Abstract
Though anti-cancer drugs have been in use since 1971, many still have a fairly low success rate in treating cancer. Since recent studies have linked tumor development to mitochondrial modifications, reversing these changes could reduce tumor size. Cancerous cells suppress their mitochondrial activities and use glycolysis rather than oxidative phosphorylation to synthesize ATP. This process is known as the Warburg Effect. Dichloroacetate (DCA), a mitochondria-targeting drug, and etoposide, a topoisomerase II inhibitor, may be able to reverse abnormal metabolic processes in cancer cells. DCA shifts ATP production back to oxidative phosphorylation and activates mitochondrial potassium channels, which consequently induce apoptosis. Etoposide inhibits catalytic activity of topoisomerase II, releasing supercoils in DNA. Effectiveness of drug treatment was assessed based on cell viability. An MTT Assay was performed on cells treated with dichloroacetate, etoposide, or after treatment with both drugs. The Western blot performed in this study was used to determine protein levels of the etoposide-treated cells. Dichloroacetate, etoposide, and both drugs together were successful in lowering cell viability of Hep G2 cells. Though each treatment resulted in apoptosis, etoposide’s MTT Assay did not clearly illustrate increasing apoptosis with higher drug dosages as expected. However, western blot analysis showed greater apoptosis after the first increase in dosage, but not the second, suggesting that lower concentrations of etoposide would need to be used in order to observe a correlation between dosage and apoptosis. Since DCA and etoposide were both effective in lowering cell viability, these treatment methods should be further investigated by testing their synergistic effects in clinical trials of various cancers.

Introduction
Research of various cancer treatments has been rapidly expanding ever since the “war on cancer” started in 1971. However, the development of targeted and effective cancer treatment drugs has not been as successful as initially expected. This lower than expected success rate can be in part attributed to cancer’s rapid mutation and adaptation rates. Another problem is that a drug developed specifically for one type of cancer may not have the same effect on a different type of cancer, since each cancer cell is slightly different and can even change according to its environment. Cancer cells differ not just from each other but also from other cells.

In regular cells, the cell cycle is carefully coordinated and has checkpoints, controlled by cyclins and cyclin-dependent kinases, to make sure that only healthy cells advance to the next steps. However, cancerous cells are mutated, allowing them to progress through the cell cycle without restraints (1). When a normal cell is damaged, it undergoes self-induced death, called apoptosis. However, cancerous cells lack the ability to undergo apoptosis and therefore exhibit the problem of continual cell division. Cancerous cells are able to avoid apoptosis and adapt quickly to anticancer drugs due to their unique metabolism. These carcinogenic cells can alter metabolism of their mitochondria, the organelle responsible for a cell’s metabolic activity and where apoptosis and energy production converge. The modified mitochondria can make the cell resistant to apoptosis (2).

Most cancerous cells transform energy using glycolysis, which does not utilize oxygen. Glycolysis produces a small amount of ATP, which is used for energy requiring reactions. This process of shifting from aerobic respiration to anaerobic respiration is known as the Warburg Effect. This process is thought to be correlated with mitochondrial dysfunction, which causes increased glucose uptake and metabolism (2).

Glycolysis is thought to be associated with resistance to apoptosis in malignant neoplasms. Several of the enzymes involved in glycolysis are also involved in apoptosis. The enzymes provide a link between metabolic sensors and apoptosis since they increase metabolic changes and make the cell compatible with the remodeled mitochondria’s glycolytic phenotype. The use of anaerobic glycolysis continues in cancerous cells because it offers resistance to apoptosis (2).

Pro-apoptotic factors such as cytochrome c and other apoptotic inducers are protected in the mitochondria, and when the mitochondrial transitory pores (MTP) open they enter the cytoplasm and induce apoptosis. If the metabolic process begins with pyruvate entering the mitochondria, MTP opening and apoptosis are suppressed (2). Dr. Otto Warburg, for whom the Warburg Effect is named, noticed that cancerous cells suppress their mitochondrial activities, and use glycolysis rather than oxidative phosphorylation for ATP production. The Warburg Effect and the increased mitochondrial activity of glutaminolysis, caused by glutamine catabolism are two important features, which characterize cancer. (3).

The generic drug, dichloroacetate (DCA), is a mitochondria-targeting drug that may have the potential to reverse the metabolic remodeling that occurs in cancer cells (2). Humans have been exposed to this drug for centuries through chlorinated drinking water (4). Dichloroacetate is able to reverse the Warburg Effect by shifting ATP production back to oxidative phosphorylation.

Dichloroacetate and Etoposide Induce Apoptosis in Hep G2 Cells

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Dichloroacetate is able to increase pyruvate dehydrogenase activity, and this increase in pyruvate causes an efflux of cytochrome c and apoptotic-inducing factors that restore oxidative phosphorylation. DCA decreases membrane potential and activates mitochondrial potassium channels, and this restoration of mitochondrial processes consequently induces apoptosis (3). Recently, the drug dichloroacetate has been effective in reducing tumor-size in the preclinical phases of breast, brain, liver, leukemia, and colorectal cancer.

A recent study targeted two aspects of cancer metabolism in breast cancer cells using arsenic trioxide and dichloroacetate. Arsenic trioxide is able to open mitochondrial transitory pores, which release cytochrome c and thereby depolarize mitochondrial membranes. Dichloroacetate is able to reverse the Warburg Effect by shifting ATP production back to oxidative phosphorylation. The restoration of the mitochondria consequently induces an increase in apoptosis. While dichloroacetate was able to reverse the shift to glycolysis, arsenic trioxide targeted the mitochondria directly. The described study was the first to use two drugs to target cellular and mitochondrial metabolism and was an effective in vitro strategy (3).

Another in vitro study conducted on cancer cells tested the synergistic effect of DCA with a combination of different platinum compounds. This study showed that when dichloroacetate was combined with platinum-based compounds, its anti-proliferative activity was increased (5). Another study involving dichloroacetate was conducted on leukemia. In the conducted experiment, dichloroacetate was delivered to target cancerous monocytes by hemoglobin (Hb), an effective drug carrier because it is already sought out by monocytes and macrophages. Hb works with dichloroacetate in THP-1 cells, a leukemia cell line, inducing apoptosis and therefore leading to cell death. A dichloroacetate and hemoglobin complex was found to be formed via peptide bonds resulting in the induction of apoptosis (6).

In response to studies conducted on the effects of dichloroacetate on glioblastoma, breast, and lung cancer cells, a study was conducted on endometrial cells. Endometrial cancer cells originate from the epithelial lining of the uterine corpus. Dichloroacetate was found to promote apoptosis among endometrial cells without significantly affecting the non-cancerous cells (7). Yet another study was conducted on colorectal cancer. This study also used 2 drugs, 5-Fu and dichloroacetate, as part of the cancer treatment. The combination of the drugs yielded greater apoptosis than either drug yielded on its own (8). An in vitro study of HeLa cells showcased the effectiveness of dichloroacetate. In this study, the in vitro effects of the drug along with CO2 were tested in a hypoxic HeLa cell line. In each experiment, dichloroacetate was once again shown to increase apoptosis (9).

The tumor microenvironment conditions and the Warburg Effect play a role in many epithelial cancers. A 2010 study showed wide applications of controlling the Warburg Effect. This new study was able to provide evidence that the Warburg effect is not just confined to epithelial cancers and their mitochondria, but is also present in the stromal Cav-1, a type of human breast cancer cell line. Because research presented in this study showed that control over the effect was possible, this process was termed the “Reverse Warburg Effect.” This finding shows the significance of the Warburg Effect in a broad range of cancers and suggests a treatment pathway if it can be effectively manipulated (10).

One characteristic that makes dichloroacetate promising as an anti-cancer drug is that it has fewer side effects than other commonly used medications. Most of its side effects are known, because dichloroacetate has been used to treat metabolic disorders in the past. While dichloroacetate has only recently been shown to be an effective treatment for cancer, it has been used to treat lactic acidosis for years. Lactic acidosis is caused by a disorder of acid and base metabolism. Dichloroacetate treats the disorder by inhibiting glycolysis and increasing oxidative phosphorylation, decreasing lactate production. In 1992, a clinical study was conducted on adults with lactic acidosis using dichloroacetate as treatment. The study showed that dichloroacetate was able to reduce hyperlactatemia and improve the acid/base balance (11).

Another drug that has recently been used in cancer treatment is etoposide. Etoposide is a topoisomerase II inhibitor. The drug unwinds the supercoiled strands and introduces DNA strand breaks by establishing a cleavable complex. However, etoposide does not lead to DNA degradation. Its action is accomplished by inhibiting the catalytic activity of topoisomerase II, which allows the disentanglement of DNA. This alteration of DNA causes proapoptotic protein Bax to be translocated into the mitochondria. This translocation results in cytochrome c release and consequently induces apoptosis. In comparison to other antitumor drugs, etoposide shows lower levels of DNA binding, suggesting that its inhibition of catalytic activity is due to a direct targeting of DNA topoisomerase II (12).

Based on the studies above, an important next step in cancer research is to evaluate the synergistic effectiveness of dichloroacetate and etoposide in Hep G2 cells, a perpetual cell line derived from hepatocellular carcinoma. Dichloroacetate and etoposide both restore mitochondrial function and consequently apoptosis, coaxing cancer cells to undergo “cell suicide.” Therefore, it was hypothesized that dichloroacetate, etoposide, and the synergistic effect of both drugs would result in reduced cell viability and apoptosis of carcinogenic Hep G2 cells, and consequently reduced tumor sizes.

Materials and Methods

**Cells and Regents:** The Hep G2 cells were purchased from American Type Culture Collection (Manassas, VA, USA). Cell culture reagents were purchased from Genesee Scientific (San Diego, CA, USA) and Caisson Labs (North Logan, UT, USA). Cell lines were maintained in Minimum Essential Medium (Earle’s) containing 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (P/S) at 37.6°C, with 5% CO2 in a humidified incubator. Etoposide (Etop), dichloroacetate (DCA), Trypan blue and MTT reagents were purchased from Sigma-Aldrich Co. Ltd (St. Louis, MO, USA). Caspase-3 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Stock solutions of DCA (1M) and Etop (0.1M) were used throughout the experiment. Briefly, 8.58 X 10^6 cells (See Figure 4) were seeded into 8-well tissue culture plates. The cells were then incubated for 48 hours at 37°C under standard growth conditions. **MTT Assay:** Cells were washed with PBS buffer and then 5 mL of trypsin was added to each flask of cells to detach them. The solution was swirled around in the
flask and was placed in the incubator for 10 minutes under standard growth conditions followed by an addition of 5 mL of medium. The mixture of trypsin, medium, and cells was swirled, collected and centrifuged at 1100 RPM for 5 minutes. Supernatant was removed and pelleted live cells were resuspended in 6 mL of medium. Trypan Blue dye was used to differentiate between dead and living cells. 10,000 cells were seeded into each of 60 wells. Wells were labeled blank, control, 10/25/50/100 mM DCA concentrations, 25/50/100/200 mM Etop concentrations, and combined 10/25/50/100 mM DCA and 50mM Etop concentrations. After a 24-hour incubation, cells were treated with the various concentrations of drugs listed. Wells were incubated for 48 hours. MTT reagent, a tetrazolium dye whose color change is used to distinguish between live and dead cells, was mixed with water to obtain a 5 mg/mL concentration. Medium was removed from the wells. Wells were washed twice with 100µL of HPSS medium. A mixture of 600µL of MTT reagent and 5400 µL of medium was made and 100µL of this solution was added to each of the 60 wells. Wells were placed in an incubator for 4 hours. Then, after the medium was discarded, 100 µL of DMSO was added to each well. The number of living and dead cells was determined using a Microplate Reader at 570 nm. Cell viability was calculated using Equation 1. Three trials were conducted.

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\text{% of dead cells} = \frac{[A \text{ sample} - A \text{ blank}]}{[A \text{ control} - A \text{ blank}]} \times 100 \quad \text{Equation 1}
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**Lysis buffer preparation:** Lysis buffer was prepared using 4 µL of 100X cocktail inhibitor enzyme and 4 µL of 0.1 M PMSF (Phenylmethylsulfonylfluoride) in RIPA buffer (25mM TrisHCl, pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS). **Protein Extraction:** Medium in the wells was aspirated and cells were washed with 1 mL of cold PBS. 50 µL of Lysis buffer was added to each well. Wells were then incubated on ice for 30 minutes. The cell lysate was collected in eppendorff tubes and centrifuged at 12,000 RPM for 15 minutes. Supernatant was collected from each tube. **Protein Assay:** Biorad Protein assay was used to determine total concentration of proteins. To separate samples, 2 for each concentration, 797 µL of water and 3 µL of the supernatant were combined. Five protein standard dilutions with concentrations of 2.5, 5, 7.5, 10, and 12.5 (mg/mL) were prepared. 800 µL of each standard were used for protein assays which were performed in duplicate. 200 µL of BioRad dye was added to each of the test tubes, which were then vortexed. Then the tubes were incubated at room temperature for 10 minutes. Absorbance, which corresponded to the amount of protein in each tube, was then measured using a spectrophotometer at a 595 nm wavelength. **Western Blot:** Cells were cultured and then subcultured. A standard western blot was performed. Sample concentrations used were control, 50mM, 100mM, and 200mM Etop. The primary antibody used was caspase 3. **Statistical Analysis:** Two sample t-tests were conducted comparing the controls with each of the drug concentrations. ANOVA tests were used to evaluate and compare the cytotoxic values of the treatments. A value of p < 0.05 was considered to reveal statistically significant differences between data sets. Three trials were conducted at each concentration for the MTT Assay and the western blot.

**Results**

The effect of dichloroacetate on cancerous tissue was significant. As the dichloroacetate concentration increased, the percentage of living cells decreased, (p-value less than 0.0000001). The cell viability was significantly lower for cells treated with 100mM dichloroacetate than for any other concentrations as shown by the MTT Assay (Figure 1). None of the cells survived this treatment. Using a two-sample t test, the untreated cells were compared to each drug treatment concentration. The p-values for all dichloroacetate treated cells were less than 0.05, indicating that the effect at each concentration was significant. Using an ANOVA test, the viability of control untreated cells and treated cells exposed to varying dichloroacetate concentrations were compared. The p-value for this comparison was 8.85E−10, supporting the hypothesis that dichloroacetate would decrease cancer cell viability.

As the etoposide concentration increased, the percentage of living cells decreased at first, but at 50 mM, 100 mM and 200 mM it appeared to increase as shown by the MTT Assay (Figure 2). In fact, lack of a trend was shown by a low R2 value of 0.37. However, when compared statistically, the viability percentages for the increasing concentrations were not significantly different from each other (ANOVA single factor p-value of 0.1014) suggesting that either 25mM of etoposide is sufficient for its maximum effect and any extra drug results in no additional cell death or that the assay was faulty. Using a two-sample t test, the viability of untreated cells was compared to their viability after they were treated with varying concentrations of the drug. The p-values for each comparison were less than 0.05. Using an ANOVA test, the cell viabilities at varying etoposide concentrations were also compared. The p-value for this analysis was less than 0.00001, so the hypothesis that etoposide would kill cells was supported.

Figure 3 shows the results of a western blot conducted to ascertain cell apoptosis after treatment with different concentrations of etoposide. Caspase 3 (CASP3: cysteine-aspartic acid protease) is sequentially activated during apoptosis. When cleaved into two subunits (16 and 12 kDa) Caspase 3 becomes activated. Therefore, as the amount of large (~35 kDa) protein decreases, the amount of cleaved (active) caspase 3 increases, resulting in greater apoptosis. As seen in Figure 3, treatment with 0 mM etoposide results in a greater amount of the full-size caspase 3 than treatment with either 50 or 200 mM of this drug, suggesting that more cancer cell death is occurring through apoptosis when higher concentrations of etoposide are used. Both the MTT Assay and Western Blot results indicate that cell growth decreases, while apoptosis increases with increasing etoposide concentrations. However, cell viability as observed using the MTT Assay appears to level off after 50 mM of etoposide while caspase 3 activity, which leads to apoptosis, continues to be affected until 100 mM of etoposide are used. It is possible that this discrepancy is due to the techniques used to detect cell death. Although both assays test for cell viability they do so via different mechanisms, which could be more or less affected by etoposide concentrations.
As shown by the MTT Assay (Figure 4), as the concentration of both drugs increased, the percentage of living cells decreased. The combinatorial treatment of dichloroacetate and etoposide showed no synergistic or additive effect compared to treatment with dichloroacetate alone. Using an ANOVA test the viability of control, untreated cells was compared to the viability of cells after being treated with varying drug concentrations. The p-value for this analysis was 0.0000001, indicating that dichloroacetate and etoposide would reduce cancer cell viability. Using a two-sample t test, the viability of untreated cells was individually compared to each drug treatment concentration. The p-value for all dichloroacetate and etoposide treated cells compared to untreated cells was less than 0.05, indicating that these drugs significantly reduced cell viability.

Figure 1. The mean cell viability and standard error of Hep G2 cells after treatment with varying dichloroacetate concentrations (n=3). The viability was calculated by comparing the number of surviving cells to the control, untreated cells. The p-value from the ANOVA test was 8.85E-08. When comparing untreated cells to each concentration (10mM, 25mM, 50mM, 100mM), all of the p-values were below 0.05.

Figure 2. The mean cell viability and standard error of Hep G2 cells after treatment with varying etoposide concentrations (n=3). The viability was calculated by comparing the number of surviving cells to the control, untreated cells. The p-value from the ANOVA test was 2.43E-06. When comparing untreated cells to each concentration (25mM, 50mM, 100mM, 200mM), all of the p-values were below 0.05.

Figure 3. Western blot of proteins from cells treated with etoposide after incubation with caspase 3 antibody. Lane 1: size standards. Lane 2: control. Lane 3: caspase 3 from cells treated with 0 mM Etop (relative intensity of 2.4x10^6). Lane 4: caspase 3 from cells treated with 50 mM of Etop (relative intensity of 9.6x10^5). Lane 5: caspase 3 from cells treated with 200 mM of Etop (relative intensity of 9.7x10^5). Size of bands: 38.5-40.7 kDa. All size and intensity estimates were calculated using Gel Doc Easy Imager (BioRad).

Figure 4. The mean cell viability and standard error of Hep G2 cells after treatment with varying dichloroacetate concentrations and 50 mM etoposide (n=3). To test for a synergistic effect of these drugs, the cell viability was calculated by comparing the number of surviving cells to the control, untreated cells. The p-value from the ANOVA test was 1.75E-08. When comparing untreated cells to each concentration (10mM, 25mM, 50 mM DCA and 50mM Etop each) the , all of the p-values were below 0.05.
Discussion
DCA, as a pyruvate dehydrogenase kinase inhibitor, can reverse the glycolytic effect and has demonstrated significant anti-cancer properties both in vitro and in vivo and some of the treatments using this drug have even shown an increase in apoptosis (2, 3, 7). In this experiment, dichloroacetate was used in combination with etoposide, a topoisoamerase inhibitor. Dichloroacetate, etoposide, and the combination of both drugs were able to lower cell viability of Hep G2 cells (see Figures 1-4). The hypothesis that dichloroacetate would be able to induce apoptosis and decrease cell viability, and that dichloroacetate and etoposide together would be more effective than etoposide alone, were supported because each treatment demonstrated decreased cell growth (p-values 8.85E-08, 1.75E-08 respectively).

Although combining dichloroacetate and etoposide did not appear to decrease cancer cell viability any more than simply treating these cells with dichloroacetate alone, a two-drug approach to cancer cells in vivo could be very beneficial. Cells in culture are usually uniform so they may be treated effectively by blocking or activating a single pathway; however, within human tissues cancerous growths are much more diverse and could benefit from the synergistic effect of several drugs acting on multiple pathways even if this effect is not seen in cultured cells. Therefore, results from the presented experiment are useful in that they show a strong effect when the drugs are used individually and together.

A possible source of error for the MTT Assay could be cell plate thickness. When etoposide was being analyzed in the Microplate reader, the data showed that etoposide did not lower the cell viability as much as expected. Since absorbance was being measured with the Microplate reader the plate thickness may have prevented detection of subtle changes in color and therefore cell viability. Another source of error to account for the etoposide results could be that the machine may not have been as sensitive to the color change produced at 50mM and 100mM concentrations.

Since both drugs were effective in lowering cell viability, they should be further investigated as potential treatment methods for various cancers. The treatments should be tested in in vivo experiments to determine the best dosage and types of cancers most adversely affected by these medications.

The treatment proposed here is an effective potential option not just because of its promising initial results, but also because of its safety. Humans have been exposed to dichloroacetate for many decades through chlorinated drinking water, and it is known to have fewer side effects than many other common drugs. Another beneficial aspect of dichloroacetate is that it has been shown to be effective in several pre-clinical trials of different types of cancers, including, thanks to this study, liver cancer. Finally, one of the most appealing aspects to patients is that the drug is generic and affordable. Since cancer is such a heterogeneous group of diseases, the more diverse and extensive the arsenal of drugs available, the more likely we are to either eradicate it or force it to kill itself.

References