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Implementation of the Chou-Talalay method for studying the *in vitro* pharmacodynamic interactions of binary and ternary drug combinations on MDA-MB-231 triple negative breast cancer cells

Ahmed Elwakeel[a], Hadeer Soudan[a], Ahmed Eldoksh[a], Manal Shalaby[b,] Maha Eldemellawy[b,c], Doaa Ghareeb[c,d], Myriam Abouseif [e], Amira Fayad[e,] Mostafa Hassan[f], Hesham Saeed[*a]

[a*]Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University, Alexandria, Egypt.

[b] Genetic Engineering and Biotechnology Research Institute (GEBRI), City for Scientific Research and Technology Applications, New Borg Al-Arab City, Alexandria, Egypt.

[c] Center of Excellence for Drug Preclinical Studies, City for Scientific Research and Technology Applications, New Borg Al-Arab City, Alexandria, Egypt.

[d] Department of Biochemistry, College of Science, Alexandria University, Alexandria, Egypt.

[e] Department of Clinical Pathology, College of Medicine, Alexandria University, Alexandria, Egypt.

[f] Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University, Alexandria, Egypt.

*****Author to whom correspondence should be addressed; E-Mail: hesham25166@alexu.edu.sa; hesham25166@hotmail.com; hesham25166@yahoo.com

Postal Address: 163 Horyya road Elshatby - P.O:832 - Postal Code: 21526 - Alexandria -Egypt.

Tel.:002035914285 Fax: 002035920956

Abstract

Triple Negative Breast Cancer (TNBC) treatment is more challenging than other subtypes of breast malignancy and due to the lack of markers for the molecularly targeted therapies (ER, PR, and HER-2/Neu), the conventional chemotherapeutic agents are still the mainstay of the therapeutic protocols of its patients. Unfortunately, the initial good response to the chemotherapy eventually turns into a refractory drug-resistance, therefore; more efficient therapeutic regimens are urgently required. Here, we examined the single and combined cytotoxicity of PU-H71, Dehydroepiandrosterone, Berberine, and Sorafenib on the MDA-MB-231 triple negative breast cancer cells after 48 hours incubation period through the neutral red uptake assay. Based on Median Effect Equation (*Chou*), Combination Index Theorem and Dose Reduction Index Equation (*Chou-Talalay*), we tested six binary combinations and four ternary ones to define and quantify their pharmaco-dynamic interactions (synergism, antagonism or additivity). The highest-to-lowest order of potency of a single drug treatment was PU-H71 > Sorafenib > Berberine > Dehydroepiandrosterone. At fractional affected level (*fa*) ≥ 0.90, almost all the actual and computer-simulated combinations exerted synergistic effects, where (PU-H71 plus Sorafenib), (Berberine plus Sorafenib) and (PU-H71 plus Berberine plus Sorafenib) were the strongest synergistic combinations with CI value < 0.30. Based on our *in vitro* combination results, we suggest subsequent downstream investigations to understand the molecular mechanisms of such promising synergistic combinations. Additionally, we recommend the application of such combinations on TNBC-xenografted animal models to effectively establish the go\no-go decision of the further application in clinical settings.

Keywords: Triple-negative breast cancer, Combination therapy, Chou-Talalay method, Synergy.

Running title:

1. Introduction

Breast cancer (BC) is the most commonly diagnosed malignancy and the major cause of cancer deaths among females in Egypt and worldwide. According to the latest GLOBOCAN 2018 report of the International Agency for Research on Cancer (IARC), approximately 2 million new cancer cases and 626,000 deaths were estimated to have occurred globally in 2018[1]. Triple negative breast cancer (TNBC) is one of the most aggressive subtypes of breast malignancy associated with poor prognosis and therapeutic outcomes[2]. It represents about 15% - 20% of breast cancer patients. Moreover, TNBC tumors are larger, poorly-differentiated and more likely to metastasize beyond the breast to initiate new ones in the brain tissue. It is defined molecularly by the lack of estrogen receptor, progesterone receptor and HER-2 overexpression[3]. Accordingly, it is unlikely to be clinically managed by the dependence on the hormonal or HER-2 targeted therapies, making the treatment more challenging. Thus, the conventional chemotherapeutic agents are still the mainstay of its therapeutic protocols[4]. Although, many TNBC patients initially respond to the chemotherapeutic protocols, the initial good response to chemotherapy eventually turns into a refractory drug resistance[5]. Hence, efficient therapeutic regimens are urgently required.

Using a single drug for the treatment of cancer, especially the aggressive forms like TNBC, is mostly worthless due to the inherent genetic instability of cancerous cells that could easily develop resistance. Thus, a cocktail of two or more drugs with different mechanisms of action is more effective and could successfully control the disease[6]. Furthermore, combination therapy reveals more, or at least the same, efficacy with lower doses of each single agent and decreases the risk of drug resistance via the simultaneous targeting of multiple signal transduction pathways responsible for the tumorigenesis in a concentric or linear modality[7]. Accordingly, combination

therapy is considered a promising choice that could alter the future roadmap towards designing more efficient therapeutic protocols in TNBC.

The first molecule used in our study was the purine-based PU-H71 (8-[(6-iodo-1,3-benzodioxol-5-yl) sulfanyl]-9-[3-(propan-2-ylamino) propyl] purin-6-amine)[8]. It is considered a novel synthetic inhibitor for the heat shock protein 90 (Hsp90), a highly-conserved 90-kDA molecular chaperone known for its role in protecting a wide assortment of mutated and over-expressed oncoproteins from misfolding and degradation[9]. PU-H71 has been reported to have anti-tumor effects with a full response in many TNBC preclinical models[10]. Such promising preclinical antineoplastic activity was attributed to the downregulation of an array of oncoproteins such as EGFR, IGF1R, HER3, c-KIT and c-Raf-1. Furthermore, it decreased the metastatic potential of TNBC cells by two mechanisms: the interaction and subsequent proteasome-mediated degradation of the NF-kB signaling pathway components, and the reduction of the Akt and ERK levels[10,11].

The second molecule was Dehydroepiandrosterone (DHEA) ((3S,8R,9S,10R,13S,14S)-3-hydroxy-10,13-dimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-17-one)[8]. It is endogenously secreted from adrenal gland and gonads as a precursor for synthesis of male and female sex hormone. DHEA is a non-competitive inhibitor of the human Glucose-6-phosphate dehydrogenase (G6PD) that can be exploited to diminish the anti-oxidant capacity of the tumor cells via alteration of $NADPH/NADP^+$ ratio[12]. Hence, in a previous study, it potentiated the effect of paclitaxel on MDA-MB-231 breast cancer cells and overcame its acquired resistance[13]. Furthermore, *in vitro* studies of its effect alone against MDA-MB-231 TNBC cell line revealed that the pharmacological doses of DHEA have anti-proliferative and anti-migratory

effects as well[14]. Although, it is not extensively studied *in vivo*[13], DHEA could be a promising agent to be included in many breast cancer treatment protocols.

The third molecule was Berberine hydrochloride (9,10-dimethoxy-5,6-dihydro[1,3]dioxolo[4,5-g]isoquino[3,2-a]isoquinolin-7-ium)[8]. It is considered one of the most extensively-studied isoquinoline molecule among the naturally occurring protoberberine alkaloids. Berberine could be found in the root, rhizome and stem bark of *Berberis vulgaris* (barberry), *Hydrastis candensis* (Gold-enseal), and *Arcangelisia flava* (Menispermacear) medicinal plants[15]. According to a systematic review published by Xiuping Chen et al. on 2009, berberine possesses multiple mechanisms of actions as an anti-cancer molecule at both *in vitro* and *in vivo* levels (from the inhibition of tumorigenic microorganisms and the expression regulation of carcinogenesis-related genes to the inhibition of multiple enzymes involved in tumorigenesis)[16]. Furthermore, specifically from a TNBC perspective, it was found to activate apoptosis through the intrinsic cytochrome-c release\caspase-9 activation-mediated mechanism in both MDA-MB-231 cell line and its correspondent mouse-xenograft model[17].

The last molecule in our study was Sorafenib tosylate (4-[4-[[4-chloro-3-(trifluoromethyl) phenyl] carbamoylamino] phenoxy]-N-methylpyridine-2-carboxamide;4-methylbenzenesulfonic acid). It is an orally bioavailable bi-aryl urea and pharmacologically-classified as a small molecule multi-kinase inhibitor for the treatment of hepatocellular carcinoma, renal cell carcinoma, and papillary and follicular thyroid cancers [8]. It exhibits a direct antiproliferative effect by inhibiting the activity of the Raf kinase isoforms (wild-type c-Raf1, B-Raf and mutant b-raf V600E) in the Ras/Raf/MEK/ERK signaling pathway. Additionally, angiogenesis could be inhibited by sorafenib through the direct blocking of the auto-phosphorylation process of several receptor tyrosine

kinases (VEGFR1, 2 and 3, PDGFRβ, Flt-3, and c-Kit)[18]. The role of sorafenib tosylate in the treatment of breast cancer was evaluated in recent studies[19,20]. It was found that several phase-I/-II and -II single-arm clinical trials had revealed a limited single-agent activity of sorafenib in breast cancer patients. Hence, it was concluded that the combination of sorafenib with other anti-cancer agents is the only way for further investigations. Furthermore, its clinical development in combination with other targeted therapies should be pursued upon further preclinical assessment[20].

Assessment of novel anticancer agents, or combinations of FDA-approved ones, depends on the integration among basic, pre-clinical and clinical research in a structured network called ‘‘the translational research process’’. It is defined as a stepwise hierarchical system of *in vitro*, *in vivo* and clinical studies, respectively. Then, it is eventually used in standard clinical treatment protocols. Although the translational process is not unidirectional in practice, but may reverse direction at any step, the development of new anti-cancer treatments, or the improvements of FDA-approved ones through combination regimens, often depends for its initial screening on cancer cells in culture[21,22]. Furthermore, there have been no published work on the combined application of PU-H71, DHEA, Berberine and Sorafenib at an *in vitro* TNBC models to achieve a promising synergistic regimen based on the Median-Effect Equation (MEE), Combination Index Equation (CIE) and Dose Reduction Index Equation (Chou-Talalay method, 1984)[23]. In this regard, we investigated whether or not these combinations could have synergistic antitumor activities in the MDA-MB-231 TNBC cell line.

2. Materials and methods

2.1. Chemicals and Biologicals:

Berberine, Neutral red dye, Dimethyl sulfoxide (DMSO) and Methanol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). PU-H71 and DHEA were purchased from Bio-Vision Inc. (Milpitas, California, USA). Sorafenib tosylate was purchased from Toronto Research Chemicals TRC (North York, Ontario, Canada). Dulbecco's Modified Eagle Medium-High glucose with glutamine (DMEM) and Fetal Bovine Serum (FBS) were purchased from Biowest (Riverside, Missouri, USA).

2.2. Cell Culture:

MDA-MB-231 breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) via VACSERA Holding Co. for biological products and vaccines (Agouza, Giza, Egypt). Under the commercial supplier's protocols, cells were cultured in DMEM supplemented with FBS to a final concentration of 10%, to make the complete growth medium, and incubated in a humidified incubator at 37 °C with an atmosphere containing 5% CO_2. Then, it was serially passaged at 80 – 90% confluency.

2.3. Cytotoxicity assay for each single drug:

Initially, to determine the ''potency'' and construct the ''dose-effect curve'' of each drug alone, as prerequisites of the Chou-Talalay method of the *in vitro* drug interaction analysis[23,24], cytotoxicity assay for each single drug was performed through the neutral red uptake method[25]. Firstly, PU-H71, DHEA and Sorafenib were dissolved in the vehicle DMSO, and berberine was dissolved in the vehicle methanol. All the solutions were kept at suitable stock concentrations.

MDA-MB-231 cancer cells were seeded at a density of 4500 cells per well in 96-well plates. Next day, different concentrations of PU-H71 (0.01 to 0.4 µM), DHEA (100 to 400 µM), Berberine (6 to 48 µM) and Sorafenib (0.25 to 10 µM) were freshly prepared in the complete culture medium and cells were treated with these drug solutions at suitable concentration intervals (at least, five different concentrations (or doses) of each drug were tested and six replicates for each drug concentration were performed). After 48 hours incubation period, drug solutions were removed, and the neutral red medium was added. Then, the incubation period was continued for another two hours after which the plates were washed with Phosphate Buffered Saline (PBS, pH = 7.4) to remove the neutral red medium from the wells. After thorough washing with PBS, the neutral red dye was extracted from the cells with the de-stain solution (50% ethanol, 49% de-ionized water and 1% glacial acetic acid). After that, the plates were vigorously shaken for 10 minutes, after which the optical density (OD) at 540 nm was measured utilizing a micro plate reader (Spectro star, BMG Labtech). Finally, the percentage inhibition (effect) was measured using the following equation[26]:

$$\% \text{ Inhibition} = \left[1 - \left(\frac{\boldsymbol{OD}\ treated\ cells - \boldsymbol{OD}\ media\ blank}{\boldsymbol{OD}\ vehicle\ control - \boldsymbol{OD}\ media\ blank}\right)\right] \times 100 \quad \textbf{(1)}$$

Where, ‘’OD treated cells’’ was the value of the mean absorbance readings of cells exposed to our tested drugs; ‘’OD vehicle control’’ was the value of the mean absorbance readings of cells exposed to the solvent compound (DMSO or Methanol) mixed with the complete culture media to act as a growth control; and finally, ‘’OD media blank’’ was the mean of absorbance readings of media minus cells to act as a neutral red non-specific binding blank.

2.4. Constructing the dose-effect curve for each single drug and determination of its parameters:

The dose-effect curve was constructed for each single drug utilizing the CompuSyn software program (ComboSyn Inc., Paramus, NJ. U.S.A.)[27]. Half-maximal inhibitory concentration (IC_{50}), (the concentration at which 50% growth inhibition is achieved), the linear correlation coefficient (r) of each dose-effect curve and the coefficient (m) signifying its shape were calculated utilizing the same software program based on the Median-Effect Equation (Ting Chou and Paul Talalay, 1984)[23] as follows:

$$\frac{fa}{fu} = \left(\frac{D}{Dm}\right)^{m} \qquad \textbf{(2)}$$

Where, D is the drug dose (or drug concentration), *fa* is the inhibited fraction (effect) by the drug dose D (i.e. percentage inhibition/100), f_u is the unaffected fraction ($f_u = 1 - fa$), D_m indicates the dose that causes 50% inhibition (IC_{50} or the median-effect signifying the drug potency), and m is the coefficient signifying the shape of the dose-effect curve where m < 1, m > 1, and m = 1 reveal flat sigmoidal, sigmoidal, and hyperbolic dose-effect curves, respectively.

The (r) value signifies the degree of conformity of the data with the mass-action law[23], where (r = 1) indicates an ideal conformity. The values of (m), (D_m), and (r) for each single drug are the dose-effect parameters and required for the implementation of Chou-Talalay method.

2.5. Cytotoxicity assay for binary and ternary drug combinations based on the constant-ratio experimental design:

To perform *in vitro* pharmacodynamic drug interaction analysis (synergism, antagonism or additivity interactions) for the selected drugs, we conducted the neutral red uptake cytotoxicity assay for different binary and ternary drug combinations. For simplicity, symbols (P), (D), (B), and (S) represent PU-H71, DHEA, Berberine and Sorafenib, respectively. The tested binary drug

combinations were (P + D), (P + B), (P + S), (D + B), (D + S) and (P + S). While the four ternary drug combinations were (P + D + B), (D + B + S), (P + D + S) and (P + B + S) (as shown in Figure 2). As recommended by Chou and Talalay[23,28], the equipotent constant-ratio drug combination design (or the diagonal scheme) was adopted in all combinations (as shown in Table 1 and Table 2), where the contribution to the combination by each single drug would be approximately the same. The cytotoxic effects of all combinations were determined after 48 hours incubation period. At least three different equipotent concentrations for each binary or ternary combination were tested in six replicates. The percentage inhibition (effect) for all combinations was measured as previously mentioned in equation (1).

2.6. Implementation of the Chou-Talalay method through determination of the Combination Index (CI) and the Dose Reduction Index (DRI) for all drug combinations:

The Combination Index value – a dimensionless quantity for the determination and quantification of the drug interaction's type – for the binary combinations was automatically calculated utilizing the CompuSyn software program based on the Combination Index Equation[23] as follows:

$$^{2}\mathbf{(CI)_x} = \frac{(\boldsymbol{D})_1}{(\boldsymbol{D_x})_1} + \frac{(\boldsymbol{D})_2}{(\boldsymbol{D_x})_2} = \frac{(\boldsymbol{D})_1}{(\boldsymbol{D_m})_1\left[{\boldsymbol{fa}}/{(\boldsymbol{1-f_a})}\right]^{\frac{1}{\boldsymbol{m_1}}}} + \frac{(\boldsymbol{D})_2}{(\boldsymbol{D_m})_2\left[{\boldsymbol{fa}}/{(\boldsymbol{1-f_a})}\right]^{\frac{1}{\boldsymbol{m_2}}}} \qquad \mathbf{(3)}$$

Where, $(D_x)_1$ is the dose of the drug D_1 alone that inhibits the growth of cells by x%, $(D_x)_2$ is the dose of the drug D_2 alone that inhibits the growth of cells by x%, $(D)_1$ and $(D)_2$ are the doses of the drugs D_1 and D_2 in combination that also inhibit the growth of cells by x%. The $(D_x)_1$ and $(D_x)_2$ values can be easily calculated from the rearrangement of the Median-Effect Equation (2) as follows:

$$D = Dm\left[\frac{fa}{(1-fa)}\right]^{\frac{1}{m}} \qquad \mathbf{(4)}$$

Using the same software, the Combination Index value for the ternary combinations was calculated automatically based on the general equation of the Combination Index for a three-drug combination at x% inhibition[23] as follows:

$$^{3}(CI)_x = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{(D)_3}{(D_x)_3} = \frac{(D)_1}{(D_m)_1\left[f_a/(1-f_a)\right]^{\frac{1}{m_1}}} + \frac{(D)_2}{(D_m)_2\left[f_a/(1-f_a)\right]^{\frac{1}{m_2}}} + \frac{(D)_3}{(D_m)_3\left[f_a/(1-f_a)\right]^{\frac{1}{m_3}}} \quad \textbf{(5)}$$

Where, $(D_x)_1$ is the dose of the drug D_1 alone that inhibits the growth of cells by x%, $(D_x)_2$ is the dose of the drug D_2 alone that inhibits the growth of cells by x% and $(D_x)_3$ is the dose of the drug D_3 alone that inhibits the growth of cells by x%. $(D)_1$, $(D)_2$ and $(D)_3$ are the doses of the same drugs in combination that inhibit the growth of cells by x%. The $(D_x)_1$, $(D_x)_2$ and $(D_x)_3$ values can be calculated from equation (4). If the CI value is equal to 1, additive effect is achieved. If the CI value is smaller than 1, synergistic interaction is achieved. If the CI value is greater than 1, the interaction type is antagonism.

The Dose Reduction Index is a dimensionless measure of how fold the dose of each drug in a synergistic combination may be reduced at a given fractional inhibition compared with the doses of each drug alone. It was calculated for binary and ternary drug combinations automatically utilizing the same program based on the Dose Reduction Index Equations[23] as follows:

$$(DRI)_1 = \frac{(D_x)_1}{D_1}, \ (DRI)_2 = \frac{(D_x)_2}{D_2}, \ (DRI)_3 = \frac{(D_x)_3}{D_3}, \ \ldots\text{etc.} \qquad \textbf{(6)}$$

Where, DRI > 1 indicates favorable dose reduction, DRI < 1 indicates unfavorable dose reduction and finally DRI = 1 indicates no dose reduction[23].

3. **Results**

3.1.Single-drug cytotoxicity assay:

After performing the neutral red uptake cytotoxicity assay for each drug alone against MDA-MB-231 cells, the CompuSyn software was used for both the generation of the single-drug dose-effect curves, and the calculation of the parameters (D_m), (m), and (r). As shown in Table 1, all the drugs inhibited the cell growth in a dose-dependent manner. The D_m values (i.e. IC_{50}) of PU-H71, Dehydroepiandrosterone, Berberine and Sorafenib were 0.155 µM, 252.2 µM, 39.7 µM and, 8.43 µM respectively. Accordingly, the highest-to-lowest order of their potency was PU-H71 > Sorafenib > Berberine > Dehydroepiandrosterone. As shown in Figure 3A, the dose-effect curves of Dehydroepiandrosterone, Berberine and Sorafenib were sigmoidal (m > 1), while the one for PU-H71 was flat sigmoidal (m < 1). The median-effect blots of all tested drugs were shown in figure 4A. All the (r) values of the curves were above 0.95, indicating an acceptable conformity with the mass-action law[23,24].

3.2.Cytotoxicity assay of binary and ternary drug combinations:

Single-drug cytotoxicity assay results fulfilled the prerequisites of the Chou-Talalay method for the initiation of the *in vitro* pharmacodynamic drug interaction analysis. Hence, we tested all the possible binary and ternary drug combinations in a constant-ratio combination design. Behaving as a third drug, the drug combinations' dose-effect curves (Figure 3B and 3C) and median-effect plots (Figure 4B and 4C) were constructed using the CompuSyn software and the parameters of those dose-effect curves [(m), (D_m), and (r)] were automatically calculated (Table 2 and 3). All the curves were sigmoidal (m > 1) with a linear correlation coefficient (r) above 0.95.

Firstly, based on the CompuSyn-calculated CI values of the actual experimental data points (Table 1 and 2), all the binary and ternary drug modulations could achieve synergistic interactions (CI < 1) at specific combination ratios with a fractional inhibition (*fa* > 0.65). Furthermore, all

combinations turned from antagonistic/additive interactions to synergistic ones in a dose-dependent manner. Secondly, based on the CompuSyn-calculated DRI values of the actual experimental data points, all the synergistic binary and ternary drug combinations achieved favorable Dose Reduction Index (DRI > 1). Interestingly, PU=H71 attained the highest Dose Reduction Index values in all the synergistic drug combinations.

3.3.Computerized simulation by CompuSyn software:

By harnessing the Median Effect Equation, the Combination Index Equation and the Dose Reduction Index Equation; and utilizing the automation character of the CompuSyn software, an algorithm was generated to simulate the calculated CI and DRI values at different *fa* levels (other than the actual experimental dose and corresponding effect values). Hence, the simulated *fa*-CI plot, *f*a-Log CI plot, *f*a-DRI plot, and *f*a-Log DRI plot for all drug combinations were constructed automatically by the software (supplementary material: CompuSyn reports). The CI and DRI values of all drug combinations at 50%, 75%, 90% and 95% effect levels are shown in Table (4). Furthermore, based on such algorithms, and for the sake of the visual comparison between the type and degree of drug interactions, the Polygonograms of binary and ternary drug combinations at different *fa* levels were automatically constructed (Figure 5 and Figure 6), where the solid line represents synergism, the dashed line represents antagonism and the degree of synergism/antagonism is represented by the thickness of the lines. Based on the simulated CI values, it was observed that all the tested combinations had antagonistic interaction with different magnitudes at inhibition level of 50%, except the (P + B) and (D + B) combinations showed a nearly additive one. At *f*a $\geq$ 0.90, all the tested combinations had synergistic interactions, where the (P + S) and (B + S) combinations showed the strongest synergistic binary ones and the (P + B + S) combination showed the best synergistic ternary one (as shown in Table 4).

4. Discussion

TNBC is the one of the most aggressive tumors among other molecular subtypes of breast malignancy due to the lack of targeted therapies and the poor prognosis of its patients. The conventional chemotherapeutic agents are still the gold standard among TNBC therapies during the early and late stages of the disease, namely Taxanes and Anthracyclines in the neoadjuvant, adjuvant or the metastatic therapeutic protocols. However, despite the endeavors to improve the clinical outcomes of chemotherapy, more than 90% of women with metastatic TNBC malignancy will die due to the disease[29]. Furthermore, although the *in vitro* cell line experiments and the subsequent *in vivo* preclinical trials of novel single-drug treatments showed promising results, it could not be extended to clinical trials because of the heterogenic nature of TNBC and the high rate of acquiring resistance. Thereafter, about 80% of the ongoing clinical trials are working on many combination therapy protocols in a massive effort to effectively control the disease[30]. As an initial step in attaining optimal combinatorial therapies, we used MDA-MB-231 cell line as a model of TNBC to perform an *in vitro* pharmacodynamics interaction analysis of PU-H71, DHEA, Berberine and Sorafenib. The data obtained were analyzed using the CompuSyn software, which provides a mechanism-independent platform to quantitatively determine the net effect of a certain combination using the CI Equation, where CI < 1, $= 1$ and > 1 represent synergistic, additive and antagonistic interaction, respectively. The purpose of our study was to provide experimental results based on *in vitro* combination experiments, therefore, it might be useful as a guide to select a certain combination to be subjected to extensive *in vivo* studies and subsequent clinical trials. Studying the pharmacodynamic interactions of those drugs is an imperative issue per se, regardless of the mechanism by which each drug acts in the combination, particularly when dealing with multi-modal drugs (e.g. PH-H71, Berberine and Sorafenib). As combination studies of multi-modal

and/or multi-targeted drugs could last for several years to qualitatively and quantitatively elucidate which mechanism shares the net effect[23]. Furthermore, other factors that may affect the combination's outcomes should be taken into consideration, for instance, alteration of a drug resistance, transportation across the cellular membrane and modifications of the metabolism[31].

Generally, our results showed several promising synergistic combinations with favorable Dose Reduction Index, which might be valuable regimens against TNBC. Although several ternary combinations showed a superior synergistic interaction than binary ones, combining more drugs do not necessarily reveal better effect. For instance, there was no need for the addition of PU-H71 to the combination (B + S) or the addition of Berberine to the combination (P + S), as it virtually did not change the net effect. Moreover, similar to previous *in vitro* combination studies[32], adding a third drug even decreased the sensitivity of the MDA-MB-231 cells to the binary combination, for example, adding the DHEA to (P + S) and (B + S) binary combinations. The validation of our results is limited to the mentioned experimental design, and definitely, more screening studies are needed to reach the optimum combination regimens, for instance, the sequential addition of drugs within our binary and ternary combinations could significantly flip the net effects. Furthermore, we recommend performing other *in vitro* pharmacodynamic interaction analysis based on the non-constant experimental design, thus more potent combinations with more favorable DRI could be achieved. Additionally, further studies could be conducted on the effect of the achieved synergistic combinations on other TNBC models and other hallmarks of cancer, not only the suppressive effect to the MDA-MB-231's unlimited replicative potential.

Conclusion:

Utilizing MDA-MB-231 tumor cells as a preclinical model for TNBC and based on a robust and mechanism-independent platform for drug interaction analyses, the Chou-Talalay method, several

promising binary and ternary synergistic drug combinations (CI<1) with favorable dose reductions (DRI > 1) were identified. Our results paved the way for the further testing of such synergistic combinations on other TNBC modalities and warranted future investigations of the primary molecular mechanisms behind their synergism.

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Conflict of Interest:

The authors declare no conflict of interest.

List of abbreviations:

BC: Breast Cancer

TNBC: Triple Negative Breast Cancer

ER: Estrogen Receptor

PR: Progesterone Receptor

HER-2/Neu: Human Epidermal Growth Factor Receptor-2

MDA-MB-231: MD Anderson Metastatic Breast adenocarcinoma triple negative cell line

CI: Combination Index

DRI: Dose Reduction Index

IARC: International Agency for Research on Cancer

FDA: Food and Drug Adminitration

Hsp90: Heat shock protein-90

EGFR: Epidermal Growth Factor Receptor

IGF1R: Insulin Like Growth Factor 1 Receptor

HER3: Human Epidermal Growth Factor Receptor-3

c-KIT: KIT proto oncogene

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

DHEA: Dehydroepiandrosterone

Akt: Serine\Threonine Protein Kinase B

Ras: Rat Sarcoma small GTPase

c-Raf-1: Rapidly Accelerated Fibrosarcoma kinase-1

MEK: Mitogen-activated protein kinase kinase

ERK: Extracellular Signal Regulated Kinase

G6PD: Glucose-6-phosphate dehydrogenase

NADPH: reduced form of Nicotinamide Adenine Dinucleotide Phosphate (NADP)

VEGFR1: Vascular Endothelial Growth Factor Receptor-1

PDGFRβ: Platelet Derived Growth Factor Receptor-β

Flt-3: fms like tyrosine kinase-3

DMSO: Dimethyl Sulfoxide

PBS: Phosphate Buffered Saline

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal Bovine Serum

MEE: Median-Effect Equation

CIE: Combination Index Equation

Figure legend:

Figure 1: Chemical structure of different drugs used in the study.

Figure 2: Schematic diagram for tested drug combinations. (A) Represents the six different binary combinations each indicated with a line of different color. (B) represents the four different ternary combinations each indicated with a triangle with different pattern.

Figure 3: The dose-effect curves of single drugs (A), binary combinations (B) and ternary combinations (C).

Figure 4: The Median-Effect Plot of single drugs (A), binary combinations (B) and ternary combinations (C).

Figure 5: The polygonograms of binary combinations at different fa levels 0.50, 0.75, 0.90, and 0.95, produced by Compusyn software.

Figure 6: The polygonograms of ternary combinations at different fa levels 0.50, 0.75, 0.90, and 0.95. Each combination indicated with a triangle with a different color.

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