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Research Article
Investigating Potential Drug Repurposing for Triple Negative Breast Cancer

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Abstract

Background and Objective: Triple Negative Breast Cancer (TNBC) neither expresses estrogen receptor and progesterone receptor nor overexpress human epidermal growth factor receptor-2. To identify compounds that have been approved by the FDA for non-oncology diseases and which may selectively inhibit TNBC cells either individually or in combination and to test combinations of these compounds for synergistic activity for killing or inhibiting the proliferation of selected TNBC cell lines was sought. **Materials and Methods:** For such compounds, a 96-well homogenous cell-based viability assay utilizing the TNBC cell lines MDA-MB-231, MDA-MB-468 and HCC1806 was employed for testing. **Results:** Using this assay, 5-azacytidine, digoxin, pyrvinium pamoate, disulfiram and albendazole were confirmed to possess inhibitory activities with average IC_{50} values of 4.5 μM, 181, 238, 138 and 348 nM in HCC1806, respectively. No synergies were identified for combinations of these compounds, except that of digoxin which may have synergistic effects in HCC1806 cells at some concentrations. Albendazole was active in three different TNBC cell lines and shown to induce apoptosis. **Conclusion:** Some selected FDA-approved non-oncology drugs have cytotoxic/anti-proliferative properties against two TNBC cell lines.

INTRODUCTION

Breast Cancer (BC) is a tumor that initiates in breast tissue of women and can initiate also in breast tissue of men with low percentage. It ranks amongst the most common cancer disease that affects women[1]. According to the American cancer society, there will be an estimated 1,665,540 new cancer cases diagnosed and 585,720 cancer deaths in 2014 in the US[2]. Worldwide, it is estimated that the numbers of women who are diagnosed with BC reach about 1 million every year, with approximately 400,000 women expected to die from this disease. Triple Negative Breast Cancer (TNBC) is a subtype of breast cancer that is characterized by absence of estrogen receptor and progesterone receptor expression as well as absence of HER-2 amplification[3]. About 20% of breast cancer cases can be considered to be triple negative subtype[4]. It is characterized by its biological aggressiveness, short survival time, poor prognosis and lack of a proven therapeutic target, in contrast to hormone receptor positive and ERBB2+ breast cancers[5]. The clinical features of TNBC patients include an increased body weight, metabolic syndrome, a worse prognosis, larger tumor size, rare histologies, elevated mitotic count, aggressive relapses and no specific proven therapeutic target[5]. The TNBC poses a great drug discovery challenge because TNBC lacks a specific therapeutic target[4]. Consequently, more and immediate research is required to develop an effective therapy for treating TNBC patients[5]. Six TNBC subtypes have been described by Chen *et al*.[4] based on gene expression studies of 587 TNBC samples, where each subtype displays a unique gene expression pattern and ontology. They also reported existing cell lines as models representing each of the TNBC subtypes. These subtypes of TNBC also displayed different sensitivities to targeted therapeutic agents[4]. One study conducted by Dent *et al*.[6], compared women with different types of breast cancer, those with triple negative breast cancer had an increased possibility of recurrence and consequently dying within 5 years of first diagnosis, but not thereafter. Furthermore, they found that the pattern of recurrence was also qualitatively different among the triple-negative group, the risk of recurrence is higher at approximately 3 years and declined sharply thereafter[6]. So far, there are limited options for treating triple negative breast cancer[4,5]. It has been noted that triple negative breast cancer subtype is unresponsive to chemotherapy and other anticancer drugs such as; ixabepilone, anthracyclines, platinum and taxanes[5]. Due to the heterogeneity of TNBC and absence of a proven molecular target, treatment of patients with triple negative breast cancer is challenging[7]. There are many clinical trials currently on-going to test the effectiveness of therapeutic compounds such as; small molecules and recombinant proteins as possible treatments for triple negative breast cancer[5,7]. Presently, standard chemotherapy is the only treatment option available for treating TNBC[7]. Recent studies by Perou *et al*.[8] have distinguished five distinct BC subgroups; luminal-A, luminal-B, Basal Like; Breast Cancer (BLBC), HER-2 and normal-like. The TNBC overlaps molecular features with BLBCs that include lack of Estrogen Receptor (ER−), Progesterone Receptor (PR) and ErbB-2/human epidermal growth factor receptor (HER-2−), but BLBC's are also has an over-expression feature of basal epithelial cells and basal cytokeratins (CK 5/6 and CK17)[8]. Drug combinations are widely used in treating disease. Using combination of drugs with different mechanisms or action may be beneficial in treating diseases more effectively. For these therapeutic benefits, drug combinations have been widely used and became an alternative choice for treating the most harmful diseases, such as; cancer and AIDS[9]. Therefore, the objective of current research is to identify selected FDA-approved compounds with activity against TNBC cell lines and identify combinations of these compounds that show synergistic activity (Fig. 1).

MATERIALS AND METHODS

Cell culture maintenance: Triple negative human breast cancer cell lines, MDA-MB-231, MDA-MB-468 and HCC 1806 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured according to specifications. This research was conducted from October, 2013-2014. The cells were grown at 37°C in a humidified 5% CO_2 atmosphere. MDA-MB-231 and MDA-MB-468 adherent cell line cells were cultured in 75 cm² vented tissue culture flasks (Corning Inc., Corning, NY) and maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) obtained from Life Technologies (Grand Island, NY, USA) and supplemented with 10% Fetal Bovine Serum (FBS), 100 units mL⁻¹ penicillin G-streptomycin and 2.0 mM glutamine. HCC1806 adherent cell line cells were cultured in 75 cm² vented tissue culture flasks (Corning Inc., Corning, NY) and maintained in RPMI medium obtained from Life Technologies (Grand Island, NY, USA) and supplemented with 10% Fetal Bovine Serum (FBS), 100 units mL⁻¹ penicillin G-streptomycin and 2.0 mM glutamine. Cells were routinely passaged at a frequency of

Aim 1

FDA screening set actives in TNBC cell line MDA-MB-231

Confirm activity with dose response for IC_{50} in MDA-MB-231

Test confirmed active compounds for potency in TNBC cell line HCC1806

Apoptosis testing of potent compounds to assess mechanism of action

Aim 2

Test combinations of active compounds for synergistic activiy in viabilit assay

Apoptosis testing of synergistic combinations for mechanism of action

Aim 3

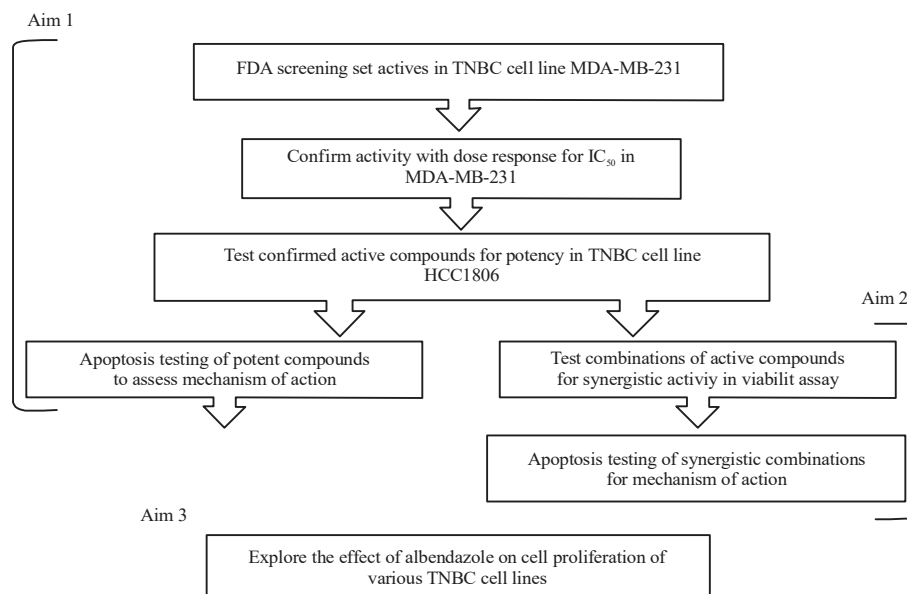Explore the effect of albendazole on cell proliferation of various TNBC cell lines

Fig. 1: Project flow chart

two times per week and cell lines harvested with trypsin following washing with Ca/Mg free Dulbecco's Phosphate Buffered Saline (DPBS).

Dose response curves and IC_{50} determination: Cells were seeded into 96 well assay plates in 90 µL medium and cultured overnight at 37°C in humidified 5% CO_2 atmosphere. Due to its rapid growth, HCC1806 cells were seeded in triplicate at 3000-5000 cells per well (90 µL) in a 96-well plate. MDA-MB-231 and MDA-MB-468 cells were plated in triplicate at 10,000 cells per well (90 µL) in a 96-well plate. Compound preparation was done by using the following procedure: 3-fold serial dilutions of initial 100% DMSO stock compound solutions were performed in 100% DMSO. The 1:100 intermediate dilutions were performed by the addition of 5 µL of diluted compound to 495 µL of suitable growth media i.e., 1:100 dilution factor. The cells were treated with 10 µL of a corresponding intermediate dilution providing a dilution factor of 1:10 and a total dilution factor of 1:1000 (0.1% final DMSO). Cells then incubated at 37°C in humidified 5% CO_2 atmosphere for 48-72 h. After 48-72 h incubation period, plates were treated with 10 µL of resazurin (PrestoBlue® reagent). Plates were shaken briefly for 30 sec by using IKA MTS4 plate shaker before the plates were returned to 37°C in 5% CO_2 for 90 min to allow viable cells to convert resazurin into fluorescent resorufin. Fluorescence was determined using 560 nm excitation and 590 nm emissions filters by using BMG PheraStar plate reader. The data obtained from plate reader were normalized using the following formula of percent activity:

$$\text{Activity (\%)} = \frac{\text{Experimental} - \text{Min}}{\text{Max} - \text{Min}} \times 100$$

where, experimental represents the effect of the compound dose Maximum represents the average fluorescence of DMSO only treated wells (solvent control). Minimum represent the average fluorescence of a "0 cell" control. The half-maximum inhibition concentrations (IC_{50}) were calculated using non-linear regression analysis with 3 or 4 parameter curve fits (GraphPad Prism v.4.0, GraphPad Software, San Diego, CA, USA).

Synergy assays: HCC1806 cells were seeded in triplicate into 96-well plates and cultured overnight using the standard protocol outlined above. Cells were treated with compounds, equal-molar mixtures of compounds were generated by mixing equal volumes of the 10 mM 100% DMSO stock solutions. This mixture was then treated as a compound with total concentration of 10 mM which was diluted as described previously (0.1% final DMSO concentration). The assay plate was incubated for 72 h. Fluorescence intensities were read at 560 nm excitation/590 nm emission after 1.5-2 h incubation. Dose response curves for each drug combination were obtained by using GraphPad prism-4 software.

Data analysis: GraphPad-Prism (version 4 and 5) was used to create IC_{50} curves (non-linear regression analysis), means, standard deviation of all replicates and graphs. Microsoft Excel was used to calculate mean, standard deviation, coefficient variation (%) and Z-factors. CompuSyn (version 1) was used to

determine synergy, this is the third generation of computer software developed by Nick Martin of MIT, Cambridge MA in 2005. This program was used in this project to quantify synergy using the Chou-Talalay method.

Flow cytometry apoptosis assay: Apoptosis FITC Annexin V detection kit was obtained from BD Pharmingen (San Jose, CA, USA). HCC1806 cells were seeded into 6-well assay plates in 2 mL volumes to give 400,000 cells/well and cultured overnight at 37°C in humidified 5% CO_2 atmosphere. Compound preparation was done using the following procedure: About 10 mM of albendazole was diluted in 100% DMSO to make 1 mM of albendazole. A 1:100 intermediate dilution was performed in the diluted material as 20 µL of the compound in 1980 µL of RPMI growth media. A 1:100 intermediate dilution was performed in the diluted material as of 20 µL of DMSO in 1980 µL of RPMI growth medium and used as control. Ten milliliter treatment stock of albendazole and diluted DMSO, as control was created by adding 1 mL of albendazole from intermediate dilution to 9 mL RPMI culture medium and the same procedure was used with DMSO. Three wells were treated with 2 mL of a corresponding final dilution providing 1 µM of albendazole as final concentration and the other three wells were treated with DMSO as control to get 0.1% of DMSO as final concentration. Cells then incubated at 37°C in humidified 5% CO_2 atmosphere for 24-48 h. After 24-48 h incubation period, plates were prepared for apoptosis assay. Cells were washed twice with cold PBS and then re-suspended in 1×binding buffer, 100 µL of the solution was transferred to 5 mL culture tube, then 5 µL of conjugated FITC Annexin V and 5 µL of Propidium Iodide (PI) were added. Then cells were vortexed and incubated for 15 min at room temperature in the dark. After 15 min, the cells were analyzed by flow cytometry. Three controls were used for flow cytometry: unstained cells, cells stained with FITC annexin V only and cells stained with PI only.

RESULTS AND DISCUSSION

Confirmation of hit compounds in MDA-MB-231: The selected compounds were identified from the Prestwick chemical library using the MDA-MB-231 cell line during previous studies in the Scott lab using High Throughput Screening (HTS) in a synthetic lethal screen. Compounds that were found to be more than 50% active have unknown anti-cancer activities and FDA approved were selected for further screening with different TNBC cell lines. Table 1 showed set of FDA approved drug and their screen hits using MDA-MB-231 with their percentage inhibition values.
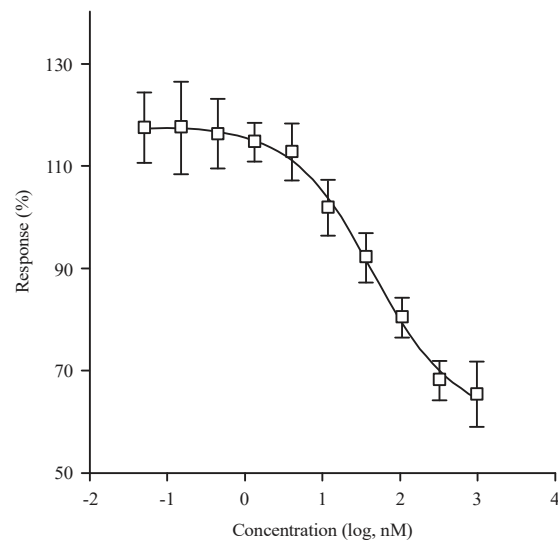

Fig. 2: Effect of dasatinib on MDA-MB-231 cell viability

Table 1: Compounds identified in a primary screen in MDA-MB-231 cells

Compounds	Inhibition (%)	N
Hydrochlorothiazide	56	1
Amlodipine besylate	55	2
Digoxin	61	2
Pyrvinium pamoate	53	2
Quinacrine	50	2
5-azacytidine	60	2
Thiethylperazine malate	58	2

In order to confirm activity of the compounds in the MDA-MB-231 cell line it was determined their IC_{50} values in a resazurin-based viability assay. The incubation time for cells treated with the test compounds was 48-72 h. Cell treatment was performed in a 96-well plate. In order to test the compounds in different TNBC cell lines and due to different growth rates between cell lines, it was optimized cell number to be plated from previous studies done in the Scott lab. Optimal cell numbers were seeded onto assay plates with different cell lines and allowed to incubate for 48-72 h. For the MDA-MB-231 cell line, 10,000 cells/wells were used, for the HCC1806 cell line, 3000-5000 cells/wells were used. Previous studies have shown that dasatinib potently inhibits the growth of most TNBC cell lines[10]. Dasatinib (das) is used at the beginning of these studies as an assay method control compound. A dose response curve for dasatinib was generated (Fig. 2). The observed IC_{50} was 46 nM which was similar to that observed by Pichot *et al.*[11], in which they arrived at an IC_{50} of 37 nM when using the same cell line. The IC_{50} of the dasatinib is a relative IC_{50}, rather than absolute. The IC_{50} curve did not get to zero, but showed a maximal ~50% inhibition effect on the cells which suggested that dasatinib is not toxic to the cells, but rather prevented cell growth. This
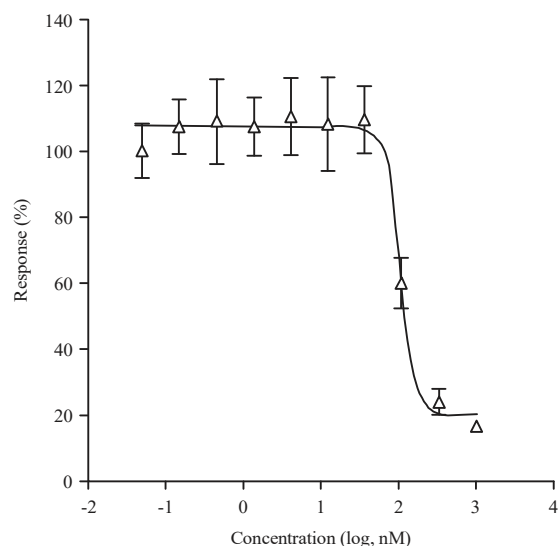
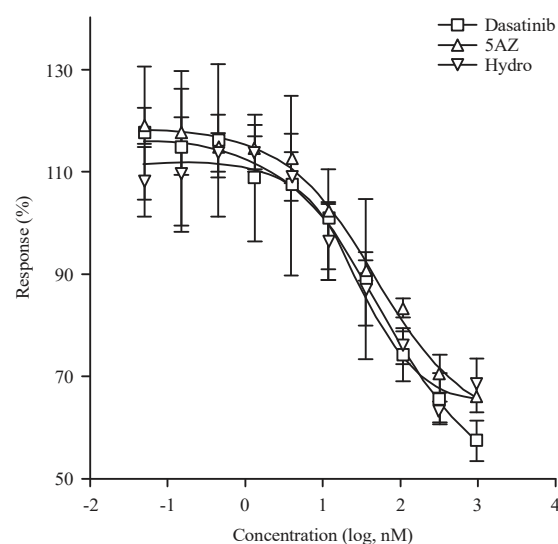Fig. 3: Effect of digoxin on MDA-MB-231 cell viability

Fig. 4: Effect of dasatinib, 5-azacytidine and hydrochlorothiazide on MDA-MB-231 cell viability

Table 2: IC$_{50s}$ of dasatinib, digoxin, emetine, pyrvinium, 5-azacytidine, hydrochlorothiazide and quinacrine in MDA-MB-231

Compounds	IC$_{50}$ (nM)$\pm$SD	N
Hydrochlorothiazide	55.0$\pm$33	2
Dasatinib	55.0$\pm$33	6
Digoxin	274.0$\pm$156	3
Pyrvinium pamoate	778.0$\pm$178	4
Quinacrine	1562.0$\pm$619	2
5-azacytidine	2325.0$\pm$122	4

data is consistent with the anti-proliferative activity that has been described for TNBC cell lines in the literature[12] and previously in the Scott lab[10].

For the purpose of current research the following compounds were selected from previous studies in the Scott lab and tested in MDA-MB-231 cell line to confirm the previous results, digoxin, 5-azacytidine, emetine dihydrochloride, quinacrine dihydrochloride, primaquine bisphosphate, pyrvinium pamoate, amlodipine besylate and thiethylperazine malate were first tested in MDA-MB-231 cell line, disulfiram was tested in HCC1806 only, albendazole and its metabolites albendazole-sulfoxide were tested in HCC1806, MDA-MB-231 and MDA-MB-468. A dose response curve for digoxin was generated (Fig. 3). The observed IC$_{50}$ was 107 nM. This was an absolute IC$_{50}$ because it gives the concentration of compound where the response is reduced in half[13]. The IC$_{50}$ indicated that digoxin is active against the MDA-MB-231 cell line.

A representative dose response in MDA-MB-231 cells is shown with a starting concentration of 1 μM. Using data normalized to in-plate controls, the average IC$_{50}$ value from 3 experiments was determined to be 274$\pm$156 nM for digoxin. A dose response curve for 5-azacytidine and hydrochlorothiazide was generated (Fig. 4). The observed relative IC$_{50}$ for this particular replicate when using 1 μM starting concentration were 51 and 31 nM, respectively. The absolute IC$_{50}$ values are >1 μM. The IC$_{50}$ values confirmed that 5-azacytidine and hydrochlorothiazide may hold some possible activity against the MDA-MB-231 cell line and selected for further testing in HCC1806.

A representative dose response in MDA-MB-231 cells is shown with a starting concentration of 1 μM. Using data normalized to in-plate controls, the average IC$_{50}$ values were determined to be 2325$\pm$122 and 55$\pm$33 nM for 5-azacytidine and hydrochlorothiazide, respectively. IC$_{50}$ average values for digoxin, pyrvinium pamoate, 5-azacytidine and hydrochlorothiazide in MDA-MB-231 were 274$\pm$156, 778$\pm$178, 2325$\pm$122 and 55$\pm$33 nM, respectively. It is necessary to mention that when 5-azacytidine was tested using starting concentration of 10 μM, it gave an average IC$_{50}$ of 2325$\pm$122 nM, but when tested using starting concentration of 1 μM, it gave an average IC$_{50}$ below 100 nM as shown in Table 2. Table 2 summarized the 6 out of 9 compounds that showed activity against the MDA-MB-231 cells in this assay, while primaquine bisphosphate, amlodipine besylate and thiethylperazine malate have not shown inhibitory effects on MDA-MB-233 and were excluded from being tested in HCC1806.

Confirmation of selected compounds activities in HCC1806:
Compounds tested in MDA-MB-231 cell line and found active against, it were tested in the HCC1806 cell line to assess
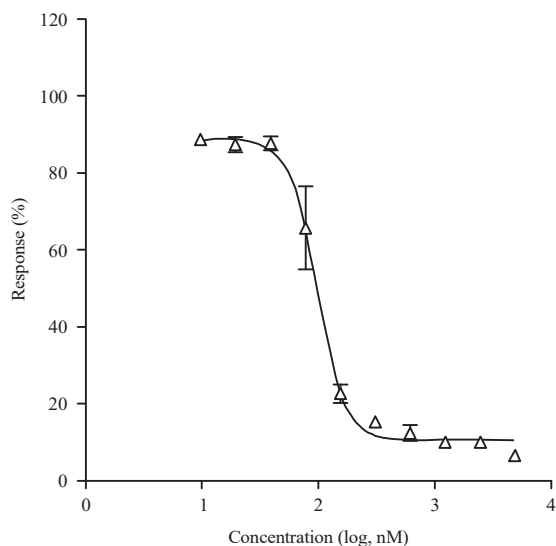
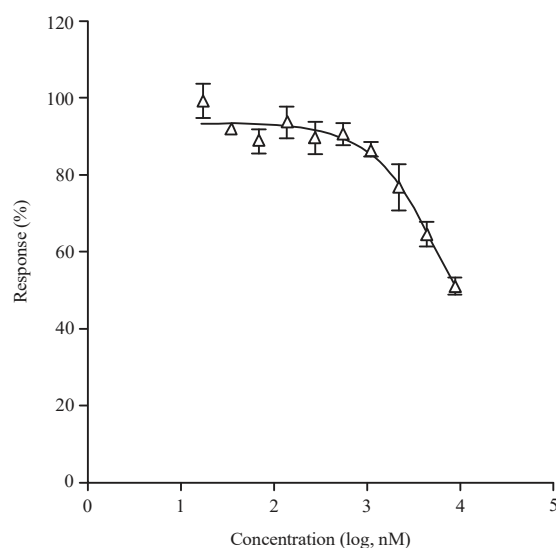Fig. 5: Effect of digoxin on HCC1806 cell viability

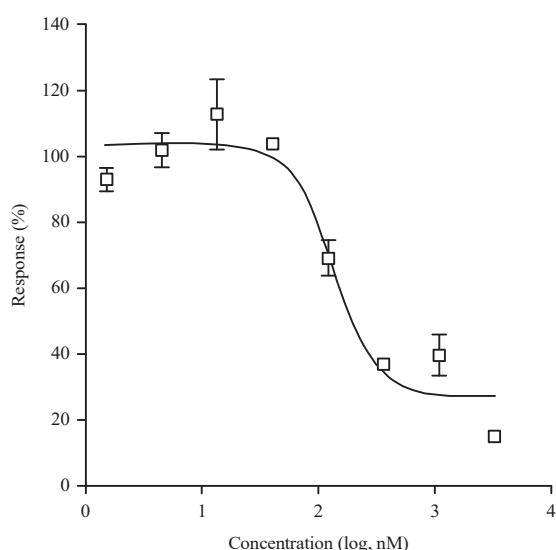Fig. 7: Effect of 5-azacytidine on HCC1806 cell viability

Fig. 6: Effect of pyrvinium on HCC1806 cell viability

activity in abroad panel of triple negative breast cancer cell lines. IC_{50} determinations were performed with these compounds. Digoxin is used to treat heart failure and abnormal heart rhythms (arrhythmias), it helps the heart work better by increasing its contractility and it helps control heart rate[14]. The dose response curve for digoxin was generated and found to be active in HCC1806 cell line with an average IC_{50} of 181±67 nM (Fig. 5). This compound achieved >80% inhibition that may indicate a cytotoxic mechanism, rather than just anti-proliferative.

A representative dose response in HCC1806 cells is shown with a starting concentration of 10 µM. Using data normalized to in-plate controls, the average IC_{50} value from

11 experiments was determined to be 181±67 nM for digoxin. A representative dose response in HCC1806 cells is shown with a starting concentration of 10 µM. Using data normalized to in-plate controls, the average IC_{50} value from 10 experiments was determined to be 138±64 nM. Pyrvinium pamoate is used in the treatment of pinworm infection[15]. A dose response curve was generated and the compound was found to be active in HCC1806 cell line with an average IC_{50} of 238±144 nM (Fig. 6). This compound again achieved >90% inhibition, this is showing some cytotoxic effect, rather than just anti-proliferative.

A representative dose response in HCC1806 cells is shown with a starting concentration of 10 µM. Using data normalized to in-plate controls, the average IC_{50} value from 5 experiments was determined to be 238±144 nM. The 5-azacitidine is drug that is FDA approved for the treatment of myelodysplastic syndrome[16]. The dose response curve for 5-azacytidine was generated and the compound was found to be active in HCC 1806 cell line with an average absolute IC_{50} value of 4515±853 nM (Fig. 7).

A representative dose response in HCC1806 cells is shown with a starting concentration of 10 µM. Using data normalized to in-plate controls, the average IC_{50} value from 4 experiments was determined to be 4515±853 nM. Disulfiram is used as alcohol deterrent[17]. This compound was tested only in HCC1806 since, it was a hit in a different high through put screen performed in the Scott lab. A dose response curve was generated and the compound was found to be active in HCC1806 cell line with an average relative IC_{50} of 138±6 nM. This is partial curve with only ~40% inhibition, showing mild anti-proliferation effect (Fig. 8).
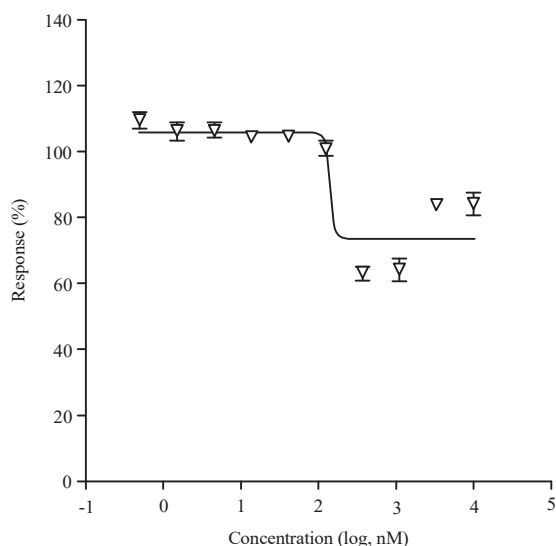
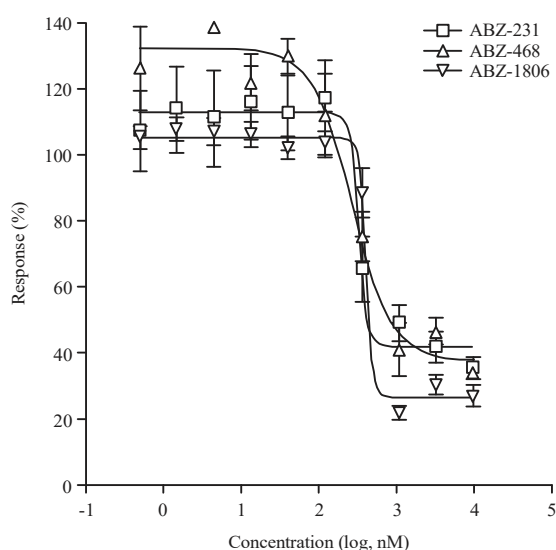Fig. 8: Effect of disulfiram on HCC1806 cell anti-proliferation

Fig. 9: Effects of albendazole on cell inhibition using MDA-MB-231, MDA-MB-468 and HCC1806

Table 3: IC$_{50s}$ of disulfiram, digoxin, pyrvinium, albendazole and 5-azacytidine in HCC1806

Compounds	IC$_{50}$ (nM)$\pm$SD	N
Disulfiram	138.0$\pm$6	4
Digoxin	181.0$\pm$67	11
Pyrvinium pamoate	238.0$\pm$144	5
Albendazole	348.0$\pm$59	7
5-azacytidine	4514.0$\pm$853	4

A representative dose response in HCC1806 cells is shown with a starting concentration of 10 µM. Using data normalized to in-plate controls, the average IC$_{50}$ value from 4 experiments was determined to be 138$\pm$6 nM.

Effect of albendazole on selected TNBC cell lines: Tubulin protein became a new target for treating cancer cells in addition to its previous proved antiparasitic target. It recently has attracted attention as antitumor agents for chemotherapeutic use. It has been shown that albendazole inhibits tubulin polymerization. Previous studies by Pourgholami *et al.*[18] have shown that albendazole inhibits growth in some hepatocellular carcinoma cell lines, in which they arrived at an IC$_{50}$ of 100 nM when using some HCC cell lines, but not any TNBC cell lines. MDB-MB-231, MDB-MB-468 and HCC1806 cell lines were all treated with albendazole. The compound was found to be active in all TNBC cell lines tested with an average IC$_{50}$ of 338, 276 and 348 nM for MDA-MB-231, MDA-MB-468 and HCC1806, respectively (Fig. 9). However, when we tested albendazole-sulfoxide, a major albendazole metabolite, no inhibitory effect on cell lines was observed. This suggested that more work may be needed to reformulate albendazole before being tested in TNBC.

A combined representative dose response in cell lines tested is shown with a starting concentration of 10 µM. Using data normalized to in-plate controls, the average IC$_{50}$ values from 3 experiments were determined to be 338 nM, 276 nM and 348 nM for MDA-MB-231, MDA-MB-468 and HCC1806, respectively. Table 3 summarized that disulfiram, emetine, digoxin, pyrvinium, albendazole and 5-azacytidine showed possible activity against the HCC1806 cells in this assay.

IC$_{50}$ determinations of combinations of compounds in HCC1806: The compounds that were confirmed actives in MDA-MB-231 and HCC1806 were further tested in combination based on their IC$_{50s}$ values generated from HCC1806. The compound that were tested included 5-azacytidine, digoxin, pyrvinium pamoate, disulfiram and albendazole.

Combination tests: HCC1806 cells were seeded at 3×10^3 cells/well in 96-well plate. After 24 h, cells were treated in triplicate with 3-fold serial dilutions of individual compounds and their combination at approximately the same ratio as their IC$_{50}$ ratios determined previously. Thus, the compounds were mixed at 1:1, 1:2 or 1:3 ratios depending on the IC$_{50s}$ of the tested individual compounds. The combined compound was formed by mixing volumes of equal concentration (10 mM) stock in 100% DMSO, so the total compound concentration remained at 10 mM. After 3 days, cells were treated with PrestoBlue® reagent and read by using PheraStar. Dose response curves were generated and IC$_{50s}$ calculated using GraphPad program. Combination Indices (Cis)
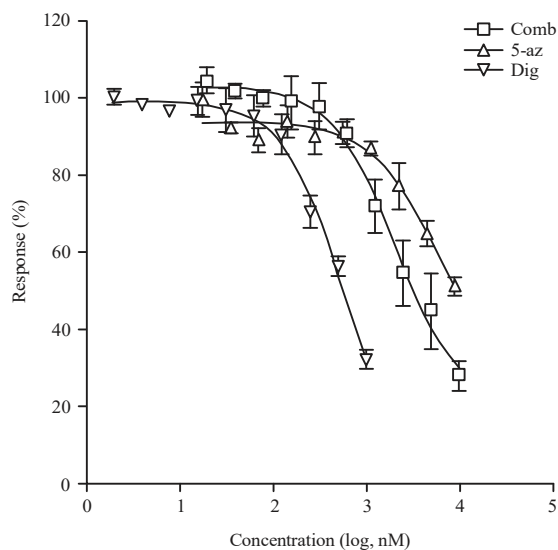
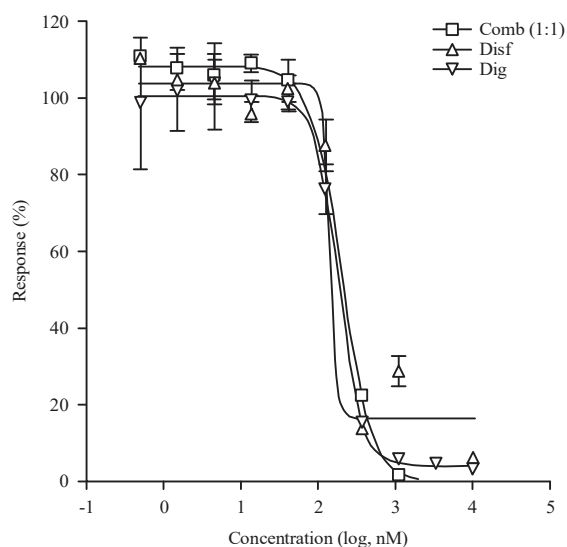Fig. 10: Effects of 5-azacytidine, digoxin and combination in HCC1806

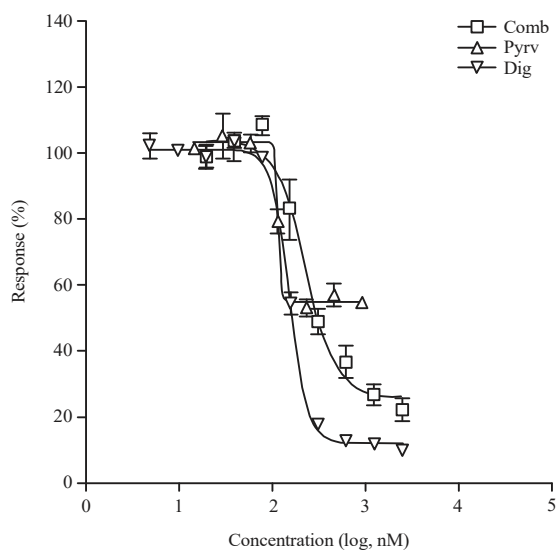Fig. 11: Effects of pyrvinium, digoxin and their combination in HCC1806

Fig. 12: Effects of disulfiram, digoxin and combination in HCC1806

were obtained using the method of Chou and Talalay when the compounds appeared to potentially have a synergistic effect. A representative dose response in HCC1806 cells is shown with a starting concentration of 10 μM. Using data normalized to in-plate controls, the average IC_{50} value from 2 experiments were determined to be 134 nM for disulfiram and 154 nM for their combination. HCC1806 cell line was treated with digoxin, 5-azacytidine and their combination in 1:3 ratio. The dose response curve was generated and the compound was found to be active in HCC1806 cell line with

IC_{50} of 5.4 μM for 5-az, 521 nM for digoxin and 2.0 μM for their combination. There was no apparent synergy observed in this combination (Fig. 10).

A representative dose response in HCC1806 cells is shown with a starting concentration of 10 μM. Using data normalized to in-plate controls, the average IC_{50} value from 2 experiments were determined to be 5.4 μM for 5-azacytidine, 521 nM for digoxin and 2.0 μM for their combination. HCC1806 cell line was treated with pyrvinium, digoxin and their combination in 1:2 ratio for their combination. A dose response curve was generated and the compounds were found to be active with IC_{50} of 117 nM for pyrvinium, 153 nM for digoxin and 235 nM for their combination. The curve did not show synergism, in this particular combination (Fig. 11).

A representative dose response in HCC1806 cells is shown with a starting concentration of 10 μM. Using data normalized to in-plate controls, the average IC_{50} value from 2 experiments were determined to be 117 nM for pyrvinium, 153 nM for digoxin and 235 nM for their combination. HCC1806 cell line was treated with digoxin, disulfiram and their combination in 1:1 ratio. A dose response curve was generated and the compounds were found to be active with IC_{50} of 144 nM for disulfiram, 181 nM for digoxin and 200 nM for their combination. The curve indicated that combination appeared to be toxic to the cells (Fig. 12).

A representative dose response in HCC1806 cells is shown with a starting concentration of 10 μM. Using data normalized to in-plate controls, the average IC_{50} value from 2 experiments were determined to be 144 nM for disulfiram, 181 nM for digoxin and 200 nM for their combination.

Investigating mechanisms of action of albendazole

Apoptosis assay: It is tested that the ability of albendazole to induce apoptosis in HCC1806 by treating cells with 1 µM of albendazole for 24 h. Cells were stained with conjugated FITC Annexin V and Propidium Iodide (PI). Cells negative for both PI and Annexin V are live cells, PI-negative and Annexin V positive are apoptotic cells, while dead cells are positive for both. The percentage of apoptotic cells in the DMSO control was found to be 3.94% (n = 6, SD =1.1), while the percentage of apoptotic cells in the treated sample was 9.2% (n = 6, SD = 1.12). The data indicated that treated cells displayed a two-fold increase in the number of apoptotic events after 24 h compared to DMSO treated cells with a $p < 0.0001$ (Fig. 13 and 14).

Cells were treated with 10 µM albendazole or control DMSO for 24 h. The graph displays the mean percentage±SD of annexin V-positive/PI-negative. There was a two-fold difference observed after 24 h of treatment (n = 6) (**$p < 0.0001$).

These graphs are representative of flow cytometry results for HCC1806 cells treated with 10 µM albendazole (right) or DMSO (left) for 24 h. The abscissa and ordinate represented the fluorescence intensity of annexin V FITC and Propidium Iodide (PI), respectively.

Homogeneous assays for screening compounds have been used extensively by pharmaceutical companies and researchers to identify novel drugs for treating diseases. A homogeneous cell-based assay was utilized towards re-purposing known drugs that have anti-cancer properties. This study sought that combinations of known drugs would have synergistic activity against TNBC. Selective combinations of these drugs were tested for synergistic activity in killing or inhibiting proliferation of TNBC cell lines. Digoxin is used to treat heart failure and abnormal heart rhythms (arrhythmias). It helps the heart work better and it helps control heart rate by its positive inotropic mechanism of increasing intracellular Ca^{2+} concentration leading to contractions of heart muscle[14]. However, it may hold greater potential than it is currently employed for. To explore this possibility, this study set out testing the compound against two TNBC cell lines, MDA-MB-231 (Fig. 5) and HCC 1806 (Fig. 5). Results from inhibition studies clearly demonstrated that MDA-MB 231 and HCC 1806 are profoundly inhibited by digoxin. This was manifested by obtaining average IC_{50s} of 274±156 and 181±67 nM in MDA-MB-231 and HCC1806, respectively. This data indicated a potential novel use for digoxin for TNBC. Data obtained by this study is consistent with a previous *in vitro*
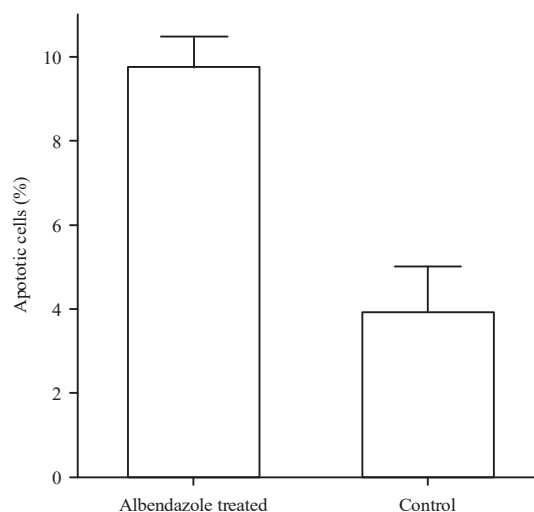

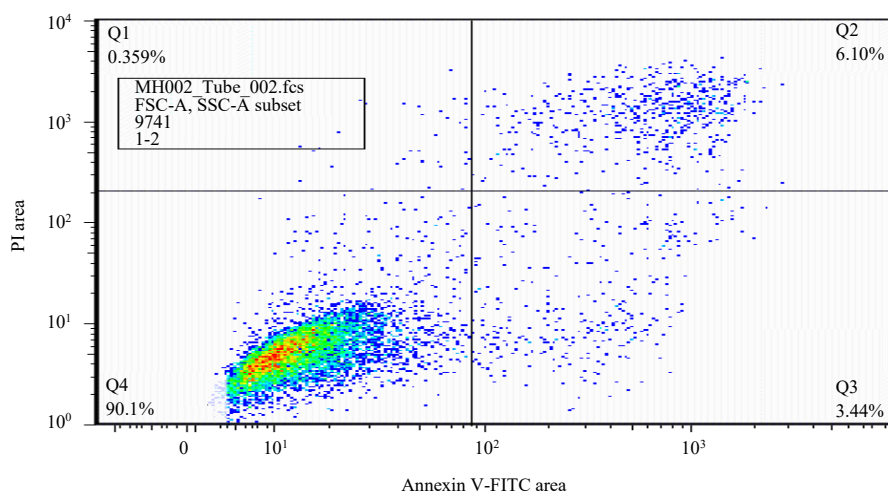Fig. 13: Induction of apoptosis in HCC1806 by albendazole

Fig. 14: Induction of apoptosis in HCC1806 by albendazole

report that digoxin was active against prostate cancer cell lines both *in vitro* and in an *in vivo* animal study[19]. Pyrvinium pamoate is used in the treatment of pinworm infection. This compound achieved >90% inhibition suggestive of a cytotoxic mechanism as opposed to just anti-proliferative. This was obtained by dose response curves using MDA-MB-231 and HCC1806 and obtaining an average IC_{50} of 778±178 (Table 2) and 238±144 nM (Fig. 6), respectively. It showed possible inhibitory effect of pyrvinium pamoate in both cell lines being tested. The combinations of digoxin with 5-azacytidine, pyrvinium and disulfiram showed antagonistic effects rather than synergistic effects. The single agent activity is consistent with reports that showed pyrvinium has anti-cancer activity in some BC cell lines, mainly SUM149 and SUM 159 cell lines[15]. However, further testing of different TNBC cell lines as well as animal model studies would be needed for further investigations into re-purposing this drug for TNBC.

5-azacytidine is used in the treatment of refractory anemia[16]. This compound has been investigated in the lab to study its potential to treat TNBC. This study tested this drug against two TNBC lines MDA-MB-231 and HCC1806. The results obtained showed possible inhibitory effect of 5-azacytidine with IC_{50} values of 2325±122 nM (Table 2) and 4514±853 nM (Fig. 7) in MDA-MB-231 and HCC1806, respectively. The 5-azacytidine is unlikely to be useful since, it is not very potent or may useful if it is combined with some other compound.

Disulfiram functions as an alcohol deterrent by interfering with ethyl alcohol metabolism causing unpleasant side effects and consequently discouraging the use of alcohol[17]. This compound has been investigated in the Scott lab for its inhibitory effect on TNBC cell lines. In addition, this activity was confirmed against TNBC cell lines as reported by Robinson *et al.*[20]. This study tested the compound mainly in HCC1806. This compound may hold possibility of being repurposed for treating TNBC. The results obtained showed inhibitory effect of disulfiram with an IC_{50} value of 138±6 nM obtained in HCC1806 (Fig. 8). This is a new use for disulfiram that increases its potential as a TNBC drug. Unfortunately, no synergism was observed in combination with any of the other compounds. However, further investigations are necessary to identify drugs that are synergistic with disulfiram. Synergism is the interaction of two or more drugs when their combined effect is greater than the sum of the effects seen when each drug is given alone. All possible hit compounds have been tested to investigate possibility of synergistic effects. More experiments would be needed to confirm synergy, possibly using two-fold serial dilutions instead of three and testing at

shorter exposure times. Also, using other assay methods to measure cell death may help determine synergy. If confirmed, this would be a novel combination of already approved drugs. However, further testing of different TNBC cell lines as well as animal model investigations would be needed. Albendazole is a drug that has been used in the treatment of parasitic diseases. It works by inhibiting the polymerization of tubulin and it's found to inhibit proliferation of some HCC cell lines *in vitro* and *in vivo* by more than 90%[18]. To explore this possibility and confirmed this screening hit, this study set out testing the drug against different TNBC cell lines. Results from inhibition studies clearly demonstrated that MDA-MB 231, MDA-MB-468 and HCC1806 are profoundly inhibited by albendazole (Fig. 9). The IC_{50} values of approximately 476 nM were obtained in all TNBC cell lines tested. This is novel data for albendazole since prior to this research, albendazole had only been tested against a single non-TNBC cell line. The potential efficacy and inhibitory activity of the drug in other forms of cancer is also under investigation[7]. However, further testing by using different TNBC cell lines *in vitro* and activity against human TNBC tumor growth in mouse models are needed to justify human trials. However, testing the metabolite of albendazole, albendazole-sulfoxide have shown no inhibitory effect on cell lines tested, suggested that perhaps the drug may be reformulated for delivery to the tumor. Moreover, albendazole has significant ability to induce programmed cell death in HCC1806 with an average of 3.94% for control and 9.2% for treated cells (Fig. 13). Hence, this data suggested that albendazole may have potential utility as an anti-TNBC agent.

CONCLUSION

The results revealed that some selected FDA-approved non-oncology drugs have cytotoxic/anti-proliferative properties against two TNBC cell lines. The results have confirmed that 5-azacitidine, digoxin, disulfiram and pyrvinium pamoate can inhibit proliferation and/or viability of TNBC cell lines MDA-MB-231 and HCC1806 with IC_{50} values ranging from 52-2077 nM. Albendazole has potential of being as anti-cancer drug for TNBC.

SIGNIFICANCE STATEMENT

This study discovers the albendazole that can be beneficial for TNBC treatment. This study will help the researcher to uncover the critical areas of safe cancer treatment that many researchers were not able to explore. Thus, a new theory on drug repositioning may be arrived at.

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