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Effect of 2-Deoxyglucose on Colorectal Cancer Cell Lines

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ABSTRACT

The third leading cause of cancer deaths in the U.S. is colon cancer. The major disadvantage of cancer chemotherapy is its non-selective toxicity to healthy cells at the therapeutic doses. A possible target selective for cancer cells is their dependence on glycolysis for cellular energy. 2-deoxyglucose (2-DG) is a glycolytic inhibitor that has been shown to be safe in both animals and humans. The molecular mechanisms for the anti-cancer effect of 2-DG cannot be explained solely by its glycolytic inhibition. In this manuscript we studied the effect of 2-DG on colon cancer cells and its possible molecular mechanism. Colon cancer cells are more susceptible to 2-DG treatment than other cancerous and non-cancerous cell lines tested. The colon cancer cells tested are SW620, SW480 and GC3/C1. In cell cycle analysis studied using propidium iodide staining of DNA followed by flow-cytometry, 2-DG induced cell cycle arrest at G0/G1 phase in SW 620 cells. 2-DG also modified the expression of various cell cycle proteins such as p21, p53, and cyclins as measured through Western Blotting. In addition to cell cycle arrest, 2-DG also induced apoptosis through activation of Caspase 3. Complementing 5-Fluorouracil (5-FU) treatment of colon cancer cells with 2-DG significantly enhanced the efficiency of 5-FU treatment up to 3.5 fold. This study showed a new molecular mechanisms for 2-DG that could be used to design novel combination therapies with other known chemotherapeutic agents for colon cancer. The addition of a well-tolerated molecule like 2-DG increases the efficiency of 5-FU, thus reducing the patient's cumulative exposure to 5-FU. This may lead to fewer dose dependent side effects and better patient outcomes.

INTRODUCTION

Colorectal cancer is the third deadliest form of cancer in the United States. It is estimated that 102,480 people will be diagnosed with colon cancer and it will be responsible for 50,830 deaths in 2013 alone (Siegel *et al.*, 2013). The most widely used chemotherapeutic agent to treat colorectal cancer, as a single agent and in combination regimens, is 5-Fluorouracil (5-FU) (Cersosimo, 2013). The greatest challenge with chemotherapeutic agents, like 5-FU, is that they cause severe systemic toxicity at their therapeutic doses. This brings up a need to develop and identify drugs that are effective as well as exhibit a safe drug profile. A new class of drugs, glycolytic inhibitors, is selective for cancer cells based on the Warburg hypothesis.

Otto Warburg observed in the early 1920's that cancer cells differ from normal cells in their primary source for energy. All cells need energy in the form of ATP for their survival, but cancer cells need more energy than healthy cells because of their uncontrollable growth. Usually, cellular energy (ATP) comes from glycolysis and the Tricarboxylic Acid cycle-coupled oxidative phosphorylation of glucose. In the presence of oxygen, healthy cells depend on oxidative phosphorylation for their main source of energy. Warburg observed that cancer cells typically produce the majority of their ATP by glycolysis, even in the presence of oxygen. Cancer cells are characterized by their higher rate of lactate release and increased glucose consumption rate. The dependency of cancer cells on glycolysis as their primary source of energy makes this enzymatic pathway a key target for drug therapies. Accordingly, various small molecule inhibitors of the glycolytic pathway have been used effectively in the past to halt the progression of cancer both *in-vitro* and *in-vivo* (Gatenby & Gillies, 2007; Knight *et al.*, 1996).

2-Deoxyglucose (2-DG) is a well-characterized small molecular inhibitor of glycolysis both in animals and humans (Maschek *et al.*, 2004; Vander Heiden, *et al.*, 2009). It is safe and well tolerated in mice and rats (Kang & Hwang, 2006). Studies have also shown that doses of 2-DG as high as 200 mg/kg are well tolerated in people (Scatena *et al.*, 2008). 2-DG is a glucose molecule in which hydrogen takes the place of the hydroxyl group on the second carbon. Similar to glucose, 2-DG gets transported into the cell by glucose transporters. It then can be phosphorylated by hexokinase to form 2-DG-P. However, unlike glucose-6-

phosphate, 2-DG-P cannot be metabolized further by phosphohexose isomerase and cannot be easily transported out of the cell. The accumulation of 2-DG-P in the cell causes feedback inhibition of hexokinase. This block of glycolysis is especially harmful to tumor cells which rely heavily on glycolysis for their ATP generation (Scatena *et al.*, 2008). However, recent research has shown that there may be other mechanisms involved with 2-DG anti-cancer effects in addition to its glycolytic inhibition. Firstly, the decline in ATP levels following 2-DG treatment in eukaryotes is moderate at best (Stein *et al.*, 2010). Secondly, tumor cells react differently to 2-DG than its fluorescent analogue 2-Fluorodeoxy-D-Glucose (FDG). Although FDG is also a stronger inhibitor of glycolysis, it is non-toxic to cells that were responsive to 2-DG (Qutub & Hunt, 2005). Taken together, these findings suggest the existence of additional cellular effects or targets for 2-DG.

In this manuscript, the additional cellular effects of 2-DG, apart from inhibiting glycolysis, in colon cancer cell lines were evaluated. The results showed that 2-DG arrests the cell cycle at G0/G1 by modulating cell cycle proteins such as p21, p53 and cyclins. This cell cycle arrest subsequently results in cell death by apoptosis. More cancer cells were killed when 5-FU treatment was complemented with a safer molecule like 2-DG.

METHODS

Cell culture and drug treatment

Three human malignant colorectal cancer cell lines (Gc3/C1, SW480 and SW620), two human breast cancer cell lines (MDA-MB-231 and MCF7), a human ovarian cancer cell line (Ovcar3), a human melanoma cell line (B16F10) and a human kidney epithelial cell line (HEK 293T) were used in this study. The cells were grown in RPMI 1640 (Ovcar3 and Gc3/C1), DMEM (MCF 7, MDA-MB-231, B16F10 and HEK293) or Leibovitz's L-15 medium (SW620 and SW480) supplemented with 10% fetal calf serum (FCS), penicillin (100U/ml), and streptomycin (100µg/ml) in a humidified incubator (37°C, 5% CO₂). A 1 M stock solution of 2-DG (Sigma, St. Louis, Mo.) was prepared in 50 mM phosphate buffered saline (PBS), stored at -20°C and diluted in the complete media to obtain the desired concentrations.

Cytotoxicity assays and the determination of IC₅₀ of 2-DG and 5-FU

The dose of 2-DG and 5-FU required to kill 50% of cells (IC₅₀) in various cell lines was determined by MTT (3,4,5-dimethylazol-2,5-diphenyl-tetrazolium bromide, Sigma) assay. Ten thousand cells were seeded per well of a 96 well plate and allowed to adhere for 24 hours. After incubating the cells for 72 hrs with various concentrations of 2-DG (0 – 25 mM) and 5-FU (0-50 μ M), the viability of the cells was assessed using MTT assay. The IC₅₀ was calculated using SigmaPlot® software by plotting the cell viability against the concentration of 2-DG. All the experiments were performed in replicates of three with three repetitions.

Efficiency of 5-FU in combination with 2-DG in colorectal cancer cell lines

The cells were treated with a range of 5-FU (0-50 μ M) and a fixed amount of 2-DG (0.25 mM for SW480 and SW620 and 0.025 mM for GC3-C1). The effectiveness of the combination therapy of 5-FU with 2-DG was determined by comparing the IC₅₀ of 5-FU alone or in combination with 2-DG. All the experiments were performed in replicates of three with three repetitions. Statistics to test for significant difference in variance was performed using the Student's t-test at P<0.05.

Protein isolation and Western blotting

Cells were treated with the various concentrations of 2-DG in a 6-well plate and incubated for different time periods following which, total cell protein was extracted using 0.25 ml cold fresh lysis buffer (1% Triton X-100, 150 mM NaCl, 0.5 mM MgCl₂, 0.2 M EGTA and 50 mM Tris-HCl (pH 7.5) with aprotinin (2 μ g/ml), DTT (2 mM), and phenylmethylsulfonyl fluoride (PMSF, 1 mM)). Proteins from the cell lysates were separated on a 10-15% polyacrylamide gel under reducing conditions (reducing, SDS-PAGE). Proteins were then transferred onto nitrocellulose membrane, and the blot was blocked with 5% non-fat milk. The protein levels were detected by immune-detection with specific antibodies. Anti-p21, anti-CDK4, anti-CDK2, anti-Caspase 3 (SantaCruz biotechnologies, CA), and anti- β tubulin antibodies (Developmental Hybridoma Bank, IA)

were used at a concentration of 1:1000 dilution. Anti-cyclin D1, A and E polyclonal antibodies (SantaCruz biotechnologies, CA) were used at a concentration of 1:250 dilution. Appropriate HRP-conjugated secondary antibodies (SantaCruz biotechnologies, CA) were incubated at room temperature at a dilution of 1:5000. The specific protein complexes were identified using a chemiluminescence detection kit (Amersham, CA).

Cell cycle analysis

The cell cycle analysis was performed following the method described in the literature (Liu, Li, & Raisch, 2010). In brief, SW620 cells were switched to media without serum for a period of 24 hours, to render them quiescent and synchronize their cell cycle phases. Following this, the cells were treated with various concentrations of 2-DG for 18 hours and harvested by trypsinization. The harvested cells were washed twice with PBS and fixed in 70% ethanol at 4°C. The nuclear DNA was stained using propidium iodide (50 µg/ml) in the presence of DNase free RNase (2U/ml). The cells were sorted depending on the DNA content using a fluorescence activated cell sorter (FACS) (BectonDickinson, San Jose, CA). The proportion of cells in each phase of the cell cycle was determined using CellFIT software (BectonDickinson, San Jose, CA).

RESULTS

1. Colorectal cancer cells are more susceptible to 2-DG treatment

The cytotoxic effect of 2-DG on various cancer and non-cancer cell lines was tested using the MTT assay. The types of cell lines tested were non-cancer kidney epithelial cell line (293T), colorectal cancer cell lines (SW620, SW480 and GC3/Cl), breast cancer cell lines (MDA-MB-231 and MCF7), ovarian cancer cell line (Ovcar 3), and melanoma cell line (B16F10). The IC_{50} of 2-DG on various cell lines is represented in Figure 1. Out of eight cell lines tested, 2-DG exerted the least cytotoxic effect on the non-cancer cell line (HEK 293, IC_{50} : 6.452 mM), which further supports its enhanced selectivity towards cancer cells. The metastatic aggressive breast cancer cell line (MDA-MB-231, IC_{50} = 1.874 mM) was more susceptible to 2-DG treatment than non-metastatic cancer cells (MCF7a, IC_{50} = 5.891mM). The IC_{50} values of the three colorectal cancer cell lines were lower than that of

other cancer cell lines tested. Since colorectal cancer cells were more susceptible to 2-DG treatment than other cancer cells tested, and to maximize the subsequent output, we chose to use colorectal cancer cells (SW620) for further analysis of 2-DG activity in the Western blotting and cell cycle analysis.

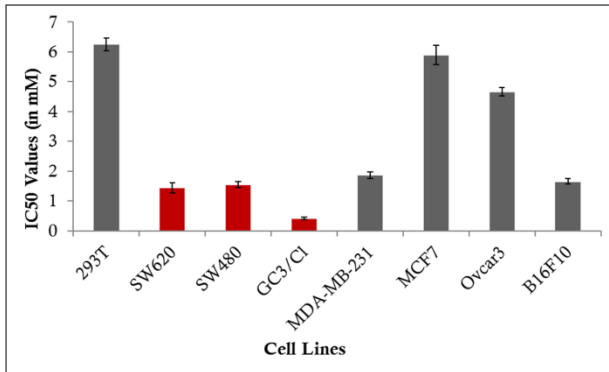


Figure 1. The IC₅₀ values of 2-DG in various cancer cell lines after 72 hour treatment at various concentrations (mM) of 2-DG. The colorectal cancer cell lines (represented in red) SW620, SW480 and GC3/CI exhibit the highest sensitivity to 2-DG treatment. The data represent the average of triplicates.

2. 2-DG inhibits cell cycle progression in colorectal cancer cell lines

The reduced viability of cancer cells after 2-DG treatment could be interpreted as a result of inhibiting cell cycle progression or inducing cell death or both. Therefore, the role of 2-DG in modulating the cell cycle progression was evaluated in SW620 cell line using flow cytometric analysis as described in the methods. Treatment of SW620 cells with 2-DG for 18 hours caused cell cycle arrest at the G₀/G₁ checkpoint (Figure 2). The percent of cells in G₀/G₁ significantly increased from 63.49 % to 80.15 % ($P < 0.05$; student T-test) after 2-DG treatment. Simultaneously the number of cells in S phase decreased from 19.11 % to 7.13 %.

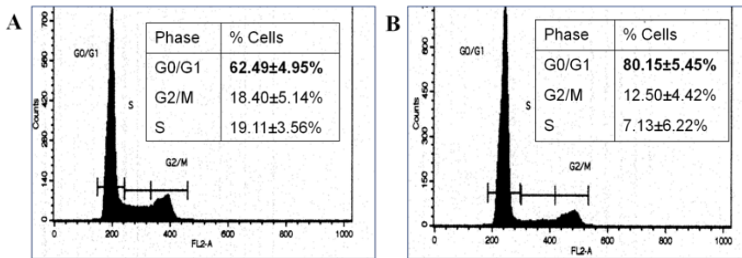


Figure 2. Cell cycle analysis of SW620 cells following 18 hour treatment with 20mM 2-DG using flow cytometry. Control cells **A**, and treated cells **B**, were fixed and stained with propidium iodide and analyzed for DNA content using flow cytometry. The data represents mean percent of cells in a particular cell cycle phase \pm standard deviation ($n=3$).

3. 2-DG increases the expression of p21 and p53 proteins

Cyclin Dependent Kinase (CDK)-inhibitory proteins, designated as CKIs (18) play a critical role in modulating the events of cell cycle. p21 is one of the key CKIs exerting a regulatory role in cell cycle progression. The expression of p21 can be p53 mediated or p53 independent. The direct effect of 2-DG on the expression of these proteins in SW620 cells was analyzed by Western Blot analysis (Figure 3 A). Treatment with 2-DG increased the expression of p21 as well as p53 in SW620 cell lines. This increase was dependent on the dose of 2-DG (Figure 3 A). To check the kinetics of p21 and p53 expression, SW620 cells were treated with 5 mM 2-DG for various time periods and the amount of p21 and p53 proteins in the cell lysate was analyzed by western blot analysis. 2-DG dependent increased expression of p21 was transient with the maximum expression occurring after 18 hours of treatment (Figure 3 B). The protein levels of p53 reached a plateau at 18 hrs following 2-DG treatment.

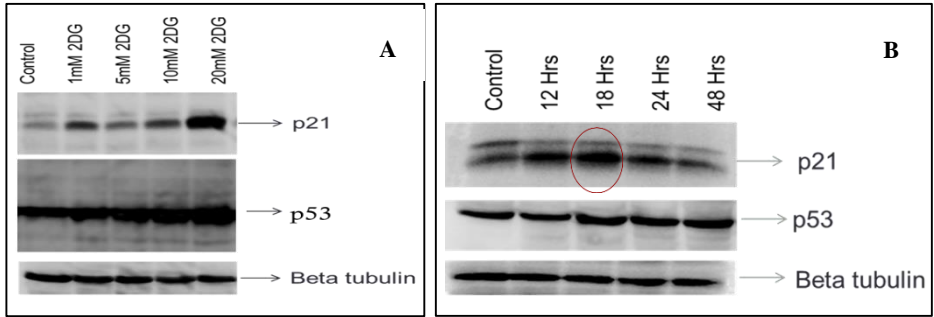


Figure 3 A. Effect of different doses of 2-DG treatment on anti-apoptotic p53 and p21 protein expression in SW620 cells. The cells were treated for 48 hours.

Figure 3 B. Transient expression of p21 in SW620 cells. Cells were treated with 5mM 2-DG for indicated time points. For both western blots, whole cell protein was extracted and ran on 12% acrylamide gel. The beta tubulin concentration was constant, showing that equal amounts of cell protein was loaded in each well. Beta-tubulin was used as a loading control in the blots.

4. 2-DG alters the expression of various cell cycle associated proteins

Expression levels of various proteins involved in cell cycle are critical for proper progression of cell cycle (Madhok *et al.*, 2010). The 2-DG treatment decreased the expression of various proteins associated with cell cycle progression (Figure 4). 2-DG treatment caused a down regulation of cyclin A, cyclin D and CDK4, whereas the levels of CDK2 and cyclin E remained unaltered.

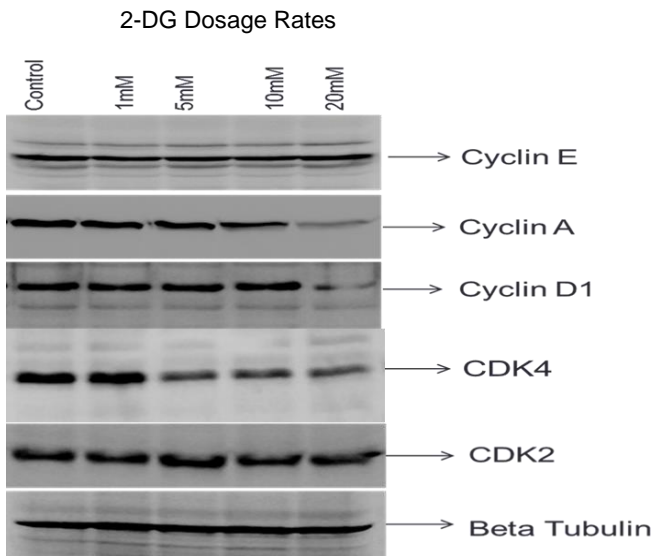


Figure 4. Relative expression of various cell cycle proteins. SW620 cells were treated with the indicated doses of 2-DG for 18 hours. The expression levels of various cell cycle associated proteins were checked by western blot analysis. The figure is a representative image of three separate experiments.

5. 2-DG causes cell death through apoptosis or cell death

Cell cycle arrest at G1/G0 phase and the transient over expression of p21 protein may direct the cells towards apoptosis (Wu *et al.*, 2002). To check whether the treatment of 2-DG causes the induction of apoptosis, SW620 cells were treated with various doses of 2-DG and apoptosis was analyzed by investigating an apoptotic marker protein called caspase 3. Caspase 3 exists in all cells, but this protein is cleaved during apoptosis and the cleaved caspase is active and acts as an effector protease during apoptosis. (Wolf *et al.*, 1999). As seen in Figure 5, there is an increase in cleaved caspase 3 with 2-DG treatment.

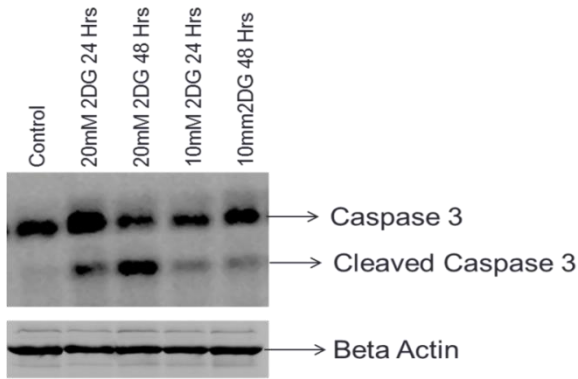


Figure 5. Expression of cleaved caspase 3 following the treatment with 2-DG in SW 620 cells. The control well was not treated with 2-DG. This is representative of three separate experiments.

6. The combination of 2-DG with 5-FU reduces the IC₅₀ of 5-FU

Once the effect of 2-DG on cell cycle and apoptosis was established, the complementary effect of 2-DG on the efficiency of 5-FU was investigated. The IC₅₀ of 5-FU was reduced by the addition of 0.25 mM of 2-DG in SW480 and SW620 while 0.025 mM of 2-DG was used in GC3/C1 for the treatment (Table 1).

Cell line	IC50		Fold enhancement provided by co-application of 5-FU and 2-DG
	5-FU alone (μM)	5-FU (μM) + 2-DG (0.25 mM)	
SW620	9.25 \pm 0.32*	5.23 \pm 0.18*	1.77
SW480	15.28 \pm 1.02*	5.17 \pm 0.54*	2.96
GC3/CI [#]	13.27 \pm 1.09*	3.75 \pm 0.38*	3.54

#The concentration of 2-DG used in GC3/CI was 0.025 mM

***Shows significant difference in variance by Student's *t*-test at $P < 0.05$**

Table 1. The IC50 values of 5-FU alone and in combination therapy with 2-DG in various cancer cell lines after 72 hour treatment at various concentrations of 5-FU. In all cases, 2-DG enhanced sensitivity compared to 5-FU alone. The data represents the average of triplicates.

DISCUSSION

Current first line treatment of colorectal cancer, 5-FU as a monotherapy or in combination therapy, has the major drawback of extensive tissue toxicity. The major side effect of 5-FU is myelosuppression, which can lead to many other health complications (DiPiro *et al.*, 2011). For this reason, developing anticancer agents with less toxicity to normal tissues would be desirable. An example of a drug with such an ideal safety profile is 2-DG. It is known to block glycolysis by inhibiting the key glycolytic enzyme hexokinase (Scatena *et al.*, 2008). However, the full array of effects of 2-DG in cancer cells cannot be completely explained by its inhibitory effects on the glycolytic pathway (Qutub & Hunt, 2005; Stein *et al.*, 2010). In this manuscript, we have identified a plausible new mechanism of 2-DG on colorectal cancer cells. Colorectal cancer cells were more susceptible to cytotoxic effects of 2-DG when compared to other cancer or non-cancer cell lines tested. The IC50 values of 2-DG in colorectal cancer cells was almost four-fold lower than in non-cancerous HEK293 cells. This finding is consistent with the fact that 2-DG is a useful therapeutic agent and exerts minimum toxic effects *in-vivo* on non-cancerous cells (Kang & Hwang, 2006). The

increased potency of 2-DG in colorectal cancer makes it an attractive candidate for such types of cancer where local high concentrations of the drug can be achieved using novel formulation strategies such as colon targeted drug delivery (Philip & Philip, 2010).

It was observed that 2-DG shifts the SW620 cells G0/G1 phase and sharply decreases the numbers in the S phase during the cell cycle analysis. The cell cycle inhibition may be attributed to the transient expression of p21, a Cyclin Dependent Kinase Inhibitor (CKI). Moreover, the transient increase in expression of p21 was independent of p53 levels and this observation might suggest the molecular effect of 2-DG on p21 levels, irrespective of p53 status. Furthermore, the expression levels of various cell cycle associated proteins such as cyclin A, cyclin D1 and CDK4 were also reduced. Such a reduced expression of CDK4 and other cell cycle-associated proteins may be responsible, in part, for the G1/G0 cell cycle arrest observed after 2-DG treatment (Figure 2). During the treatment of SW620 with 2-DG, increased amounts of cleaved caspase were seen in the treated versus the untreated cells. An expression of cleaved caspase 3 such as this indicates that the cells are undergoing apoptosis. The implications for these findings would be the ability to identify potential new synergistic combination therapies of 2-DG with other chemotherapeutic agents that have similar apoptosis inducing mechanism. 2-DG has had very limited success as a single agent in treating cancer (Kurtoglu *et al.*, 2007). What is promising though are the studies in which 2-DG is used in combination therapy. In mice that bear human osteosarcoma or non-small cell lung cancers, 2-DG has been shown to drastically increase the anticancer efficacy of adriamycin and paclitaxel (Maschek *et al.*, 2004). As far as potential for combination therapy in colon cancer, a recent paper has found that the drug lupulone induces the expression of p53 in both SW480 and SW620 cells through activation TRAIL death receptor signaling pathways (Lamy *et al.*, 2010). Since 2-DG and lupulone both induce p53 expression, this brings up the potential of a synergistic combination therapy. By identifying other drugs that have similar mechanisms in colorectal cancer, more combination therapies can be tested.

2-DG is a very safe molecule for cancer treatment, however, it is very challenging to achieve the therapeutic concentration of 2-DG in a clinical setting (Figure 1: IC50 in SW620 is ~1.436 mM). Therefore, we have proposed to use 2-DG as an adjunct therapy at concentrations from 0.025 mM to 0.25 mM with existing chemotherapy to enhance the efficiency of chemotherapy. The complementary therapy of 5-FU with 2-DG was also

established by analyzing the increased efficiency of 5-FU killing of the colorectal cancer cell lines in the presence or absence of 2-DG. The IC50s of FU decreased 1.77 to 3.54 fold, depending on the cell line used, when 0.025-0.25 mM 2-DG was co-administered. 2-DG is a safe drug in large concentrations in both humans and animals (Scatena *et al.*, 2008). By using small concentrations of 2-DG, and the range used was monumentally smaller than the IC50 of our normal cell (Figure 1: IC50 in HEK 293T is ~6.251 mM), a drop in the amount of 5-FU needed to kill half of the cancer cells sharply decreased. The implications for this finding would be that doses of 5-FU required to treat colon cancer could be reduced when 2-DG is given with 5-FU. This will potentially reduce side effects of 5-FU as the side effects depend on the dose of 5-FU. This could lead to better health outcomes and an improved quality of life in colon cancer patients.

Future studies are needed to establish independence of the plausible new mechanism of 2-DG from glycolytic inhibition in these cell lines. In vivo experiments will also be necessary to see if these effects of 2-DG hold true in an animal model. In vivo experiments will allow us to design a dosage form system that could release the combination therapy of 5-FU with 2-DG once it reaches the colon. This would allow the medication to be released and be in the highest concentration in the colon. A successful development in this dosage form would further reduce chemotherapy exposure to other parts of the body not necessitating chemotherapy.

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