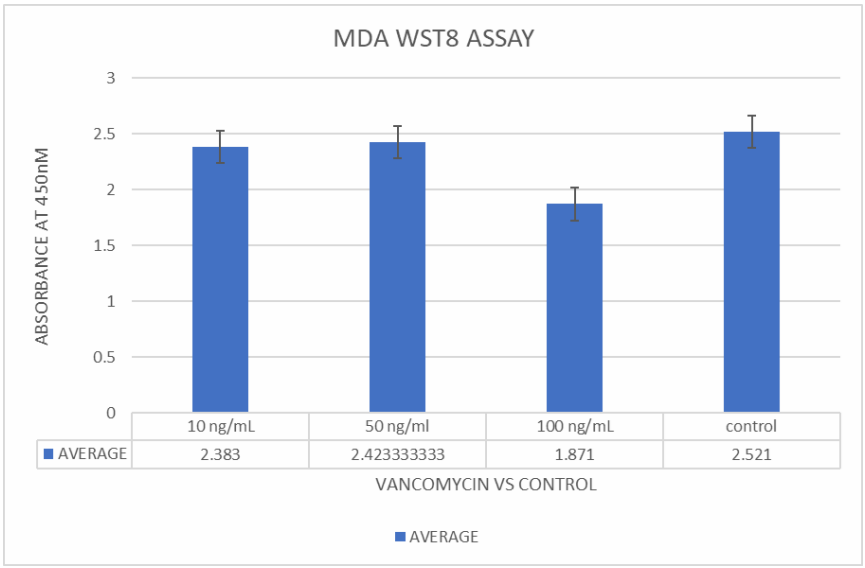
WST-8 absorbance results of MDA cells treated by Vancomycin vs. Control at Absorbance of 450nm. The higher arbitrary unit (AU) reading equates to higher number of viable cells, IC50 was determined to be at 100ng/ml of Vancomycin as shown in Figure 1.

As shown in Figure-2 the spheroid plate treated with vancomycin had less spheroid development than the control, proving that vancomycin can help prevent the formation of spheroid development in MDA cancer cells.

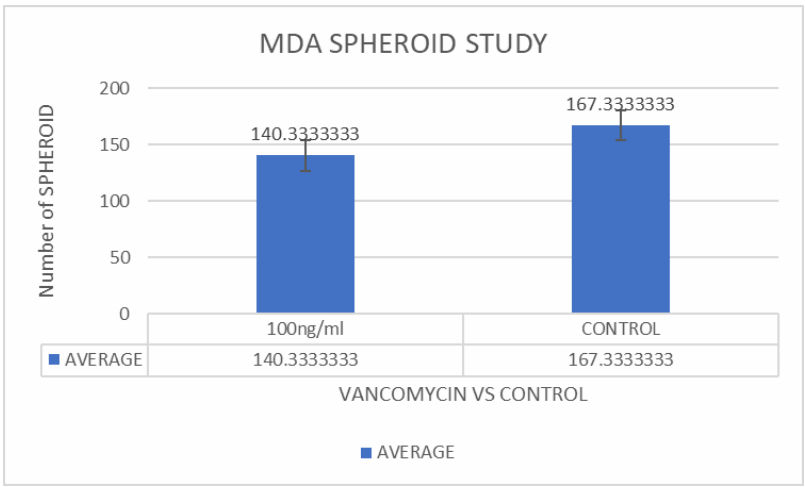
Vancomycin treatment developed more Reactive Oxygenated Species accumulation in the MDA cells as shown in Figure 3.

Vancomycin treatment showed to significantly suppress EZH2 expression when calculating the ΔCt values in the treated samples vs the control as shown in Figure 4.

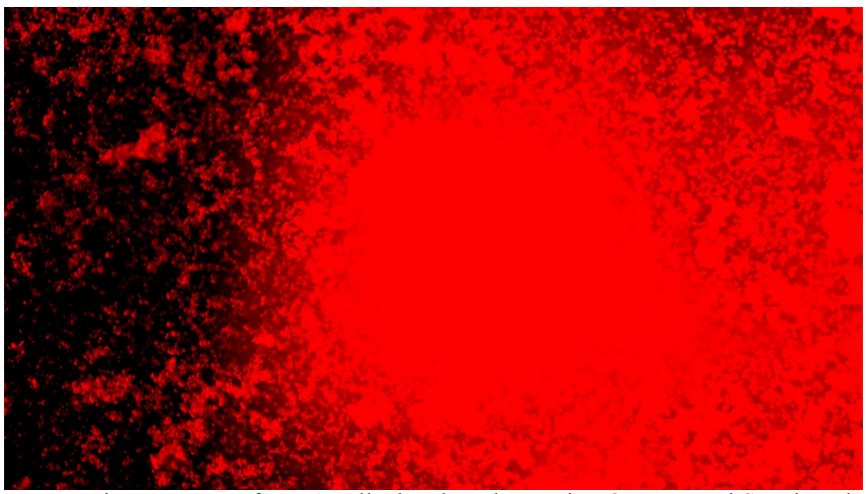
In the ELISA study, EZH2 protein expression was more in the control compared to the vancomycin treated at a concentration of 100 ng/ ml as shown in Figure 5.



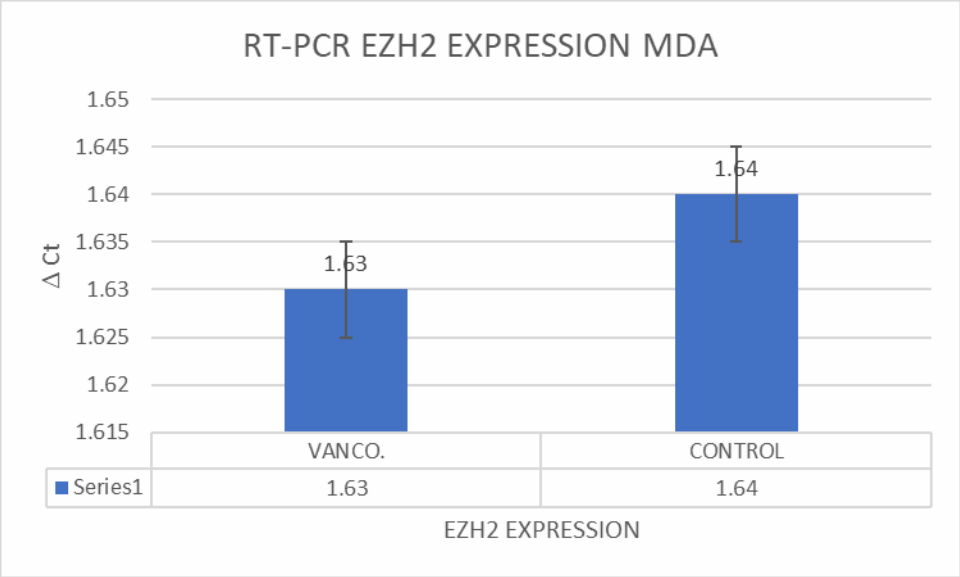
Figure#1 : : WST-8 absorbance assay results of MDA cells treated with Vancomycin vs. Control. Absorbance was determined at 450nm. The higher arbitrary unit (AU) reading equates to higher number of viable cells. The IC50 of Vancomycin was determined to be@100 ng/ml.



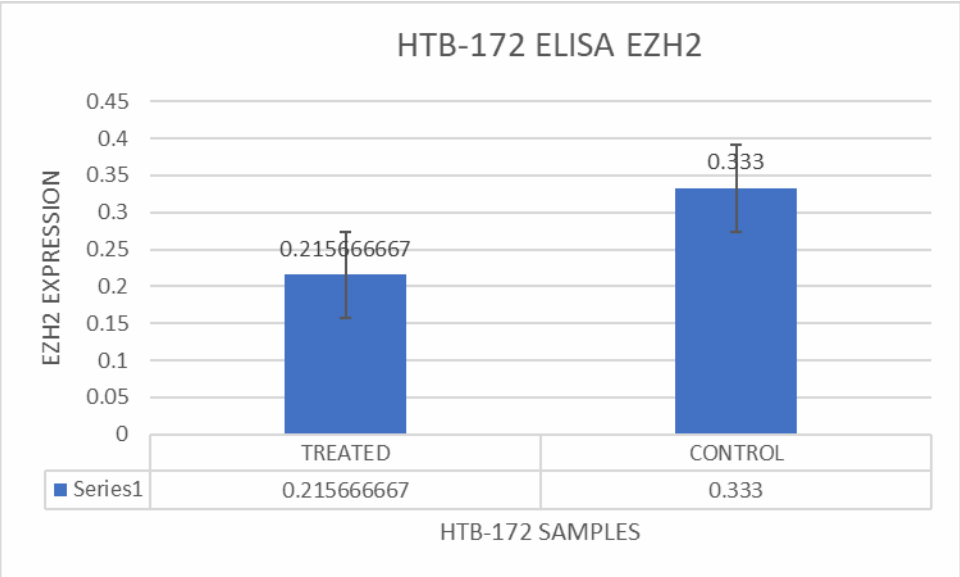
Figure#2 The number of Spheroids in MDA cell line treated with Vancomycin vs. Control after an 18-day interval. As shown in Figure, the MDA plate that was treated with vancomycin is preventing spheroids formation throughout the 18-day interval.



Figure#3: The vancomycin treatment of MDA cells developed Reactive Oxygenated Species The elevated ROS level breaks the redox homeostasis and consequently causes cancer cell death.



Figure#4 The MDA treated Vancomycin cells had shown to significantly suppress EZH2 expression when calculating the ΔCt values in the treated samples vs the control.



Figure#5 In this figure EZH2 protein expression is shown to be more in the control compared to the vancomycin treated at a concentration of 100 ng/ ml.

IV. Discussion:

Triple Negative Breast Cancer (TNBC) is a heterogeneous disease that based on immunohistochemistry (IHC) is estrogen receptor (ER) negative, progesterone receptor (PR) negative and human epidermal growth factor receptor 2 (HER2) negative. TNBC is typically observed in young AA women and Hispanic women who carry a mutation in the BRCA1 gene. TNBC is characterized by a distinct molecular profile, aggressive nature and lack of targeted therapies. The 5-year survival rate for triple-negative breast cancer (TNBC) is 77% when all stages are combined. However, this rate varies significantly depending on the stage of the cancer: 91% for localized TNBC, 66% for regional TNBC, and 12% for distant TNBC(17,18).

Drug repurposing in cancer treatment offers several advantages, including a faster and cheaper route to drug development, leveraging existing safety and pharmacokinetic data, and potentially overcoming drug resistance. Repurposing can also lead to personalized treatment strategies and the identification of novel drug combinations(19,20).

Our investigation by molecular docking studies identified Vancomycin as the potential drug to bind to the oncogene EZH2, we treated TNBC cell lines MDA-MB-231 VIM RFP to test for cytotoxicity, spheroid formation and ROS generation and the drug successfully induced cell death, reduced spheroid formation and

increased ROS generation; we then tested the drugs efficacy in reducing EZH2 gene expression by RT-PCR and protein production by ELISA assay, Vancomycin was able to downregulate EZH2 RNA expression and resultant translation product.

The EZH2 oncoprotein leads carcinoma to Epithelial Mesenchymal Transition and cancer stem cell formation, hence it would be beneficial to investigate the genes connected to the various EZH2 signaling pathways, including the RAF1, MAP2K1/2, ERK1/2, CaMKII, RPS6KA1, IL1B, and CASP1 genes,since Vancomycin is a FDA approved drug and have very minimal harmful side effects, therapeutic advantage of this drug while using synergistically with other anticancer drugs in clinical trials will be the ultimate goal of our future cancer translational research from bench to patient bedside.

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