Is Glycolysis Inhibition a Good Method for Cancer Treatment?

By Monica Siu

Inside the cells of the human body, there is a nucleus. The nucleus contains genes which contain instructions on how to act and when to grow, divide, and die. If there is some fault in the genes, the cell is generally capable of repairing "damaged" genes by different mechanisms such as: excision repair, mismatch repair, double-strand break repair, and cross link repair. (1)

When a cell is mutated, it is unable to interpret the instructions from its genes. It generally grows rapidly and without being able to repair the DNA damage, it will be not be able to self-destruct. These mutated cells will grow exponentially, leading to cancer. (2) The cancer cells, if large enough, can eventually replace normal tissue, and possibly crowd and push the tissue aside. Cancer cells can easily spread when they travel through the body via the bloodstream or lymph vessels. (3)

Common cancer treatments include: surgery, the use of medicines or drugs, radiation, heat, and stem cell transplants. (2) Each of the treatment methods are dependent on the location as well as the condition of the tumor, and at what stage the tumor is. Surgery is the ideal method, particularly finding the tumor and extracting it out, but the difficulty lies upon cancer cells spreading to other parts of the body through the bloodstream or lymph vessels. (4) Researchers have been trying to find new treatments via therapy. One such example is the inhibition of glycolysis.

From a recent experiment, chemotherapy was combined with a protein that inhibits glycolysis. (5) It is known that cancer cells use glycolysis rather than oxidative phosphorylation to produce energy. The researchers wanted to find a way to further enhance the effects of battling cancer cells. The researchers used 2-deoxyglucose (2DG), a competitor inhibitor of glucose transport, because both use the same transporters. Once phosphorylated by hexokinase, 2DG was converted into 2DG-6-phosphate which does not further metabolize in the glycolysis pathway. 2DG not only immensely lowers the amount of product formed by glycolysis, but it also prevents the N-glycosylation of proteins, resulting in the unfolding of proteins. N-glycosylation is where the sugar molecule glycan binds to a nitrogen atom of a protein. (6) It is an extremely important linkage for the protein's structure and function

Flow cytometry and fluorescence were used to measure the percentage of apoptotic cells that were present. (7) Apoptotic cells are cells that are programmed for self-destruction. Lymphoma cells were first isolated and then treated with etoposide (ETO), a DNA damaging cytotoxic anticancer drug, in the presence and absence of 2DG; the absence of 2DG was the control. Propidium iodide was then added to the cells as a substrate to determine the apoptotic cells by fluorescence. Data was taken immediately and resulted in where 2DG combined with greater amounts of ETO had a higher percentage of PI; PI correlates to the percentage of apoptotic cells (8). To confirm the results, the amount of DEVD-ase activity was measured using fluorescence as well. Caspase-3 cleaves DEVD at the site where the amino acid sequence: aspartate-glutamate-valine-aspartate occurs, hence DEVD. This cleavage happens during apoptosis. (9) By measuring the DEVD-ase activity, the activity of apoptosis is also measured.

Wild-type C57BL/6 mice were injected with lymphotic cells. Lymphotic cells are white blood cells in the immune system of a vertebrate. They were then injected with either PBS (control), 2DG, ETO, or a combination of ETO and 2DG three times a week for three weeks. The weight of the lower abdominal lymph nodes were compared after the third week of treatment. The mice injected with 2DG alone and PBS were the heaviest. The combination of ETO and 2DG, and ETO alone was able to reduce the lymph node enlargement, meaning they were successful to treating the immune response. The survival rate of the mice was also recorded after the last injection. The combination of ETO and 2DG, and ETO alone had the longest survival rate of up to 147 days and 84 days, respectively.

The researchers wanted to look further into how ETO alone and the combination of ETO and 2DG compared in the activation of cytotoxic T cells. A new set of wild-type mice were injected with either ETO alone or ETO and 2DG for a week. The researchers isolated the CD8+T cells from the spleen and then incubated the cells with tumor target cells. From the results, the cells from the cotreated mice were more efficient in killing tumor cells compared to the mice treated with ETO only.

The researchers concluded that the combination of glycolysis inhibition and a cytotoxic agent can induce apoptosis and increase the life span of the mice. The results showed 2DG and ETO resulted in the longest survival rate for the mice and had the highest activation of cytotoxic T cells, when compared to ETO itself. This showed a combination of chemotherapy with a substrate causing the inhibition of glycolysis had favorable results in comparison to chemotherapy itself, thus supporting glycolysis inhibition can potentially be a treatment to cancer. However, the researchers did caution future experiments may discover the tumor relapsing in a more aggressive form.

Another research experiment showed how HuR, an mRNA-stabilizing protein, can be targeted by the β-TrCP1 protein to cause degradation due to the response to glycolysis inhibition. (10) It is known that in cancer therapy, HuR can stabilize and/or alter translation rates of target mRNA, thereby regulating the expression of proteins implicated in oncogenesis and tumor progression. HuR is highly abundant during the presence of a tumor.

The levels of HuR were measured in the presence and absence of glucose in the cytoplasm. β-actin was used as a control because β-actin is unaffected by glucose. (11) This was done by quantitative real time PCR (qRT-PCR), using flurosecent probes to measure the amplification of RNA. (12) In the absence of glucose, the HuR levels showed a significant decrease in comparison to the presence of glucose. The percentage of mRNA expression with and without glucose was measured afterwards. The results showed glucose had no effect on the mRNA expression. Then, the rate of decrease of HuR in the absence of glucose was measured. The glucose transport inhibitors used were 2DG and CG-5 which both showed consistent results of the decrease in HuR levels. Then the decreasing rate of HuR in the presence of CG-5 was compared in the cytoplasm and nucleus. CG-5 showed a higher depletion rate of HuR in the cytoplasm than the nucleus. 2DG was again used to confirm glucose inhibition cause HuR to have a faster rate of depletion in the cytoplasm than the nucleus, which proved true. Afterwards, the researchers used leptomycin B to examine its effect on HuR depletion, considering leptomycin blocks nuclear export of proteins by alkylating and inhibiting the protein chromosomal region maintanence/exportin 1 (CRM1). (13) Results showed leptomycin B blocked CG-5's facilitation of HuR depletion.

Western blotting was used to determine how the presence of CG-5 affected β-TrCP1 and β-TrCP2. The two β-TrCPs are very similar except β-TrCP1 has roughly 25 more amino acid residue than β-TrCP2. Results showed the presence of CG-5 caused the amount of β-TrCP1 to increase. However, there was no effect on the β-TrCP2. This suggests that β-TrCP1 has a unique role in the presence of CG-5.

Coimmunoprecipitation analysis and GST pulldown assay were done to verify the physical interactions between β-TrCP1 and HuR. Coimmunoprecipitation and GST pulldown assay are both methods used to determine physical interaction between multiple proteins. In coimmunoprecipitation, an antibody is used and mixed in with a protein mixture. The antibody binds to a specific protein, thus forming precipitate. (14) In a GST pulldown assay, a GST marker binds to the protein of interest from a protein mixture. After transcription and translation, the GST protein and protein of interest fuse together as one protein. The protein mixture goes through beads where the GST-protein of interest fused protein will stick to the beads. Then the fused protein gets washed with free GST to detach the

protein of interest from the beads. (15) DMSO was used as the control to see the effects of CG-5 to the unknown interactions. The result was CG-5 increased the binding of β-TrCP1 and HuR, relative to DMSO.

The researchers concluded that glycolysis inhibition causes HuR to translocate to the cytoplasm where it is targeted by β-TrCp1 for degradation. It is still under investigation if a glucose transport inhibitor can be used as a cancer preventative.

Glycolysis inhibition has other perks than battling cancer. It can have positive effects like increased fat disposition in adipose tissue as well as increase protection against insulin resistance and inflammatory responses. (16) There was an experiment performed which focused on how the overexpression of inducible 6-phosphofructo-2-kinase (iPFK2) can increase fat disposition in the adipose tissue but at the same time protects against diet-induced insulin resistance and inflammatory responses. It is known that iPFK2 produces fructose 2,6-bisphosphate which is the most important activator for the glycolytic enzyme 6-phosphofructo-1-kinase. 6-phosphofructo-1-kinase is a critical enzyme in the phosphorylation of fructose 6-phosphate into fructose 1,6-bisphosphate, one of the important products formed before becoming pyruvate. Pyruvate is an important factor to energy production in living cells with the presence of oxygen, otherwise an important factor to producing lactate with the absence of oxygen. It can also branch off to form fructose 2,6-bisphosphate, which controls insulin production, a regulator of sugar levels in the blood.

There were two types of mice, Tg mice and the control. The Tg mice had overexpressed PFKFB3/iPFK2 in their adipose tissue by using a aP2-PFKFB3 cDNA transgene, a process which transfers genetic material from one organism to another. (17) The wild type (WT) littermates were the controls. To confirm the Tg mice had the overexpression in their adipose tissue, Western Blot analysis was used to determine the amount of iPFK2 in the white adipose tissue, liver, muscle, and bone marrow. In Western blot, the protein mixture is put into gel electrophoresis where it is separated, then the results get transferred to a membrane which produces a band of each protein. The membrane get incubated and then labeled with an antibody-specific label which has a specific binding for the protein of interest. The membrane gets washed, resulting in the removal of the unbound antibody. Only the bound antibody to the protein of interest remains. The bound antibodies then become detected visually in a charged coupled device. (18)

At 5-6 weeks of age, the mice were either given a high fat diet (HFD) or low fat diet (LFD). This was done for a 12 week period. Insulin, glucose, and plasma levels were tested by analyzing blood samples. 4 hours after the feeding, the mice would be injected with either insulin or D-glucose. The amount of insulin given to the mice depended on their diet; LFD mice were given .5 units/kg of insulin while the HFD mice were give 1 unit/ kg of insulin. The mice injected with D-glucose were given 2 g/kg of glucose, regardless of their diet. Blood samples were taken from the mice's tail vein to test for insulin in the blood. Blood was analyzed before the injection and 15, 30, 45, and 60 minutes after the injection, whereas collection to test for glucose was before the injection and 30, 60, 90, and 120 minutes after the injection. 5 hours after the feeding, the mice were injected with 500 mg/kg of tyloxapol. Blood samples were taken at 20, 40, 60, and 80 minutes after the injection to measure the amount of triglyceride in the plasma levels.

After 4 hours after the last feeding, 12 weeks into the experiment, blood and tissue samples were collected. Lipids from the adipose and liver tissue, and plasma were taken out using a 2:1 volume to volume ratio of chloroform to methanol solution, and then separated by thin layer chromatography. The lipid fractions were measured using capillary gas chromatography. The results showed there was an increase in adipose fatty acids but no effect in the liver fatty acids. However, one notable observation

the scientist notice was an increase in palmitoleate levels in the Tg mice relative to the WT rats (control) in both the adipose and liver tissues. Palmitoleate is a monounsaturated fatty acid which is healthier than saturated fatty acid.

The researchers concluded that PFKFB3/iPFK2 promotes adipocyte glycolysis and glycolysis-derived lipogenesis after taking a look at how there was an increase in fatty acids in the adipose tissue. There was increasing levels of palmitoleate from both the adipose and liver tissues. Palmitoleate has been proven to be a beneficial fatty acid, as it has evidently been shown to increase insulin sensitivity by suppressing inflammation. (19) Thus overexpression of PRKFB3/iPFK2 in adipocytes help with lowering inflammatory responses in both adipose and liver tissues.

As promising as glycolysis inhibition seems, it could exhibit negative responses. In a particular experiment, it was found that glycolysis inhibition caused moderate mitochondrial dysfunction without having neuronal death. This could lead to low levels of aconitase activity, causing damage to the nervous system, muscle malfunctions, and exercise intolerance. (20) Injecting iodoacetate (IOA) into the test subject is a known method to inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme which enhances neural vulnerability to excitotoxity. Excitotoxicity is a process by which the overreaction of receptors for the excitatory neurotransmitter damages and even kills neurons. (21) This particular experiment was similar to one previously done by Rodriguez et al., only smaller dosages of IOA were administered to the rats than the dosages given in Rodriguez's experiment. Rodriguez's experiment resulted in neuronal death of the rats, whereas in this particular experiment, the researchers' goal was to record mitochondrial dysfunction without causing neuronal death to the rats. The scientists decided to only administer IOA for 3 days compared to Rodriguez's 5 day approach before collecting data on the rats. There has been no prior research on the correlation between glycolysis inhibition and mitochondrial function without causing neuronal death.

Male Wistar rats were injected daily with 25 mg/kg of IOA for 3 days. 24 hours after their last injection, they were killed to isolate their brain mitochondria, done in the same manner as Mirandola et al. (22) Their brains were removed within a minute and put into an ice-cold 10 mL isolation buffer. The cortex, striatum, and cerebellum were dissected and then cut into smaller fragments by surgical scissors, then washed in the isolation buffer. The tissues were homogenized and centrifuged for 3 minutes. The supernatant was recentrifuged afterwards for 8 minutes. Then the pellet that was formed was put back into the isolation buffer. It was once again centrifuged for 10 minutes. The products were the supernatant, a dark layered pellet, and a light layered pellet. The light layer pellet as well as the supernatant were discarded while the dark pellet was put back into the isolation buffer. It was centrifuged another time for 10 minutes at 12000g. The supernatant was discarded and the resulting pellet was washed and put back into the isolation buffer.

The amount of GAPDH was measured by using the spectrophotometric determination of NADH at 340 nm. GAPDH catalyzes glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate. Within the reaction, NAD+ is converted into NADH, thus correlates the higher NADH levels to higher GAPDH levels.

The scientists concluded the decrease in GAPDH activity caused a decrease in oxygen uptake in the brain. Different factors were recorded to see how the decrease in oxygen affected the brain, including reactive oxygen species productions, glutathione, lipoperoxidation, and aconitase activity. A decrease in GAPDH leads to lower levels of aconitase activity. Low levels of aconitase activity can cause damage to the nervous system, muscle malfunctions, and exercise intolerance. The overall experiment provided evidence that IOA can cause moderate mitochondrial dysfunction without having neuronal

death. This is evidence glycolysis inhibition may not be an ideal treatment because of its negative effects to the brain.

Another experiment done by researchers found glycolysis inhibition can be caused by poly(ADP-ribose) polymerase-1 (PARP-1) activation which inhibits hexokinase. (23) PARP-1 is known to cause disorders associated with necrotic cell death, premature death of the cell by a cell's own enzymes. (24) "Parthanatos" is a specialized name for the necrotic cell death caused by PARP-1 activation. The mechanism for parthanatos is a PARP-1 gets activated, causing the release of apoptosis inducing factor (AIF) from the mitochondria which goes into the nucleus and causes nuclear condensation, DNA fragmentation, and cell death. Parthanatos requires the consumption of NAD+. In the past, it was believed cell death was caused by energy collapse of NAD+ and ATP declination. However, there have been many recent studies evidently proving cell death is independent from cellular energy but dependent on PAR signaling.

Real time analysis of glycolytic flux, a method where glucose (basal glycolysis), oligomycin (maximal glycolysis), and 2-deoxyglucose (reserve glycolysis) are each added to the medium, was used to analyze the PARP-1 activation levels on glycolysis. One of the sugars previously stated was added to the medium and analyzed every 15 minutes. N-methyl-N-nitroso-N'-nitroguandidine (MNNG), an activator of PARP, was to be injected into mouse cortical neurons, along with DMSO or a combination of MNNG and 3,4-dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ), a PARP inhibitor. The percentage of extracellular acidification rate (ECAR) was measured between the different mice to detect and quantitate the glycolytic flux in the cancer cells. According to the data, MNNG with DPQ had similar results to DMSO of hardly any change in the percentage of ECAR, while MNNG alone had the significant decrease in the percentage of ECAR in all three stages of the glycolytic flux. To prove whether glycolysis defects was because of the PAR formation, the PAR-degrading enzyme (PARG) was transduced into the MNNG treated mice neurons and the lactate production in the mice were measured. The mice treated with MNNG alone had a 58% reduction in lactate levels while the mice with MNNG and PARG, MNNG and DPQ, and DMSO all had hardly any reduction in lactate levels, suggesting PARP-1 activation does cause defects in glycolysis.

Oxygen consumption rate (OCR) was used to determine if PARP activation causes mitochondrial dysfunction by measuring the OCR per mole every minute. The MNNG treated mice showed a decrease in OCR after 15 minutes while MNNG with DPQ treated mice had little decrease in OCR. The amount of protons were monitored but there was no sudden decrease in protons. This suggested change in the mitochondrial OCR may have been due to the unavailability of substrates for mitochondrial oxidative phosphorylation rather than mitochondrial dysfunction. To prove this assumption, pyruvate was added to the medium and the OCR was once again measured. The OCR levels were preserved significantly thus proving PAR causes a reduction in mitochondrial function due to the inadequate amount of substrate available.

NAD+ levels were monitored after the neurons were treated with MNNG, again by flux glycolysis. Reduction in NAD+ occurred after the reductions from glycolytic and mitochondrial function occurred. To confirm reduction in NAD+ levels didn't have an effect on glycolysis or mitochondrial function, FK866, a noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, was used to decrease NAD+ levels independent of PARP activation. PAR immunoblot analysis was used to confirm FK866 had no effect on activating PARP. FK866 is a glycosyltransferase and a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, an enzyme that promotes B cell maturation and inhibits neutrophil cell death. Results showed FK866 failed to reduce ATP levels but

caused a decrease in NAD+ levels, compared to MNNG which led to PAR activation and reduced ATP levels. From the flux glycolysis methods, there was no effect to adding NAD+ when compared to DMSO.

Hexokinase (HK) ezymatic assay was used to determine whether PARP inhibits HK activity. HK is an enzyme essential to initiating glycolysis because it is the first step in glycolysis where it phosphorylates a glucose molecule to allow it to precede further in the mechanism. MNNG treated neurons were immunoprecipitated with a PAR antibody, where a solution containing an specifically binding antibody will cause the protein of interest to precipitate. (25) PAR coimmunoprecipitated HK from the MNNG neurons, thus concluding the decrease HK activity is dependent on PARP.

The researchers concluded PARP activation leads to the inhibition of glycolysis, the decrease in mitochondrial function due to inadequate amounts of substrates, and HK inhibition. NAD+ proved not to have anything to do with these conclusions. PARP can lead to disorder associating with necrotic cell death, including Parkinson disease, Alzheimer's disease, autoimmune encephalomyelitis, and multiple sclerosis. This shows possible methods of inhibition of glycolysis can lead to potential risks if used as a treatment.

There are a lot of research conducted on finding the cure for cancer. Glycolysis inhibition is a potential method that may soon be seen in the future. It provided desirable results when: glycolysis inhibition substrate was combined with chemotherapy to result in an antitumor immune response and increased lifespan; the highly abundant mRNA-stabilizing protein HuR gets translocated to the cytoplasm where it is targeted by β-TrCp1 for degradation; and glycolysis inhibition causes increased fat disposition in adipose tissue as well as protection against insulin resistance and inflammatory responses. The undesirable results of glycolysis inhibition include: a decrease in GAPDH activity caused a decrease in oxygen uptake in the different parts of the brain, leading to a damaged nervous system, muscle malfunctions, and exercise intolerance; and PARP activation leads to a decrease in mitochondrial function and can lead to disorder associating with necrotic cell death. It seems glycolysis inhibition can potentially be a treatment for cancer but with many neurological risks. However, further research must be provided to see if the pros can outweigh the cons.

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