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# The Cytotoxic Effect of 2-Deoxy-D-Glucose Combination with 5-Fluorourasil and NO-Aspirin on Mammary Adenocacinoma Cell Line

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**Abstract:**

2-deoxy-D-glucose (2DG) and NO-Aspirin (NO-ASA) are new anticancer agents that are under intense clinical investigation for their remarkable cytotoxic activity. Combining 2DG, which targets glucose metabolism, with other agents mainly the DNA- and the mitochondrial-damaging agents represent a promising chemotherapeutic strategy. In this study, we investigated the cytotoxic effects of 2DG, 5-fluorourasil (5FU), and NO-ASA on AMN3 breast cancer model, in addition to the cytotoxic effects of 2DG combination with 5FU and NO-ASA on the same cells. The cytotoxic activity of 2DG, 5FU, and NO-ASA was measured by using the MTT assay at 24, 48, and 72 hr. Then 2DG was combined with 5FU and NO-ASA in a constant concentration ratio based on their corresponding IC50s and the inhibition of cell growth was measured by MTT assay at 72hr. Median effect analysis was conducted to determine the cytotoxic activity of the combinations. 2DG, 5FU, and NO-ASA were found to exert a significant dose- and time-dependant growth inhibition on AMN3 cells. The mean combination index values reveal an additive effect for both combinations. This study demonstrated that 2DG and NO-ASA are capable of inhibiting the breast tumor growth effectively. It also shows that 2DG/5FU and 2DG/NO-ASA combinations result in mean additive effects with good dose reduction index values that have the advantages of reducing the toxicity, adverse effects, and the drug resistance in cancer patients.

**Key words:** 2-deoxy-D-glucose (2DG), NO-Aspirin (NO-ASA), 5-fluorourasil (5FU), Glucose metabolism, Median effect analysis, Breast cancer

## 1. Introduction

In the beginning of the 20th century, Otto Warburg, discovered that cancer cells show a higher rate of glycolysis than the normal cells and that they use the fermentation pathway to produce necessary energy, even in the presence of oxygen (Warburg 1956). Targeting cancer cell by glycolytic inhibitors results in glucose deprivation and thus, inducing cytotoxicity via mechanisms involving oxidative stress in tumor cells, relative to normal cells (Spitz *et al.* 2000). 2-Deoxy-D-Glucose (2DG) is a glucose analog that is recently under intense investigation in cancer therapy. It competes with native glucose for entry into the glycolytic pathway, once inside the cell, it is phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate (2DG6P). Unlike glucose, 2-DG cannot be further metabolized and begins to accumulate inside the cell. This accumulation will effectively inhibit the glycolysis process to further metabolize its natural substrate and depresses energy production. The depression in cellular ATP levels leads to inhibition of cell cycle progression and eventual cell death. Additionally, 2DG can inhibit the metabolism of glucose in the pentose phosphate pathways (Liu *et al*. 2001). 2-DG found also to alter *N* -linked glycosylation leading to unfolded protein responses and induces changes in gene expression and phosphorylation status of proteins involved in signaling, cell cycle control, DNA repair, calcium influx, and apoptosis that are involved in carcinogenesis (Kurtoglu *et al.* 2007).
For this, 2-DG has been suggested to inhibit the proliferation of tumor cells and induce cell death. Many studies have indeed found that 2-DG inhibits the growth of tumor cells *in vitro* (Dwarakanath *et al*. 2005 and Coleman *et al.* 2008). The inhibition of tumor growth *in vivo* by 2DG was found to require daily administration of 2-DG causing systemic toxicity, this problem limits its use as a primary therapeutic in the treatment of cancer (Dwarakanath 2009).
On the other hand, non-steroidal anti inflammatory drugs (NSAIDs) have been proved by many preclinical and clinical studies to posses antineoplastic properties and reported to reduce the risk of cancers of colon, breast, esophagus, stomach, lung, prostate, urinary bladder and ovary (Harris *et al*. 2005; Evrim *et al*. 2014). Despite all

these beneficial antineoplastic properties, cyclooxygenase-1 (COX-1) and/or cyclooxygenase-2 (COX-2) inhibition and depletion of physiologically important prostaglandins by NSAIDs is associated with gastrointestinal, renal and cardiovascular toxicities that limit the use of NSAIDs for chemoprevention purposes (Borer *et al.* 2005). The other major limitation is that the available anti-inflammatory drugs do not completely protect against disease progression (Niv *et al.* 1994).

Nitric oxide-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs) represent a class of compounds designed based on the known properties of NSAIDs and those of the Nitric oxide (NO), a molecule that plays an important role in the body. The ability of NO-NSAIDs to release NO is their defining property. NO-NSAIDs exhibit reduced gastrointestinal adverse effects relative to the conventional NSAIDs (Borhade *et al.* 2012), in addition to cardiovascular and renal safety, as well as enhancing the anti-inflammatory, analgesic and anti-thrombotic properties relative to the parent drugs (Burgaud *et al.* 2002). Many studies evaluated the effect of NO–NSAIDs on the tumor growth of many cell lines and showed that they are much more potent than the conventional NSAIDs (Chinthalapally *et al.* 2006). Nitric oxide-donating aspirin (NO-ASA) is a promising chemopreventive agent against many types of cancer. It consists of traditional ASA to which a NO-releasing moiety is bound through a spacer (Rigas *et al.* 2004). Compared with ASA, NO-ASA is 1,000-fold more potent in inhibiting the growth of many cell lines like colon, pancreatic, prostate, lung, tongue, breast and hematological tumors (Kashfi *et al.* 2002; Ouyang *et al*. 2006; Iris *et al*. 2011). It is now clear that NO-ASA targets multiple signaling mechanisms in the neoplastic cell, including modulation of NO synthesis and cell signaling mediated by the NF-kB, Wnt, mitogen-activated protein kinases and other pathways (Hundley *et al.* 2006; Hui *et al.* 2009). Many studies show that NO-ASA induces apoptosis through a series of steps that begins with the generation of an oxidative stress state, which activates the intrinsic apoptosis pathway, accompanied by disruption of adherent junctions and inhibition of Wnt signaling (Gao *et al.* 2005).

In this study, we want to demonstrate if a combination of glycolytic inhibitors with DNA-damaging agents and agents that cause mitochondrial oxidative damage could enhance the tumor cell death *in vitro*. Many studies have indicated that glycolytic inhibition could potentiate or synergize the cytotoxic drugs to induce cell death.

## 2.Materials and methods

### *2.1. Chemicals*

2-Deoxy-D-glucose (2DG) was obtained from Santa Cruz (USA); and 5-Fluorouracil (5FU), **2-(Acetyloxy) benzoic acid 4-(nitroxymethyl) phenyl ester (NO-aspirin**, NCX 4040), and Methyl thiazolyl tetrazolium (MTT) were obtained from Sigma (USA). RPMI-1640 medium was obtained from Gibco (USA). A murine mammary adenocarcinoma AMN3 cell line was obtained from ICCMGR (Baghdad, Iraq). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 µM L-glutamine and 1% penicillin/streptomycin.

### *2.2. Growth inhibition tests*

The MTT assay was done to measure the growth inhibition (GI) (Stephanie *et al.* 2008). In brief, AMN3 cells at concentrations of 5000 cells/well were seeded in 96-well plates. After overnight culture, cells were treated with 2DG, 5FU, and NO-ASA. Growth inhibition was measured after 24, 48, and 72 hr in $CO_2$ incubator at 37°C using the MTT assay and a microplate reader at 570 nm. The $IC_{50}$ (the concentration required for 50% inhibition) is then determined for each drug and according to it, constant ratio drug combinations is determined by taking two-fold serial dilutions above and below the $IC_{50}$ value of each drug and then again the growth inhibition was measured for combinations after 72 hr using the MTT assay and a microplate reader at 570 nm. The combinations tested are 2DG plus 5FU, and 2DG plus NO-ASA. The percentage of growth inhibition was calculated using the following equation (GI%) = (A-B)/Ax100, Where A is the mean optical density of untreated wells and B is the optical density of treated wells.

### *2.3. Median effect analysis*

This method, proposed by Chou and Talalay, is used to determine if there is any synergy, additive, or antagonism effect in drug combination (Chou 2010). The drugs were combined in constant concentration ratio based on their corresponding $IC_{50}$s at 72hr. $IC_{50}$s were (1050.94, 2.15828, and 2.72820) µg/ml for 2DG, 5FU, and NO-ASA, respectively. This method, using the combination index (*CI*) equation, allows quantitative assessment of the drug

interactions. The *CI* value allows the classification of the antitumor activity of the drugs in combination (Table 1). *F*a is the fraction of cell death induced by drug treatment and ranges from 0-1, with 0 meaning no cell killing and 1 representing 100% of cell killing. The AMN3 cells were treated with serial dilutions of each drug alone, or with 2DG/5FU combination at a fixed ratio of 460:1, and 2DG/NO-ASA at a ratio of 320:1. Four dilutions, depending on the $IC_{50}$, (serial dilution factor=2) of each drug in combination were tested in three independent experiments with triplicate samples. The CI was calculated based on: CI = $(D)1 / (D_x)1 + (D)2 / (D_x)2$, where (Dx)1 and (Dx)2 are the doses of drug 1 and drug 2, alone, inhibiting 'x%', whereas (D1) is the dose of drug 1 in combination, and (D2) the dose of drug 2 in combination that gives the experimentally observed 'x' inhibition. Furthermore, the dose reduction index (DRI) was evaluated as follows: (DRI)1 = (Dx)1/(D)1 and (DRI)2 = (Dx)2/(D)2 where DRI>1, which showed that combinations could result in reduced drug doses compared with the doses for each drug alone. Classical isobolograms were also constructed by plotting drugs concentrations (alone and in combination) that inhibits 50%, 60%, 70% AMN3 cell viability.

### *2.4. Statistical analysis*

The data from the MTT assay were expressed as mean+/-standard deviation (SD). Dose–effect curve parameters, CI values, CI plot, DRI values, DRI plot, and classical isobologram were determined by CompuSyn program (Compusyn Inc, Paramus, NJ, USA). A *p*-value of <0.05 denoted a statistically significant difference.

Table 1. Description and symbols of synergism, additive, and antagonism in drug combination studies analyzed with the combination index method.

| Range of CI | Description | Graded symbols |
|---|---|---|
| <0.1 | Very strong synergism | +++++ |
| 0.1–0.3 | Strong synergism | ++++ |
| 0.3–0.7 | Synergism | +++ |
| 0.7–0.85 | Moderate synergism | ++ |
| 0.85–0.90 | Slight synergism | + |
| 0.90–1.10 | Nearly additive | ± |
| 1.10–1.20 | Slight antagonism | _ |
| 1.20–1.45 | Moderate antagonism | _ _ |
| 1.45–3.3 | Antagonism | _ _ _ |
| 3.3–10 | Strong antagonism | _ _ _ _ |
| >10 | Very strong antagonism | _ _ _ _ _ |

## 3.Results

### *3.1.Single Drug Treatment with 2DG, 5FU, and NO-ASA*

To determine the effects of each drug on the growth of AMN3 cells, cells were treated with decreasing concentrations of 5-DG (8000, 4000, 2000, 1000, 500, 250, and 125) µg/ml for 24, 48, and 72 hr (Fig.1A), 5FU (50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125) µg/ml for 24, 48, and 72 hr (Fig. 1B), NO-ASA (50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125) µg/ml for 24, 48, and 72 hr (Fig. 1C). The results indicate that 2DG, 5FU, and NO-ASA were effective inhibitors of AMN3 cell growth when each one used as a single agent and that the cell growth was inhibited by all drugs in a dose- and time-dependent manner.

 

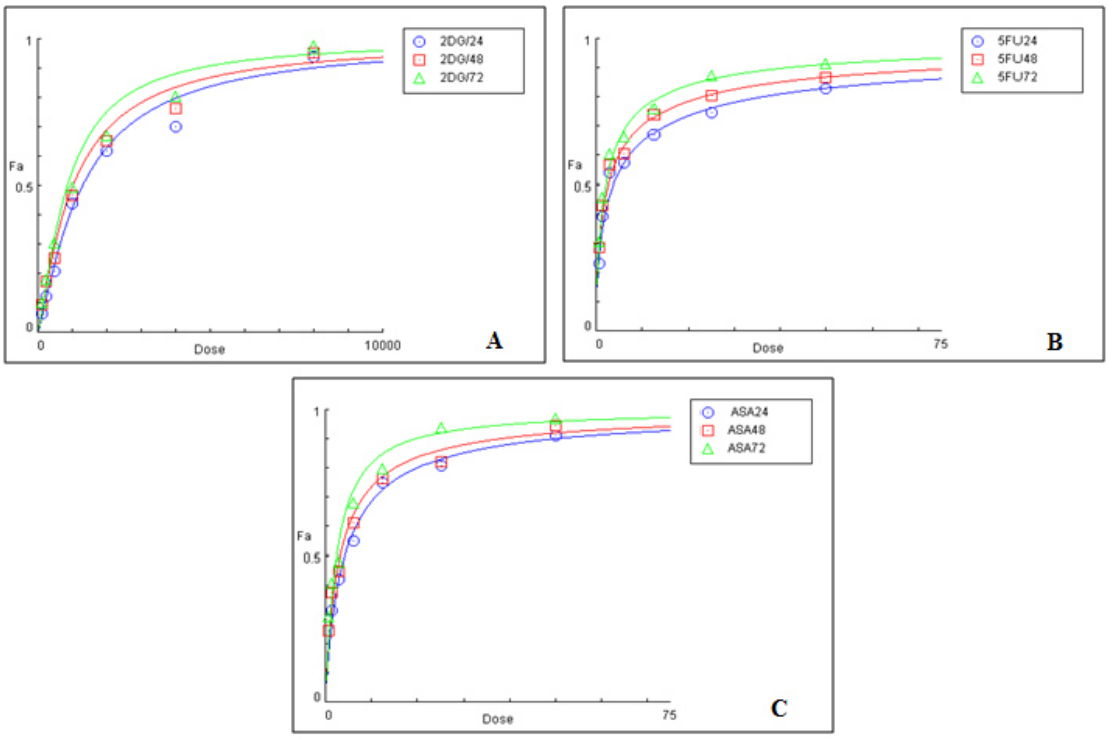


Figure 1. The dose-effect curves for 2DG (A), 5FU (B), and NO-ASA (C) after 24, 48, and 72 hr of exposure, where Fa refers to the fraction affected. The data show that 2DG, 5FU, and NO-ASA inhibit AMN3 cell proliferation in a dose- and time-dependent manner. Cell viability was determined using the MTT assay.

*3.2.Combination Treatment*

*3.2.1. 2DG and 5FU combination*

The effect of 2DG combined with 5-FU on cell viability was investigated using the MTT assay. For these studies, AMN3 cells were treated with 2DG (4000, 2000, 1000 and 500) µg/ml and 5FU (6.25, 3.125, 1.5625, and 0.78125) µg/ml for 72 hr. 2DG/5FU combination resulted in a decrease in cell viability which was greater than either 2DG or 5FU alone (Fig.2A). CI values were calculated and analyzed by compusyn program at the $IC_{50}$, $IC_{60}$ and $IC_{70}$, in addition to the DRI values, CI plot (Fig. 2B), DRI plot (Fig. 2C), and the Isobologram (Fig. 2D). The results showed that the CI values were 0.94966 +/- 0.11210 at the $IC_{50}$; that reveals additive effect, 0.91037 +/- 0.08397 at the $IC_{60}$; that also reveals additive effect, and 0.87631 +/- 0.09015 at the $IC_{70}$; that reveals slight synergy. The DRI values were 1.87416 for 2DG and 2.46330 for 5FU at the $IC_{50}$, 1.83512 for 2DG and 2.80932 for 5FU at the $IC_{60}$, and 1.79351 for 2DG and 3.24194 for 5FU at the $IC_{70}$. The greater DRI value (>1) indicates a greater dose reduction for a given therapeutic effect.

*3.2.2. 2DG and NO-ASA combination*

The effect of 2DG combined with NO-ASA on cell viability was investigated using the MTT assay. For these studies, AMN3 cells were treated with 2DG (4000, 2000, 1000 and 500) µg/ml and NO-ASA (12.5, 6.25, 3.125, and 1.5625) µg/ml for 72 hr. 2DG/NO-ASA combination resulted in a reduction in cell viability which was greater than either 2DG or NO-ASA alone (Fig. 3A). CI values were calculated and analyzed by compusyn program at the $IC_{50}$, $IC_{60}$ and $IC_{70}$, in addition to the DRI values, CI plot (Fig. 3B), DRI plot (Fig. 3C), and the Isobologram (Fig. 3D). The results showed that the CI values were 1.14184 +/- 0.07791 at the $IC_{50}$, 1.05963 +/- 0.05197 at the $IC_{60}$, and 0.97895 +/- 0.03666 at the $IC_{70}$. This reveals a moderate antagonism at the $IC_{50}$ and an additive effect at the $IC_{60}$ and $IC_{70}$. The DRI values were 1.93219 for 2DG and 1.60509 for NO-ASA at the $IC_{50}$, 1.99083 for 2DG and 1.7966 for NO-ASA at the $IC_{60}$, and 2.05675 for 2DG and 2.03145 for NO-ASA at the $IC_{70}$. The greater DRI value (>1) indicates a greater dose reduction for a given therapeutic effect.

 

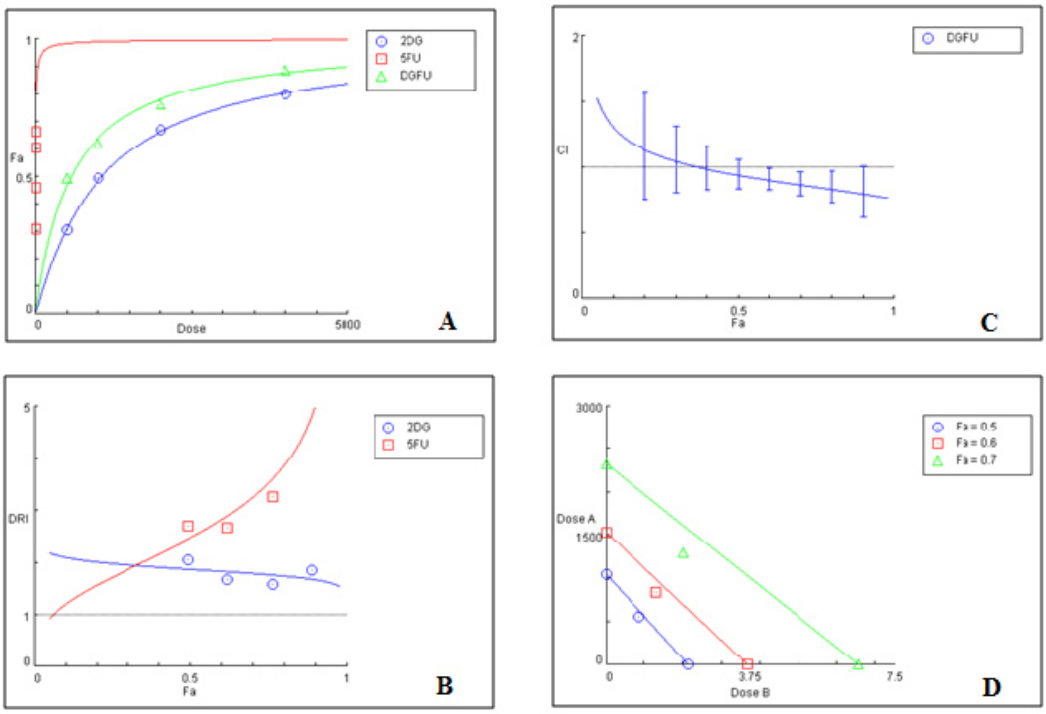


Figure 2. 2DG and 5FU combination treatment. (A) Dose-effect curves for 2DG, 5FU, and their combination after 72 hr of exposure. (B) CI plot: The CI is plotted as a function of fa by computer simulation from fa = 0.10 to 0.95. CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively. The vertical bars indicate 95% confidence intervals based on SDA using compusyn software. (C) DRI plot for combination: DRI values at different fa values for each drug in the combination plotted by computer simulation from fa = 0.10 to 0.95. DRI >1 is beneficial. The greater DRI value indicates a greater dose reduction for a given therapeutic effect. (D) Isobologram for Combination: classic isobologram at $IC_{50}$, $IC_{60}$, and $IC_{70}$. If the combination data points fall on the hypothenuse, an additive effect is indicated. If the combination data points fall on the lower left or on the upper right, synergism or antagonism is indicated, respectively.

 

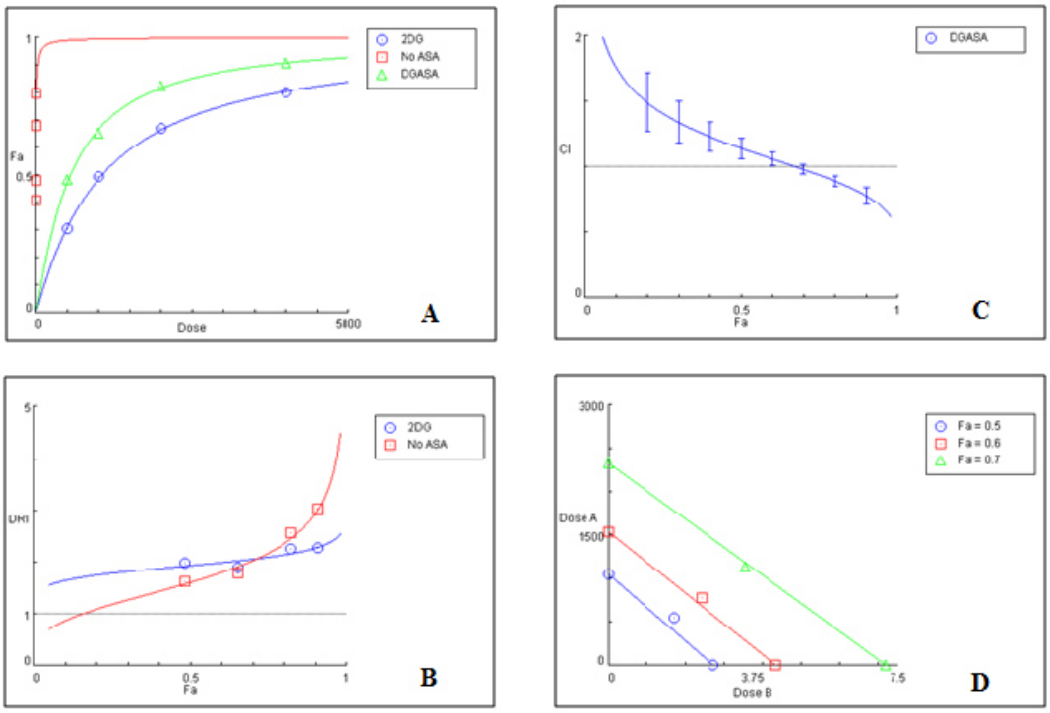


Figure 3. 2DG and NO-ASA combination treatment. (A) Dose-effect curves for 2DG, NO-ASA, and their combination after 72 hr of exposure. (B) CI plot: The CI is plotted as a function of fa by computer simulation from fa = 0.10 to 0.95. CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively. The vertical bars indicate 95% confidence intervals based on SDA using compusyn software. (C) DRI plot for combination: DRI values at different fa values for each drug in the combination plotted by computer simulation from fa = 0.10 to 0.95. DRI >1 is beneficial. The greater DRI value indicates a greater dose reduction for a given therapeutic effect. (D) Isobologram for Combination: classic isobologram at $IC_{50}$, $IC_{60}$, and $IC_{70}$. If the combination data points fall on the hypothenuse, an additive effect is indicated. If the combination data points fall on the lower left or on the upper right, synergism or antagonism is indicated, respectively.

## 4.Discussion

Breast cancer is one of the most frequently diagnosed cancers. Worldwide, it accounts for 25% of all cancer cases among women in 2012 and about 29% in 2013 (Rebecca *et al.* 2013). In breast cancer patients, metastasis is the main cause of mortality. Breast cancer is usually treated with surgery, which may be followed by chemotherapy or radiation therapy, or both (Saini *et al.* 2011). The current treatments, including cytotoxic chemotherapy, are rarely curative. Nowadays, there is a clear need for the development of new cytotoxic agents with novel mechanisms.

AMN3 mammary adenocarcinoma cell line is a transplantable tumor cell line that is derived from female BALB/c mice. It is characterized by its highly invasive, tumorigenic, and metastatic potential. It can spontaneously metastasize from the primary tumor site in the mammary gland to several body sites like lymph nodes, blood, bone, lung, brain, and liver. This cell line very closely resembles human breast cancer and shares with it many characteristics, mainly the metastatic properties, and the growth characteristics (AlShamery 2003). The AMN3 breast cancer model represents a valid and suitable experimental animal model for human breast cancer and it has been widely used in studies for evaluating antitumor effects of many cancer therapeutics.

One of the main characteristic features of cancer is the unregulated cellular metabolism. Targeting altered cancer cell metabolism represents a viable therapeutic strategy that could arrest the cell growth and induce apoptosis in cancer cells (Zhao *et al.* 2012). In this study, the results showed that 2DG is capable of reducing the *in vitro* growth of AMN3 cell line. In particular, (850-1300) µg/ml could inhibit ~50% of the cell growth after 24-72 hr of exposure.

Combining glycolysis inhibition by using 2DG with DNA damaging agents and agents that cause mitochondrial oxidative damage could enhance the tumor cell death *in vitro* (Cheng *et al.* 2012). According to Warburg theory, there is an insufficient cellular respiration that insults the mitochondria in tumor cells and leads to the formation

of reactive oxygen species (ROS) byproducts. The cancer cell compensates for this by increasing the rate of glycolysis and the pentose phosphate pathway activity to produce pyruvate and NADH. Thus, 2DG by inhibition of glucose metabolism is expected to sensitize cancer cells to agents that increase levels of hydroperoxides (Simons *et al.* 2009).

Additionally, the use of multiple drugs with different mechanisms of action may target the effect against cancer and help to treat it more effectively. The possible favorable outcomes from drug combination include increasing the efficacy, decreasing the dose to avoid toxicity, minimizing or slowing down the development of drug resistance, and providing selective synergism against target. For these therapeutic benefits, drug combinations have been widely used and became the leading choice for treating the most dreadful diseases like cancer (Chou 2006).

5FU is an anticancer drug that mainly inhibits thymidylate synthase (TS) enzyme and subsequently the synthesis of the pyrimidine thymidine, which is a nucleoside required for DNA replication. It has been given for many cancers such as breast, colorectal, stomach, oesophageal, pancreatic, and skin cancers , but 5FU has many severe and intolerable side effects that limits its application (Hector *et al.* 2006).

Combining 2DG with the DNA damaging drug 5FU in this study, results in a significant growth inhibition effect with a combination index values, indicating nearly additive effect at the $IC_{50}$ and $IC_{60}$ and a slight synergistic effect at the $IC_{70}$. If we calculate the mean of the combination indices, we get mean CI of 0.91211. This value indicates nearly additive effect on AMN3 cell line. This combination also results in a greater dose reduction for both 2DG and 5FU. When DRI is more than 1, this allows a dose-reduction that leads to toxicity reduction in the therapeutic applications. This combination reduces the $IC_{50}$s, 1.87 folds for 2DG, and 2.46 folds for 5FU.

NO-ASA, is a chemotherapeutic agent that leads to induction of oxidative stress and generation of ROS, in addition to its effect on other cellular pathways like, NF-kB, Wnt, mitogen-activated protein kinases and other pathways, that contributes to its effect against tumor (Kashfi *et al.* 2007). In this study, NO-ASA was found to exert a significant and a strong cytotoxic effect on AMN3 cells. The growth inhibition was dose- and time-dependant. The $IC_{50}$ values range from (2.4-4.2) µg/ml after 24-72 hr of exposure.

2DG combination with NO-ASA, is predicted by mechanism to give a beneficial outcomes. The results show that 2DG and NO-ASA combination, gives a significant growth inhibitory effect, comparing it with each drug alone. The combination index values indicate moderate antagonism at the $IC_{50}$ and nearly additive effect values at the $IC_{60}$, and the $IC_{70}$. If we calculate the mean of the combination indices, we get mean CI of 1.06014. This value indicates additive effect on AMN3 cell line. This combination results in a greater dose reduction for both 2DG and NO-ASA. The $IC_{50}$s reduces 1.93 folds for 2DG and 1.6 folds for the NO-ASA.

## 5.Conclusion

This present study demonstrated that 2DG and NO-ASA are cytotoxic to the AMN3 breast cancer model, and are capable of inhibiting the tumor growth effectively. The combinations of 2DG with 5FU and 2DG with NO-ASA produce mean additive effects and both combinations give a good degree of dose reduction, mainly for 5FU. This has the advantages of reducing the toxicity, the adverse effects, and the drug resistance in cancer patients.

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