Can the Antidiabetic Drug Phenformin Slow Tumor Growth in Non-Small Cell Lung Cancer?

Summary and Aims

Lung cancer is one of the deadliest cancers worldwide and has a survival rate of less than five years after diagnosis. Treating lung cancer is also incredibly difficult due to its aggressiveness and most cancers often acquire resistance to treatment. One of the hallmarks of cancer, mitochondrial dysfunction, is a possible direction for identifying drug targets for cancer. The metabolic shift frequently seen in many cancers, known as the Warburg Effect, describes how cancer cells prefer aerobic glycolysis instead of mitochondrial respiration for energy production and consumption. Non-small cell lung cancer (NSCLC) is known to exhibit the Warburg Effect, however, in recent years there seems to be conflicting evidence as to whether the Warburg Effect is the rule when it comes to mitochondrial dysfunction in cancers. Instead, it appears that cancer cells may use aerobic glycolysis or mitochondrial respiration or both to maintain energy demands. For example, one study found that suppressing mitochondrial respiration by inhibiting a regulator of an important component for mitochondrial respiration, Complex I, reduced NSCLC oncogenesis which contradicts the Warburg Effect (1). They also found that genes important for Complex I function were overexpressed which indicates that cancer cells do not always shift towards aerobic glycolysis, they may instead enhance oxidative phosphorylation (OXPHOS). Another study showed that pancreatic cancer often presents with high OXPHOS tumors and that the discontinued antidiabetic drug, Phenformin, had an antitumor effect. Phenformin is an inhibitor of Complex I which suppresses mitochondrial respiration and leads to downstream suppression of mechanistic target of rapamycin (mTOR), an important regulator of growth factors, cell proliferation, and survival. Therefore, the overall aim of our study is to analyze the metabolic changes in the NSCLC cell line NCIH358 and determine if Phenformin will have a similar antitumor effect in NSCLC.

In our study we aim to:

1. **Confirm high Complex I expression in NCIH358 cell culture.**
   We will culture NCIH358 cells and measure the expression of Complex I with cell-based Sandwich ELISA. Then we will compare the expression of Complex I with a human bronchial epithelial cell line (16HBE14o).
2. **Measure mitochondrial function in NCIH358.**
   We will measure mitochondrial function with mitochondrial respiration and glycolysis stress tests. These measure the rate of oxygen consumption and the rate of extracellular acidification.
3. **Treat athymic nude mice with NCIH358 xenografts with phenformin to observe the effects on the tumors.**
   Once we have determined the energy shift preferences of NCIH358 cells, we will inject the cells into athymic nude mice to create tumors. Then we will treat them with phenformin and the chemotherapeutic drug gemcitabine to observe the effects on the tumors.

Background

Lung Cancer Overview: Lung cancer is one of the deadliest cancer worldwide, accounting for approximately 18% of cancer related deaths in 2020 with a survival rate of less than 5 years following diagnosis (2). One of the biggest reasons for low survival rate and poor prognosis is due to late diagnosis because clinical symptoms often have a late presentation (3). Furthermore, lung cancer is difficult to treat due to its aggressive nature and acquired resistance to chemotherapy (4). Therefore,

finding new ways to target and treat cancer is desirable. The most common cancer treatments that are in clinical use typically target DNA, growth factor pathways, and regulators of the cell cycle (5). However, there are hallmarks of cancer other than uncontrolled growth and proliferation such as mitochondrial dysfunction. Targeting components of mitochondria for the treatment of cancer is an attractive possibility due to the frequently seen metabolic shift in cancers cells known as the Warburg Effect (6).

The Warburg Effect: Since the 1920s, metabolic differences between healthy cells and cancer cells have been observed (6). What is known today as the Warburg Effect describes the shift in energy demands and metabolic changes in cancer cells. Cancer cells metabolize significantly more glucose than normal mammalian cells, but rather than utilizing mitochondrial oxidative phosphorylation (OXPHOS), cancer cells often shift to aerobic glycolysis. Aerobic glycolysis is an alternative pathway for generating adenosine triphosphate (ATP) that also generates lactate in the presence of oxygen. Aerobic glycolysis is considered to be counter-intuitive for cancer cell energy demands due to the significantly lower ATP production compared to OXPHOS. However, the rate of aerobic glycolysis is much faster compared to OXPHOS which may be beneficial for rapid cell proliferation (7). Despite there being significant evidence to support the Warburg Effect, there is conflicting evidence indicating that the Warburg Effect is not necessarily the rule. The more recent view of altered cancer cell metabolism is a combination of aerobic glycolysis and OXPHOS states (8). Some cancers, such as non-small cell lung cancer (NSCLC), have also shown an upregulation in Complex I in the mitochondrial OXPHOS pathway which ultimately contradicts the Warburg Effect (9). Other studies have shown that targeting Complex I in pancreatic cancer and melanoma have reduced tumor growth (1, 10). Furthermore, suppressing mitochondrial respiration through inhibition of Complex I leads to downstream inhibition of mechanistic target of rapamycin (mTOR) (11). mTOR is an important metabolic sensor and regulator of growth factors, cell proliferation, and survival. In many cancers, mTOR is often dysregulated which can lead to uncontrolled oncogenesis.

Phenformin as a Possible Anticancer Drug: In the last few years Phenformin, a discontinued antidiabetic drug, has been studied for its potential as an anticancer drug (12). Phenformin is known to inhibit Complex I in the OXPHOS pathway in mitochondria which has the downstream effect of inhibiting mTOR. Because Complex I is upregulated in pancreatic cancer, the study *Masoud et. al.* proposed to inhibit Complex I with phenformin in combination with the chemotherapy drug, gemcitabine (1). They found that by inhibiting Complex I, phenformin enhanced the antitumor effects of gemcitabine, indicating that phenformin could be used to treat high OXPHOS tumors. Another study suggested that high OXPHOS tumors could exist in non-small cell lung cancer (NSCLC) as well which is contradictory to other previous evidence that lung cancer typically shifts towards aerobic glycolysis (13). The study *Rao et. al.* found that knocking out apoptosis inducible factor (AIF), a posttranscriptional regulator of Complex I, reduced NSCLC oncogenesis (9). AIF is also a trigger for apoptosis when released by mitochondria. Their hypothesis was that AIF knockout would enhance glycolysis in lung cancer cells and increase proliferation which would be in congruence with the Warburg Effect. Instead, they found that the AIF knockout impaired the growth of lung cancer cells. Therefore, they tested the expression levels of Complex I in NSCLC tissue from human patients and found that genes encoding for Complex I were overexpressed. However, the OXPHOS pathway was not entirely inhibited by knocking out AIF, therefore, future study on inhibiting Complex I in NSCLC is required. Therefore, we propose to inhibit Complex I with phenformin in a lung cancer cell line model and hypothesize a similar antitumor effect that was shown in *Masoud et. al.*

Methods

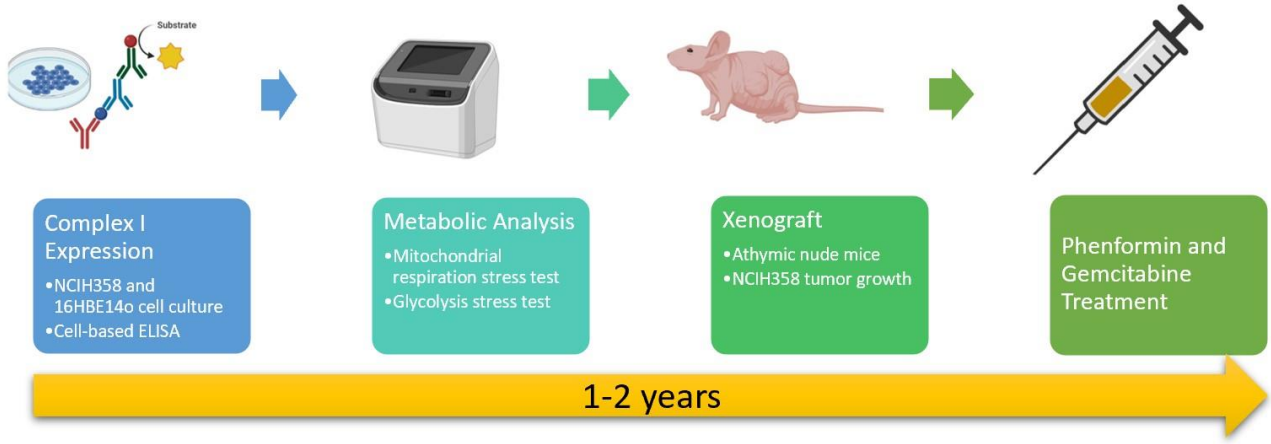

Figure 1: Experimental timeline. The figure outlines the methods we will use and the approximate timeline of our project. This figure was created using Biorender.com and Powerpoint.

Aim 1: Confirm high Complex I expression in NCIH358 cell culture.

Cell Culturing and Complex I Expression: In our study we will culture cells from an established human NSCLC cell line called NCIH385 purchased from the American Type Culture Collection (ATCC). We will use the NCIH385 cell line for measuring Complex I expression levels, metabolic analysis and to make xenografts (Fig. 1). We will also culture cells from an established human bronchial epithelial cell line 16HBE14o purchased from Sigma Aldrich to compare Complex I expression levels with NCIH385. The cell culturing period should take approximately two months. After making our cultures we will perform cell-based sandwich ELISA to measure Complex I expression in each cell type (Fig. 1). We expect NCIH385 to have higher levels of Complex I compared to 16HBE14o. If the expression levels of Complex I are not higher in NCIH358 than 16HBE14o, but are still at a similar level, we will continue to proceed with metabolic analysis outlined in the next section. We will proceed because a normal level of Complex I expression in NCIH358 cells may still produce effective OXPHOS compared to glycolysis. However, if Complex I expression is found to be lower in NCIH358 cells, we may need to evaluate the expression of other complexes in the OXPHOS pathway or determine another way to inhibit mTOR since the effects of phenformin may be less effective with lower Complex I expression.

Aim 2: Measure mitochondrial function in NCIH358 cell culture.

Metabolic Analysis: Mitochondrial Respiration Stress Test and Glycolysis Stress Test: Once we have confirmed that Complex I has higher expression in NCIH358 than 16HBE14o, we will perform a metabolic analysis in the NCIH358 cell line (Fig. 1). The first metabolic experiment we will perform is a mitochondrial respiration stress test (Agilent). We will use the mitochondrial respiration stress test to determine mitochondrial function by measuring the rate of oxygen consumption under different parameters. Such parameters include basal respiration, ATP-linked respiration, and spare respiratory capacity. The results of these parameters will indicate the efficiency of OXPHOS in the mitochondria of NCIH358 cells. Because *Rao et. al.* found that impairing the OXPHOS pathway in NSCLC oncogenesis and *Masoud et. al.* found OXPHOS and mitochondrial function to be efficient in high OXPHOS pancreatic tumors, we expect NCIH358 cells to have functional OXPHOS.

We will also perform a glycolysis stress test to measure the capability of the glycolytic pathway in NCIH358 cells (Agilent). The glycolysis stress test blocks mitochondrial respiration to push cells towards glycolysis to measure the rate of extracellular acidification. This test will indicate the

capacity of NCIH358 cells to shift to the glycolytic pathway for energy production and survival. Because *Rao et. al.* found that impairing the function of the OXPHOS pathway reduced NSCLC cell proliferation, we expect NCIH358 to have a low capacity for glycolysis. However, both the mitochondrial respiration and glycolysis stress tests may produce alternative outcomes due to the unpredictable nature of cancer cells. While our study has built its hypothesis around the possibility of high OXPHOS tumors in NSCLC, there is still a chance that our study will produce opposite results and instead favour the Warburg Effect. If this is the case, we will treat our NCIH358 cell cultures with phenformin and then do a cell-based sandwich ELISA of the quantity of activated AMP-activated kinase (AMPK) before xenografting them to athymic nude mice. Activation of AMPK is important for the downstream inhibition of mTOR and Phenformin is known to activate AMPK through inhibition of Complex I (11). However, it is possible that there are unknown mechanisms of Phenformin that lead to the activation of AMPK.

Aim 3: Treat athymic nude mice with NCIH358 xenografts with phenformin to observe the effects on the tumors.

Phenformin and Gemcitabine Treatment: Following the metabolic analyses, we will prepare NCIH358 cultures to be xenografted to athymic nude mice (n=44) (Fig. 1). We will obtain ethical approval from the Swedish Ethical Review Authority for the use of our animal model. We have chosen to use a xenograft instead of a lung cancer mouse model to better reflect human NSCLC conditions. We will measure the growth of the xenograft on a weekly basis and the health of the mice on a daily basis. We expect that the tumor should be visible after approximately 6 weeks (14). Once tumors start to be visible, we will monitor their size before we begin treatment with phenformin and gemcitabine. The treatment will occur over a period of two months (Fig. 1). We will divide the mice into 4 groups (n=11 per group). Group 1 will receive no treatment, group 2 will receive phenformin only, group 3 will receive gemcitabine only, and group 4 will receive phenformin and gemcitabine. Dividing the mice into groups will allow us to compare each treatment strategy to determine which one had the highest antitumor effect. We predict that group 4 will have the highest antitumor effect because *Masoud et. al.* found phenformin to increase the antitumor effect of gemcitabine. However, if we do not achieve successful results, we may have to consider another Complex I inhibitor for future study such as BAY 87-2243 which has shown antitumor effect in melanoma (10). Overall, we expect the entire project to be approximately 1-2 years (Fig. 1).

Significance

Since the discovery of the Warburg Effect, it has been observed in many cancers. However, it is important to be critical of this observation because there are studies that have shown cancer cells, like NSCLC and pancreatic cancer, exhibiting high OXPHOS instead of shifting to aerobic glycolysis. Therefore, it is important that we find treatments that could target mitochondrial respiration and/or aerobic glycolysis in cancer to reduce the energy supply. Furthermore, in the future if metabolic cancer treatments become more common for clinical use, it is important to know the direction of metabolic shift the cancer has taken to determine which type of drug to use. We also believe that in general, studying drugs that already exist for their potential in treating other diseases, such as Phenformin's potential in cancer treatment, is great for reducing costs that go into identifying new compounds and targets.

References

1. Masoud R, Reyes-Castellanos G, Lac S, Garcia J, Dou S, Shintu L, et al. Targeting Mitochondrial Complex I Overcomes Chemoresistance in High OXPHOS Pancreatic Cancer. Cell Reports Medicine. 2020;8.
2. Sung H, Ferlay J, Siegel R, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021;17:209-49.
3. Huang H, Yang Y, Zhu Y, Chen H, Yang T, Zhang L, et al. Blood Protein Biomarkers in Lung Cancer. Cancer Letters. 2022;551.
4. Zugazagoitia J, Guedes C, Ponce S, Ferrer I, Molina-Pinelo S, Paz-Ares L. Current Challenges in Cancer Treatment. Clinical Therapeutics. 2016;38(7):1551-66.
5. DeRidder L, Rubinson DA, Langer R, Giovanni T. The Past, Present, and Future of Chemotherapy with a Focus on Individualization of Drug Dosing. Journal of Controlled Release. 2022;352:840-60.
6. Liberti MV, Locasale JW. The Warburg Effect: How does it Benefit Cancer Cells? Trends in Biochemical Sciences. 2016;41(3):211-8.
7. Lunt SY, Vander Heiden MG. Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation. Annual Reviews of Cell and Developmental Biology. 2011;27:441-64.
8. Jia D, Park JH, Jung KH, Levine H, Kaipparettu BA. Elucidating the Metabolic Plasticity of Cancer: Mitochondrial Reprogramming and Hybrid Metabolic States. Cells. 2018;7(3).
9. Rao S, Mondragon L, Pranjic B, Hanada T, Stoll G, Kocher T, et al. AIF-Regulated Oxidative Phosphorylation Supports Lung Cancer Development. Cell Research. 2018;29:579-91.
10. Schöckel L, Glasauer A, Basit F, Bitschar K, Truong H, Erdmann G, et al. Targeting Mitochondrial Complex I Using BAY 87-2243 Reduces Melanoma Tumor Growth. Cancer and Metabolism. 2015;3(11).
11. Jaidee R, Kongpetch S, Senggunprai L, Prawan A, Kukongviriyapan U, Kukongviriyapan V. Phenformin Inhibits Proliferation, Invasion, and Angiogenesis of Cholangiocarcinoma Cells via AMPK-mTOR and HIF-1A Pathways. Naunyn-Schmiedeberg's Archives of Pharmacology. 2020;393:1681-90.
12. Rubino MEG, Carillo E, Alcala GR, Dominguez-Martin A, Marchal JA, Boulaiz H. Phenformin as an Anticancer Agent: Challenges and Prospects. International Journal of Molecular Sciences. 2019;20(13).
13. Li X-B, Gu J-D, Zhou Q-H. Review of Aerobic Glycolysis and its Key Enzymes - New Targets for Lung Cancer Therapy. Thoracic Cancer. 2015;6(1):17-24.
14. Szadvari I, Krizanova O, Babula P. Athymic Nude Mice as an Experimental Model for Cancer Treatment. Physiological Research. 2016;65.

Reference Defences:

1. Masoud et. al.: This reference was one of the main articles that inspired the proposal. In this study they found that Phenformin had an antitumor effect on a pancreatic cancer model. This was very helpful for finding good methods to use and sparking the idea to try this in lung cancer.
2. Rao et. al.: This reference was the other main article that inspired this proposal. Their findings and suggestions for future research helped to build a foundation for why Phenformin could work in lung cancer as well. It also triggered me to explore more about the Warburg Effect and its conflicting evidence, as well as mitochondrial dysfunction.

3. Jaidee et. al.: This reference was really helpful for understanding the mechanisms of Phenformin and the signalling pathways involved like mTOR and AMPK. The study was helpful for thinking of an alternative method for my second aim.
4. Schöckel et. al.: This reference was interesting to read and was used to show that inhibiting Complex I in other cancers has shown antitumor effects and not just in lung cancer. However, they did not use Phenformin. They used BAY 87-2243 which could be something that could be used for future study.
5. Liberti et. al.: This reference was about the Warburg Effect in cancer cells. It was really helpful for getting a baseline knowledge of the Warburg Effect. It also triggered me to explore further as to how cancer cells meet energy demands when aerobic glycolysis is less efficient.
6. Lunt et. al.: This reference was helpful for gaining a more detailed understanding of the metabolic shifts in cancer. It was also helpful for gaining an in depth understanding of how mitochondrial dysfunction contributes to cancer development.