Master's Thesis 2023/2024

Targeting metabolic dependencies in cancer cells *in vitro*

Serine and glycine deprivation in the serine biosynthesis pathway in lung cancer cells via treatment with NCT-503

JULIA SAHLÉN

Department of Biology and Biological Engineering
Chalmers University of Technology
Gothenburg, Sweden 2023/2024

Inst. Of Clinical Sciences, Dep. Surgery
Sahlgrenska Center for Cancer Research

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JULIA SAHLÉN

Department of Biology and Biological engineering

Chalmers University of Technology

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Supervisors:

Volkan Sayin, PhD, Associate Professor, Principal Investigator

Sahlgrenska Center for Cancer Research

Institute of Clinical Sciences

Department of Surgery

University of Gothenburg

Group Volkan Sayin

volkan.sayin@wlab.gu.se

Samantha Alvarez, Senior Researcher

Sahlgrenska Center for Cancer Research

Institute of Clinical Sciences

Department of Surgery

University of Gothenburg

Group Volkan Sayin

samantha.alvarez@gu.se

Examiner:

Mikael Molin, Associate Professor/Senior Researcher

Department of Biology and Biological Engineering

Division of Systems and Synthetic Biology

Chalmers University of Technology

mikael.molin@chalmers.se

Abstract

Lung adenocarcinoma (LUAD) is a prevalent and aggressive form of lung cancer. Targeting metabolic pathways, particularly the serine and glycine biosynthesis pathways, represents a promising therapeutic strategy. Phosphoglycerate dehydrogenase (PHGDH), a key enzyme in serine biosynthesis, is frequently overexpressed in LUAD. NCT-503, a PHGDH inhibitor, has emerged as a potential therapeutic agent against LUAD.

This study aimed to evaluate the efficacy of NCT-503 in inhibiting cancer cell viability and altering metabolic pathways in LUAD cell lines. Cell Titer Glow (CTG) assays were employed to assess cell viability across varying drug concentrations, while uptake assays measured the absorption and secretion of serine and glycine in treated and untreated cells. Experiments were conducted on two LUAD cell lines, A1 and Y2, representing elder and younger populations, respectively.

CTG assays revealed a dose-dependent response to NCT-503, with significant cell viability reduction at higher drug concentrations (200 µM). Interestingly, the A1 cell line showed higher sensitivity to lower concentrations (<100 µM) compared to Y2. Uptake assays presented complex and varied responses; Y2 cells exhibited decreased serine secretion and glycine absorption post-treatment, while A1 cells showed increased secretion of these metabolites. Notably, the patterns of nutrient uptake were inconsistent across different trials, indicating potential variability in cellular responses to NCT-503.

The study confirmed that PHGDH inhibition by NCT-503 effectively hinders cancer cell growth, particularly in the A1 cell line. However, the discrepancies between CTG and uptake assays highlight the complexity of cellular metabolic responses. Variability in results suggests the need for further investigation to ensure reliability and reproducibility. Factors such as cell age, genetics, and experimental conditions may influence the drug's efficacy. The study highlights the necessity for ongoing research, including repeated uptake assays, intracellular metabolite analysis, and *in vivo* trials, to validate NCT-503's therapeutic potential and ensure minimal side effects.

NCT-503 shows promise as a therapeutic agent against LUAD, particularly for elder patients, by effectively inhibiting cancer cell viability through PHGDH inhibition. However, the complexity of cellular responses and potential laboratory errors necessitate further research to confirm these findings and optimize treatment protocols.

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List of acronyms

SCLC- Small cell lung cancer

NSCLC- Non small cell lung cancer

PHGDH- Phosphoglycerate dehydrogenase

Y2- Lung primary tumor culture from a young mouse

A1- Lung primary tumor culture from an aged mouse

TMS- Trimethylsilyl

TBDMS- Tert-butyldimethylsilyl

AIS- Adenocarcinoma in situ

MIA- Minimally invasive adenocarcinoma

GC-MS- Gas chromatography- mass spectrometry

THF- Tetrahydrofolate

GSH- Glutathione

1C unit- One carbon unit

ROS- Reactive oxygen species

CTG- CellTiter-Glo® Luminescent Cell Viability Assay

PBS- Fetal Bovine Serum

LUAD- Lung adenocarcinoma

Table of contents

1. Introduction-- 6
   1.1. Aims-- 6
   1.2. Limitations-- 7
   1.3. Ethical and social aspects-- 7
2. Theory--- 9
   2.1. Lung cancer--- 9
   2.2. Metabolomics and Isotope tracing--- 10
   2.3. Amino acids-- 11
   2.4. Amino acid starvation--- 11
   2.5. Glycine cleavage system-- 12
   2.6. One- carbon metabolism and serine biosynthesis---------------------------- 12
   2.7. NCT-503-- 14
   2.8. K-RAS mutation and Trp53 mutation -- 14
3. Method and materials -- 16
   3.1. CellTiter-Glo® Luminescent Cell Viability Assay--------------------------- 16
   3.2. Extracellular uptake assay using targeted metabolomics-------------------- 21
4. Results -- 25
   4.1. Results from CellTiter-Glo® Luminescent Cell Viability Assay ------------- 25
   4.2. Results from Extracellular uptake assay using targeted metabolomics ------- 32
5. Discussion--- 37
   5.1. CTG assays suggests that NCT-503 effectively inhibits cancer cell viability- 37
   5.2. Serine and Glycine Uptake Patterns Reflect Varied Cellular Responses in Lung Cancer-- 38
   5.3. Discrepancies Between CTG and Uptake Assays in Evaluating NCT-503 Efficacy--- 39
   5.4. Lack of significant results-- 40
   5.5. Sources of error--- 41
   5.6. Further investigation-- 42
6. Conclusion--- 45
7. References--- 46

1. Introduction

Today, lung cancer holds the unenviable position of being responsible for the highest number of cancer-related deaths worldwide. In the year 2020, lung cancer resulted in as many as 1.8 million deaths [1]. It presently accounts for the highest death rates across both genders. Lung cancer, as of now, affects lung cells by initiating the growth of abnormal cells in an uncontrolled manner [1].

There exist two primary categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Currently, NSCLC prevails as the most common, constituting approximately 80% of all lung cancer cases [2].

This project focuses on NSCLC, more specifically on the adenocarcinoma subtype. In this thesis, we employ primary cultures collected from primary lung tumors of young and old KP mice, labeled as Y2 and A1 respectively, where Y2 is a cell line from young mice and A1 from aged mice. KP mice are mice with the KrasLSL-G12D/+ mutation, which is an activated form of the Kras gene, and the Trp53fl/fl mutation, where the Trp53 (p53) gene can be conditionally deleted.

In many studies, cre-mediated activation of the KP mutant alleles is typically initiated when mice reach 2 months of age, equivalent to a human age of 15 years. However, this age does not adequately represent the age range of lung cancer patients in the EU, which primarily falls within the 45-70+ age group. In 2020, there were 315,165 new cases for this age group, while patients under 45 accounted for only 3,162 new cases [3]. As experiments involving old mice are scarce, this thesis encompasses the response of treatments on aged primary lung tumor cultures from these aged mice.

The existing treatments for lung adenocarcinoma encompass surgeries, radiation, immunotherapy, chemotherapy, and targeted therapy. In all three approaches, drugs are currently administered to bolster the immune system, eliminate cancer cells throughout the body, or specifically target cancerous cells [4]. To uncover and develop new drugs capable of pinpointing cancerous cells, in-depth exploration of cancer metabolism is imperative.

The overarching objective of this master's thesis is to study the response of primary mouse cells, encompassing both young and old, to the inhibition of one-carbon metabolism and the response to serine depletion.

1.1. Aims

This project presently focuses on conducting a comprehensive examination of the response exhibited by primary mouse cells when subjected to inhibition of one-carbon metabolism. This investigation employs various methodologies, encompassing serine and glycine deprivation, along with the inhibition of serine biosynthesis via treatment with NCT-503, a PHGDH (phosphoglycerate dehydrogenase) inhibitor [5]. NCT-503 was developed as a potent PHGDH inhibitor with the purpose of studying PHGDH's biology and its role in serine synthesis and one-carbon metabolism [6]. The primary objective is to determine whether such inhibition currently impacts the viability, proliferation, and overall metabolic behavior of primary mouse cells. We will be using two cell lines, one derived from young mice (Y2) and

the other from aged mice (A1). This is aimed at assessing potential differences in the response between the two cell lines. Given that most experiments are presently conducted on young mice, despite the fact that the most affected group in terms of lung cancer is the older generation, there is a need for experiments involving old mice to make the results more comparable to real-life scenarios.

Concurrently, we intend to investigate the metabolic adaptations that occur in response to serine depletion using metabolomics techniques. We will conduct an extracellular serine/glycine uptake assay to gain insights into the rate at which cells secrete or consume these amino acids from the surrounding culture medium, providing a dynamic perspective on nutrient uptake.

These efforts will enhance our comprehension of cellular metabolism and refine the precision of metabolomics analyses, shedding light on metabolic adaptations and serine utilization in response to altered availability.

1.2. Limitations

This study is currently utilizing mouse primary cultures rather than human cells, and this approach may pose limitations in directly translating the findings to human physiology and diseases. Discrepancies between the responses of mouse and human cells could potentially affect the applicability of the results to human health.

Another constraint pertains to the project's focus on a single metabolic pathway. Given the intricate interconnection of cellular metabolism, alterations in one pathway may impact others. Consequently, interactions between multiple pathways might be inadvertently overlooked, although this thesis ensures they are accounted for.

Furthermore, it's important to note that only *in vitro* experiments are being conducted, and this setup may not entirely replicate the complexity of an *in vivo* environment. Consequently, this difference can lead to variations in metabolic behaviors and discrepancies in cellular responses.

1.3. Social and ethical aspects

The project's findings potentially contribute to our understanding of cancer metabolism and therapy. Insights from basic research may eventually guide clinical applications that impact patient care and healthcare practices, thus contributing to broader societal well-being.

The project's outcomes may form the basis for public discussions concerning scientific advancements, cancer research, and ethical considerations related to animal experimentation. Open dialogue and effective communication can promote better public comprehension of the research's significance and ethical complexities.

This thesis exclusively utilizes *in vitro* work, with experiments conducted on cells directly derived from mice by the Sahlgrenska Center for Cancer Research. Although *in vivo* work is not part of this project, mice have been employed to create the cells. If promising results emerge during this thesis, there may be potential for future *in vivo* serine experiments. Consequently, ethical dilemmas associated with animal testing could be considered in the

future. The drug development process adheres to the 3 Rs principle (replace, reduce, refine) in animal research. This involves initiating experiments *in vitro* and in silico, reducing the reliance on animal testing, and minimizing the number of animals required. As a result, the positive impact the findings could have on our well-being aligns with the potential negative effects on animals.

2. Theory

2.1. Lung cancer

Lung cancer is broadly categorized into two main types: Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC), each characterized by distinct features.

SCLC is identified by its small, round cell appearance and tends to exhibit rapid growth and early metastasis, commonly originating in the central bronchi of the lungs. Strongly associated with cigarette smoking, SCLC primarily presents as small cell carcinoma.

On the other hand, NSCLC comprises various subtypes, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. NSCLC cells are larger and may have diverse shapes, with tumors growing more slowly and often initiating in different areas of the lungs, including the outer regions. While smoking is a risk factor for NSCLC, a significant number of cases occur in non-smokers [4].

Lung adenocarcinoma

The most common subtype of cancer is adenocarcinoma and is formed in glandular, secretory cells. This type of cancer can be found within many organs and tissues, for example in the breasts, prostate, stomach, pancreas and lungs. Over 30% of all lung cancers are adenocarcinoma. Around half of adenocarcinoma are the NSCLC subtype. The tumors are often found in the outer parts of the lungs, and it’s a slow growing type that usually evolves from mucosal glands and for lung cancer the tumors most often start to form in the alveoli [4]. Even though lung cancers are highly connected to smoking, both in current and previous smokers, adenocarcinoma is the subtype of lung cancer that is mostly found in non-smoking patients. The 5-year survival depends on if the cancer is only located in the lungs or if it has spread to nearby or distant areas in the body and can vary from 7-63% chance of survival [4].

Stages of lung adenocarcinoma

There are four types or stages of lung adenocarcinoma: adenocarcinoma in situ (AIS), minimally invasive adenocarcinoma (MIA), invasive adenocarcinoma, and variants of adenocarcinoma. Since both AIS and MIA are less invasive, they have better survival chance if detected early [7]. The current treatments for lung adenocarcinoma are surgeries, radiation, immunotherapy, chemotherapy, and targeted therapy. For all three therapies, drugs are used in ways to either help the immune system, destroy cancer cells through the whole body, or specifically target the cancer cells [4]. To discover and develop new drugs that can target the cancerous cells, the metabolism of cancer must be carefully studied. The study of metabolism via metabolomics, which involves profiling and quantifying a broad range of small molecules in biological samples, offers valuable insights into the dynamic nature of cellular metabolism. Metabolomics can be optimized to investigate specific pathways of interest, allowing researchers to delve into the intricate details of how particular metabolic processes function and adapt under varying conditions. Deprivation of amino acids and glucose or inhibition of metabolic pathways can have profound effects on cell proliferation and tumor growth, particularly in rapid dividing cancer cells.

Aerobic glycolysis
The ability of cancer cells to convert glucose to lactate even in the presence of plenty of oxygen is a characteristic of aerobic glycolysis. This is not the case for normal cells, which produce ATP largely through aerobic oxidative phosphorylation. Aerobic glycolysis helps rapidly proliferating cancer cells in a number of ways, despite being less efficient in terms of ATP yield (producing only 2 ATP molecules per glucose molecule compared to up to 36 ATP molecules in oxidative phosphorylation). For example, it produces ATP quickly enough to meet the high energy demands of cancerous cells, and its intermediates, such as glucose-6-phosphate and pyruvate, are diverted into biosynthetic pathways to generate nucleotides, amino acids, and lipids required for cell growth and division.

Since glycolysis produces less ATP than oxidative phosphorylation, cancer cells frequently bo ost their rates of glucose absorption and glycolysis.
Glycolytic enzymes and glucose transporters like GLUT1 are upregulated in order to do this. Different cancer types and settings may require different cancer cells to rely on mitochondrial oxidative phosphorylation.

The Warburg effect explains how genetic alterations, oncogene activation (like MYC), loss of tumor suppressors (like p53), and stability of hypoxia-inducible factor (HIF-1) cause cancer cells to prefer aerobic glycolysis even in the presence of enough oxygen [8].

2.2. Metabolomics and Isotope tracing

Metabolomics is a rapidly growing field that aims to comprehensively measure and analyze the small molecule metabolites in biological systems. The metabolome is the complete set of metabolites in a cell, tissue, or organism, and it reflects the underlying biochemical processes and physiological state. Metabolomics offers valuable insights into a range of applications, including disease mechanisms, drug discovery, and personalized medicine. Gas chromatography-mass spectrometry (GC-MS) is a widely used technique in metabolomics due to its high sensitivity, selectivity, and reproducibility. GC-MS separates and identifies metabolites based on their chemical properties and mass-to-charge ratio. The sample is first derivatized to increase volatility and stability, and then injected into the GC column, where the metabolites are separated based on their boiling points. The separated metabolites are then ionized and detected by the mass spectrometer, which generates a mass spectrum that can be used to identify the metabolites. However, GC-MS has limitations, such as the inability to distinguish between isomers, which are molecules with the same chemical formula but different structures. To overcome this limitation, researchers often use stable isotope tracing, which involves feeding cells or organisms with a labeled precursor molecule, such as glucose or amino acids, that is incorporated into metabolic pathways. By tracking the fate of the labeled atoms, researchers can infer the activity of specific metabolic pathways and identify novel metabolites. Isotope tracing can also reveal metabolic fluxes, or the rate at which metabolites are produced and consumed, which is important for understanding metabolic regulation [9].

2.3. Amino acids

Amino acids are the building blocks of proteins and play a crucial role in protein synthesis. There are 20 different types of amino acids, each with a unique chemical structure and function. During protein synthesis, amino acids are linked together through peptide bonds to form polypeptide chains. These chains then fold into specific three-dimensional structures to form functional proteins. The process of protein synthesis is regulated by a complex network of signaling pathways and enzymes [10].
Protein synthesis is one of the most fundamental cellular processes and is usually under strict regulation. It is the process by which cells build proteins, which are essential for many cellular functions, including structural support, enzymatic catalysis, and cell signaling. Protein synthesis is generally under sophisticated and dynamic regulation to meet the ever-changing demands of a cell. Changes in protein synthesis activity are associated with various cellular responses to environmental changes such as nutrient/oxygen deprivation or to alterations such as pathological mutations in cancer cells. Protein synthesis is usually under strict regulation, and changes in protein synthesis activity are always associated with the fate of cancer cells. Mutations in translation regulation in cancer cells can lead to elevated global protein production, promoting tumor progression and cell survival. In the case of lung cancer, for example, dysregulated protein synthesis patterns have been observed, which may contribute to the development and progression of the disease [11].

All cells need amino acids, but cancer cells have an increased demand for amino acids and can become auxotrophic, which means they can't survive without a certain amino acid. While cancer cells often use upregulated amino acid transporters, targeting these can be challenging. Inhibiting enzymes in amino acid synthesis pathways, such as PHGDH for serine biosynthesis, holds more promise. Amino acid depletion can also be achieved by limiting the supply in the cell culture media. This strategy, especially when cancer cells rely on external sources of specific amino acids, can lead to growth arrest and apoptosis [12].

2.4. Amino acid starvation

When cells experience amino acid starvation, they activate several adaptive mechanisms to maintain homeostasis. These mechanisms involve autophagy, general amino acid control (GAAC), eIF2 kinases, and pathways related to the uptake and synthesis of amino acids [13].

Autophagy

Autophagy is a process where cells degrade and recycle their own components to survive under stress conditions like nutrient deprivation. It involves the formation of double-membrane vesicles, known as autophagosomes, which engulf cytoplasmic materials and fuse with lysosomes for degradation and recycling of the contents. mTOR (mechanistic Target of Rapamycin) is a central regulator of cell growth and metabolism, responding to nutrient availability. Under normal conditions, mTOR inhibits autophagy by phosphorylating ULK1 (Unc-51 Like Autophagy Activating Kinase 1) and other autophagy-related proteins. When cells experience amino acid starvation, including serine deprivation, mTOR activity is reduced, leading to the activation of autophagy. This process helps to recycle cellular

components and provide essential nutrients, including amino acids, to maintain cellular metabolism and survival [14].

General amino acid control

The GAAC pathway, primarily regulated by the kinase GCN2, responds to amino acid deficiency. When serine biosynthesis is impaired due to PHGDH inhibition, uncharged tRNAs accumulate, activating GCN2. Activated GCN2 phosphorylates eIF2α, leading to a reduction in global protein synthesis while selectively enhancing the translation of stress response genes, including those involved in amino acid biosynthesis and transport. This adaptive response helps cells cope with the stress caused by the shortage of amino acids such as serine [14].

eIF2 kinases

eIF2 kinases, including GCN2, PERK, PKR, and HRI, play significant roles in cellular stress responses. Inhibition of PHGDH and the resulting serine deprivation activate these kinases, particularly GCN2, which phosphorylates eIF2α. This phosphorylation reduces general protein synthesis to conserve resources and selectively enhances the translation of specific mRNAs necessary for stress adaptation and survival. This mechanism is critical for managing the cellular stress induced by amino acid deprivation [14].

2.5. Glycine cleavage system

The glycine cleavage system is a vital component of the one-carbon metabolic network, a complex cycle dependent on folate reactions. This system, driven by serine/glycine metabolism, provides essential one-carbon units for nucleotide and cellular component synthesis. Comprising four enzymes, it catalyzes the oxidative cleavage of glycine, generating CO2, NH3, and a methylene group linked to tetrahydrofolate (THF). This 1C unit is valuable for producing nucleotides, amino acids, and other critical cellular components [15].

2.6. One- carbon metabolism and serine biosynthesis

One-carbon metabolism is a complex network, mediated by the cofactor folate, that supports a lot of physiological processes such as biosynthesis of nucleotides and specific amino acids, amino acid homeostasis, epigenetic maintenance, and redox defense [16].

Serine biosynthesis is an important key factor in one-carbon metabolism. Furthermore, serine metabolism is intricately connected with the one-carbon metabolism pathway, contributing to the biosynthesis of essential molecules, such as glutathione, DNA/histone methylation, and purines. These interconnected pathways are crucial for supporting the anabolic needs of cancer cells.

Serine is an amino acid that is important for the conversion to glycine, which is required for many biosynthetic pathways because it provides 1C-units [13]. Enzymes linking serine to formate exists in both mitochondria and cytosol, where the biosynthesis of serine takes place

in the cytosol. This is due to the high cytosolic NADPH/NADP+ ratio which favors the flux from formate towards serine [16].

Serine biosynthesis is a critical metabolic pathway with profound implications in cancer biology. This pathway is responsible for the de novo production of serine from glucose through a series of enzymatic reactions. These reactions involve key steps performed by specific enzymes:

- PHGDH (Phosphoglycerate Dehydrogenase) plays a vital role by catalyzing the conversion of 3-phosphoglycerate (3-PG) into phosphohydroxy pyruvate (P-Pyr), utilizing NAD as a cofactor.
- PSAT1 (Phosphoserine Aminotransferase) transaminates P-Pyr to phosphoserine (P-Ser) with the assistance of glutamate as the nitrogen donor.
- PSPH (Phosphoserine Phosphatase) is responsible for the hydrolysis of P-Ser, ultimately yielding serine.

In cancer cells, serine biosynthesis is often upregulated, and one of the key enzymes involved, PHGDH, is frequently amplified. Inhibiting PHGDH can lead to reduced proliferation in cancer cells that depend on serine synthesis, making it a promising target for anti-cancer therapies [15].

Understanding the role of serine and glycine metabolism in cancer biology is of paramount importance. It not only influences the synthesis of crucial cellular components but also modulates cellular antioxidant capacity, which has a direct impact on tumor homeostasis. Research in this field offers potential avenues for drug development, dietary interventions, and the identification of biomarkers to enable targeted anti-metabolic therapies in cancer treatment.

Genetic and functional data strongly suggest that abnormal activation of the serine biosynthetic pathway is a "critical factor in cancer development" [17]. Serine and glycine serve as building blocks for essential cellular components, such as proteins, nucleic acids, and lipids. Controlling serine biosynthesis, particularly through the inhibition of PHGDH, which catalyzes the conversion of 3-phosphoglycerate to 3-phosphohydroxypyruvate, has a significant impact on cellular antioxidant capacity, indicating its role in maintaining tumor balance. Inhibiting PHGDH not only results in the depletion of glutathione (GSH) since serine is a precursor for GSH synthesis but also induces oxidative stress and disrupts cellular metabolism, potentially compromising antioxidant capacity [18].

These insights provide promising opportunities for the development of drugs, dietary strategies, and biomarkers aimed at targeting metabolic therapies in the context of cancer treatment [15].

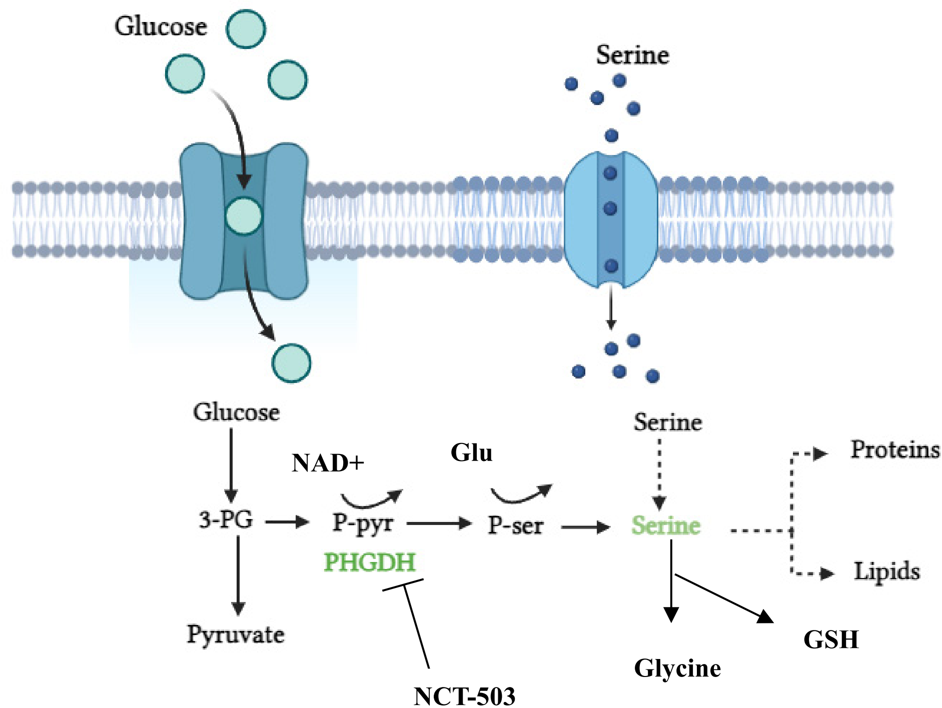

Fig. 1. Serine biosynthesis pathways [17]. (Picture created in biorender.com)

2.7. NCT-503

NCT-503 is a selective inhibitor of PHGDH, which is an enzyme involved in the serine biosynthesis pathway. Cancer cells have an increased demand for serine which support their rapid growth and proliferation. By inhibiting PHGDH, the serine synthesis is inhibited from 3-phosoglycerate, and the serine production is reduced. The inhibition aims to target cancer cells while sparing normal cells [19].

Cancer cells often rely on aerobic glycolysis (the Warburg effect) to meet their energy and biosynthetic needs, leading to altered ATP levels. By inhibiting PHGDH, NCT-503 disrupts serine production, impacting various metabolic processes, including nucleotide synthesis and redox balance. This disruption can affect ATP production, as cancer cells balance between glycolysis and mitochondrial oxidative phosphorylation to maintain their energy levels. Targeting these metabolic adaptations with NCT-503 offers a promising therapeutic strategy, as it exploits the unique dependencies of cancer cells on altered metabolic pathways [8].

2.8. K-RAS mutation and Trp53 mutation

K-RAS Mutation

A protein involved in cell signaling pathways that control cell growth and proliferation is encoded by the Kras gene. Kras mutations, like the G12D mutation, cause the Kras protein to become constitutively activated, which accelerates unchecked cell proliferation and encourages the growth of tumors. Kras mutations are common in a number of malignancies, including lung cancer, where they are detected in a sizable number of instances [20].

Trp53 Mutation

The Trp53 gene, sometimes referred to as p53, is a tumor suppressor gene that controls the course of the cell cycle, DNA repair, and programmed cell death in response to cellular stress or damage to DNA. When Trp53 is mutated, its tumor suppressor function is lost, which enables cells to avoid regular growth control systems and pick up more mutations that aid in the development of tumors. Mutations in Trp53 are frequently observed in many cancer types, including lung cancer [20].

3. Methods and materials

In this chapter, the experiments and their execution were elucidated. The cell lines and the drug, as well as the various methods employed throughout the thesis, were described.

Y2; A1

The GEMM, the Kras2LSL/+Trp53fl/fl (KP) mouse model with Cre-mediated activation, was utilized by the Sayin lab at Sahlgrenska Center for Cancer Research. A Cre-encoding virus was administered into the lungs of young (3 months) and aged (>18 m, corresponding to an average human age of 60 years old) KP mice by the Sayin lab. This action resulted in the activation of KRASG12D expression and simultaneous inactivation of p53 in the recipient mice, leading to the formation of lung adenocarcinoma (LUAD). Lung tumors from these young or aged mice were isolated for primary cultures. In this thesis, the cell line Y2 originated from young mice, while A1 was derived from aged mice.

NCT-503

For the thesis, the stock solution was provided by the Sayin lab. NCT-503, in powder format, was purchased from MedChemExpress®. The stock solution, with a concentration of 100 mM, utilized in the experiments, was prepared at the Sayin lab in Dimethylsulfoxid (DMSO) solvent and was stored at -20ºC, following the established protocol [21]. The drug was thawed before its utilization.

3.1. CellTiter-Glo® Luminescent Cell Viability Assay

The CellTiter-Glo® Luminescent Cell Viability Assay was employed as a highly efficient method for the assessment of cell viability through the quantification of ATP levels, thus indicating the presence of metabolically active cells. It was optimized for 96-well plate formats, rendering it suitable for high-throughput screening, cell proliferation studies, and cytotoxicity assays. A long-lasting luminescent signal, directly correlated with cell count, was generated by the assay. This extended signal half-life simplified the processing steps and minimized pipetting errors when compared to alternative ATP measurement methods [20].

The CTG was conducted three times to evaluate the PHGDH inhibitor NCT-503.

The first CTG experiment involved testing NCT-503 concentrations of 6.25, 12.5, 25, 50, and 100 (µM) and was carried out from 230918 to 230922. These concentrations were chosen based on the results of previous successful experiments with NCT-503.

The second CTG experiment assessed NCT-503 concentrations of 6.25, 12.5, 25, 50, 100, and 200 (µM) and was conducted from 231002 to 231006. The selection of concentrations was informed by the findings from the first CTG experiment.

The third CTG experiment replicated the second one and took place from 231009 to 231013.

The fourth CTG experiment also replicated the second and third one and took place from 231106 to 231110.

3.1.1. Preparing media

In order to maximize the growth and proliferation of cells, the preparation of the media was deemed important, involving the addition of crucial nutrients. A total of 500 ml of RPMI Medium 1640 (1X), without L-Glutamine (provided by gibco), was utilized. This medium was prepared by combining 50 ml of Fetal Bovine Serum, labeled as Value FBS (gibco), along with 5.5 ml of L-glutamine and 0.55 ml of Gentamicin (10mg/ml, gibco). The media, once prepared, was employed for all the experiments conducted throughout this thesis. The prepared RPMI media was referred to as "media" in the whole chapter 3.1. CellTiter-Glo® Luminescent Cell Viability Assay.

3.1.2. Cell culturing

The cells were consistently maintained in media stored within an incubator located in a cell culture lab. Routine checks were conducted to assess confluency, and when the cell population reached 80-100% confluence, they were subjected to splitting. Additionally, samples of 500 µl from the media were collected on a weekly basis to check for mycoplasma contamination.

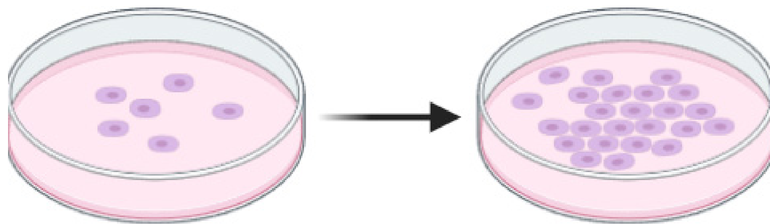

Fig. 2. Increasing cell viability. (Picture made in biorender.com)

For cell splitting, the following steps were followed:

Initially, the cells were rinsed with 3 ml of PBS (FisherSci). After aspirating the PBS, the cells were treated with 1.5 ml of trypsin (ThermoFisher) and placed in an incubator for a period of five minutes. Subsequently, 3.5 ml of media were introduced to the cells, and the entire contents were transferred to a 14 ml tube. A volume of 250 µl was extracted from the tube and placed into a new plate. Finally,10 ml of media was added to the plate.

3.1.3. Plating cells

The process commenced with the removal of the media from the cells. Subsequently, the cells were washed with 3 ml of PBS and treated with 1.5 ml of trypsin. The cells were then placed in an incubator for several minutes to facilitate the detachment of cells from the bottom of the plate. Cells, now treated with trypsin, were transferred to a 14ml tube along with 3.5 ml of media.

To assess cell viability and concentration, 10 µl was extracted from the 14 ml tube and pipetted into a 1.5 ml tube, where it was combined with 10 µl of trypan blue stain. Following this, 10 µl from this mixture was deposited onto a counting slide and subsequently lived cells were tallied using the counting machine. Further calculations were conducted to ensure an adequate quantity of cells, specifically 5000 cells in each well of the multi-well plate, based on earlier studies done at Sayin lab.

On 230918: The cells were counted, yielding a count of $3{,}97 \times 10^6$ cells/ml for Y2 and $1{,}67 \times 10^6$ cells/ml for A1. Thirty-six wells were plated for each cell line, although calculations were conducted for 40 wells, resulting in a total of 200,000 cells required for each cell line. From the Y2 cell line 14 ml tube, 50 µl were extracted, while 112 µl were obtained from the A1 cell line 14 ml tube. Subsequently, these cells were diluted with media to reach a total volume of 4000 µl separately. 100 µl of this mixture was added to each inner well, with five of the outer wells containing 200 µl of media only, and the remaining outer wells containing an equivalent amount of PBS.

On 231002: The procedure mirrored the first time. The amount taken from A1 tube was 56 µl, and from Y2 tube, it was 67 µl based on the cell counts.

On 231009: The procedure was identical to the first and second times. The amount taken from A1 tube was 69 µl, and from Y2 tube, it was 39 µl based on the cell counts.

On 231106: The procedure was identical to the first, second and third times. The amount taken from A1 tube was 42 µl, and from Y2 tube, it was 42 µl based on the cell counts.

3.1.4. Adding NCT-503

The day following the plating of the cells, the inhibitor was introduced into the wells. Additionally, the solvent DMSO was added to certain wells for each cell line to assess its toxicity.

On 230919:

The final volume in each well was set at 200 µl, and a total of 10 wells were prepared for each concentration, with four allocated for each cell line and two additional wells. Five tubes were labeled, and 2000 µl of media were placed in the first tube, while half of that amount was placed in tubes two through five. For the 100 µM concentration, 4 µl of the stock solution were added to the 2000 µl of media in the first tube. Subsequently, serial dilutions were carried out by transferring 1000 µl from the first tube to the next tube, containing 1000 µl of media. This process was repeated until the last tube contained 2000 µl.

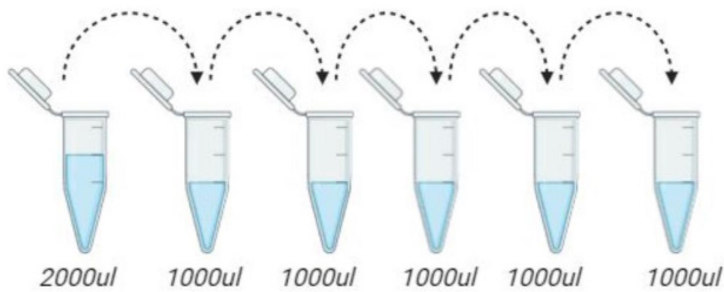

Fig. 3. Serial dilutions. (Picture made in biorender.com)

Next, 100 µl from the first tube were dispensed into each well in the upper rows of the plate requiring treatment, with the same procedure applied to all the tubes in the rows below. The wells without treatment, which contained cells and media, received only media. To assess toxicity, 2 µl of DMSO were diluted in 1000 µl of media and then added to 12 wells, with 100 µl in each well.

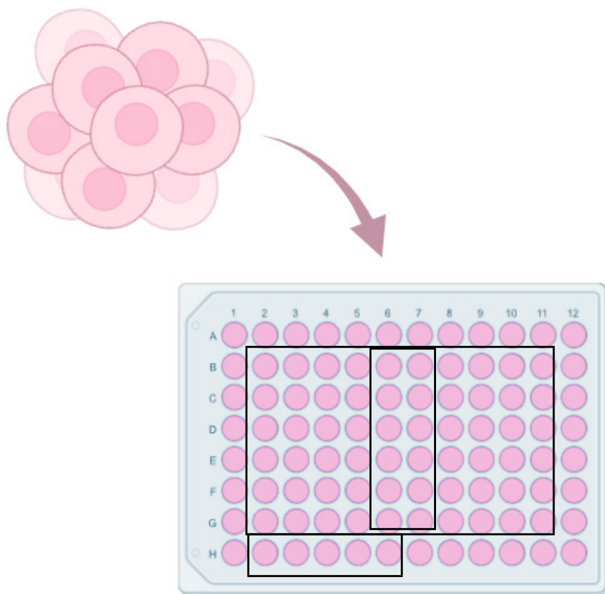

Fig. 4. The plate design for the first CTG experiment. At the bottom of the plate, five wells were solely filled with media. In the outer part of the plate, all other wells were filled with PBS. The middle columns of the plate were occupied by cells from both the Y2 and A1 cell lines, combined with media and DMSO. On the left side of the plate, cells from the Y2 line were placed alongside media and treated with NCT-503. The concentrations ranged from the highest at the top to the lowest concentration, which, in this case, was no drug at all, at the bottom of the plate. Conversely, on the right side of the plate, cells from the A1 line were combined with media and subjected to drug treatment. The concentrations were arranged from the highest at the top to the lowest at the bottom of the plate.

On 231003:

The final volume in each well was established at 200 µl, and a total of 10 wells were prepared for each concentration, with four allocated for each cell line and two additional wells. Six tubes were labeled, and 2000 µl of media were placed in the first tube, with half of that amount placed in tubes two through six. For the 100 µM concentration, 8 µl of the stock solution were added to the 2000 µl of media in the first tube. Subsequently, serial dilutions were executed by transferring 1000 µl from the first tube to the next tube, containing 1000 µl of media. This procedure was repeated until the last tube contained 2000 µl.

Next, 100 µl from the first tube were dispensed into each well in the upper rows of the plate requiring treatment, with the same procedure applied to all the tubes in the rows below. The wells without treatment, containing cells and media, were supplied with only media. To assess toxicity, 4 µl of DMSO were diluted in 1000 µl of media and then added to 6 wells, with 100 µl in each well.

On 231010: The exact same steps were repeated as in the second experiment since it was an exact replication of it, employing the same concentrations and layout.

On 231107: The exact same steps were repeated as in the second and third experiment since it was an exact replication of it, employing the same concentrations and layout.

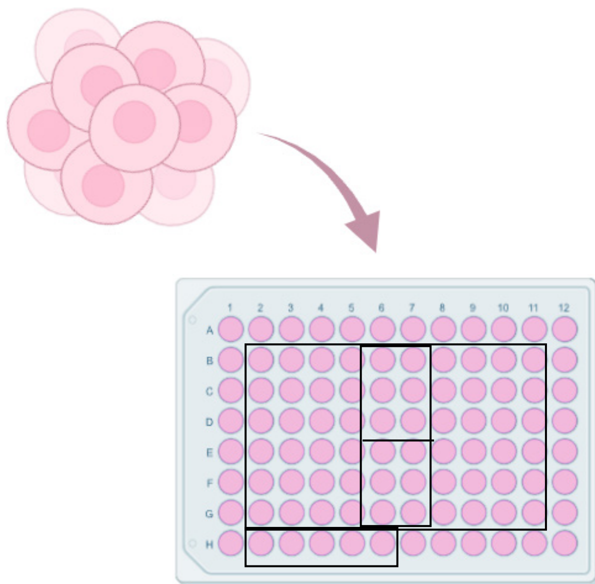

Fig. 5. Design of plate for the third CTG experiment. The second and fourth one looked the same. At the bottom of the plate, five wells contained only media. The outer part of the plate was filled with PBS in all other wells. The middle columns of the plate contained cells from both the Y2 and A1 cell lines, combined with media. In the last six wells of these middle columns, DMSO was also added. On the left side of the plate, cells from the Y2 line were placed alongside media and treated with NCT-503. The concentrations ranged from the highest at the top to the lowest concentration at the bottom of the plate. On the right side of the plate, cells from the A1 line were combined with media and subjected to drug treatment. The concentrations were arranged from the highest at the top to the lowest at the bottom of the plate.

3.1.5. CTG readings

Three days after the drug was added, the plate was read. The cytology of the cells was also examined under a microscope.

The plate was inverted to eliminate all the media, and it was substituted with 50 µl of fresh media in each well. Afterward, 10 µl of the thawed CTG reagent was introduced into every well. The reagent was consistently wrapped in foil to prevent exposure to light, as was the plate after the reagent's addition. The plate was placed on a Belly Dancer for 10 minutes. ATP levels were measured using a spectrometer.

3.1.6. Analysis of data

The data obtained from the CTG readings comprised the ATP levels of each well. In Excel, the average of the blank readings was subtracted. Subsequently, the raw data was normalized in Excel, either to the solvent DMSO or, in certain instances, to the lowest concentration. Using GraphPad Prism 10, the graphs were generated by inputting the normalized data.

Additionally, a t-test was conducted for all CTG experiments utilizing GraphPad Prism 10, to see if there was any significant difference between young and aged cell lines for each concentration tested.

3.2. Extracellular uptake assay using targeted metabolomics

An uptake assay using targeted metabolomics involves measuring the uptake of specific metabolites by cells or tissues and analyzing the changes in their concentration. In this thesis we are only looking at cells, not tissues. This assay provides valuable information about how cells utilize external metabolites and the impact of various factors on their uptake and metabolism. Uptake assays using targeted metabolomics are valuable tools for studying cellular metabolism, understanding disease mechanisms, and developing targeted therapeutic interventions.

For the first experiment one plate of the cells, Y2 and A1, got treated with 25µM NCT-503, one plate with cells was the control plate without the drug and one plate with only media without cells, 3 wells. Three replicates of each condition.

For the second and third experiments, the layout was the same, except that the cells got treated with 50µM NCT-503 instead of 25µM NCT-503.

3.2.1. Dialyze FBS

The FBS is dialyzed to remove all molecules below 10,000 Da, to not disturb the GCMS and avoid unwanted effects. A tube is soaked in 500 ml of autoclaved milliQ water for 30 minutes. 1 l of PBS is poured into a beaker. Weight-clips are

placed at the bottom of the tube and 10 ml of FBS is added to it and a normal clip is placed on the top of the tube to avoid leakage. The beaker is placed on a shaker during night with a stir bar in it. The second day the PBS is replaced since it has reached equilibrium and more molecules need to be taken away, the beaker is placed on the shaker again one more night. The third day the FBS is collected and filtered with 0.2 µm filter and put into -20ºC.

3.2.2. Sample preparations

The sample preparations consist of first plating the cells in a 6-well plate, add the drug and then collect the media for uptake assay, also add methanol and norvaline to the samples.

240115: Two 6-well plates were used the first day and 250 000 cells were plated into each well. The cells were plated with 1 ml of normal prepared RPMI media in each well. Plates were put in an incubator.

240116: Media with dialyzed FBS was prepared. For the control plate the media was changed to media with dialyzed FBS in it, 1 ml in each well. For the other plate the media was taken away. The NCT-503 was added to the media with dialyzed FBS, making a concentration of 25µM, then 1 ml was added to each well. A third plate was made with just the media with dialyzed FBS, without cells, 1 ml in each well. Plates were put in an incubator.

240117: All media was collected into separate marked tubes and cells were counted. New tubes were marked and 4 µl from each sample with the collected media were transferred to each tube. 2.2 ml of methanol was mixed with 2.2 µl of norvaline over ice. Then 100 µl from that mixture was transferred into each tube.

The steps were repeated for second and third experiment, making a concentration of 50µM NCT-503 instead, at the dates 240311-240313 and 240320-240322.

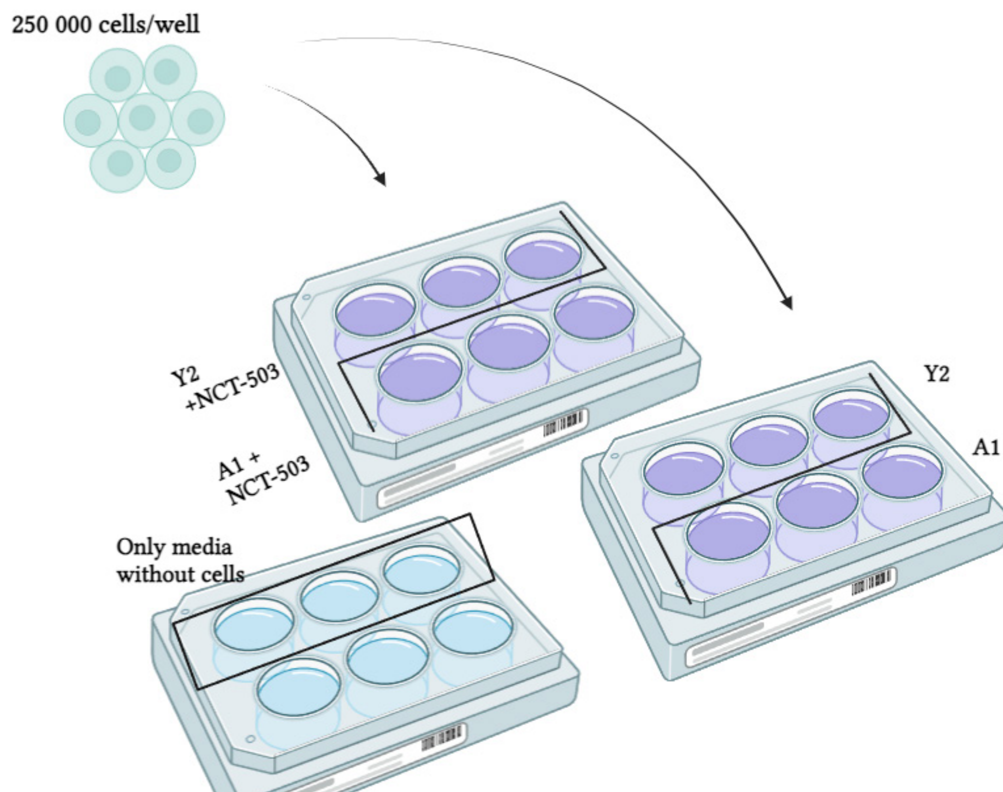

Fig. 6. Design of the six well plates, one that has both cells and drug added to it, one with only cells, three replicate of each cell line, Y2 respective A1 and one plate with only media without the cells in three wells.

3.2.3. Processing samples

The samples were placed on a speedvac for one hour, with the lid open to evaporate the methanol. A solution of 20 mg MOX (methoxamine) reagent and 1 ml pyridine was made. 20 µl was added into each sample and the samples was placed on a heatblock of 37ºC for one hour then centrifuged for 10 seconds. 20 µl of TBDMS (Tert-butyldimethylsilyl) was added into each sample, the samples were placed on a heatblock again for one hour and then centrifuged for 10 seconds. 35 µl from each sample was transferred to GCMS tubes and then placed on the GCMS.

Gas chromatography- Mass spectrometry

Samples were analyzed using an Agilent 7890A gas chromatograph coupled with an Agilent 5997B mass spectrometer, featuring an Agilent Technologies DB-35MS column. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. Samples were injected in split mode (split 1:1) at 270°C, with a one microliter injection volume. The GC oven temperature was initially held at 100°C for one minute, then increased to 300°C at a rate of 3.5°C/min. Subsequently, it was ramped up to 320°C at 20°C/min and held for five minutes. The quadrupole and MS source temperatures were set at 150°C and 230°C, respectively. The MS system operated

at 70 eV under electron impact ionization, scanning an ion range of 10–650 m/z. Mass isotope distributions were determined by combining the relevant ion fragments for each metabolite using MATLAB, taking into account their natural abundance.

3.2.4. Analyzing data

The data was collected from the GCMS 24 hours later. Data was corrected using MATLAB (predone script), then the data was normalized in Excel and graphs made using GraphPad Prism 10. A t-test was done in Excel and stars manually drawn in GraphPad Prism 10.

4. Results

4.1. Results from CellTiter-Glo® Luminescent Cell Viability Assay

The study utilized the CellTiter-Glo® (CTG) Luminescent Cell Viability Assay, a highly efficient method for assessing cell viability by quantifying ATP levels, indicative of metabolically active cells. Optimized for 96-well plate formats, the assay is well-suited for high-throughput screening, cell proliferation studies, and cytotoxicity assays. The assay's long-lasting luminescent signal, directly correlated with cell count, simplifies processing steps and reduces pipetting errors compared to alternative ATP measurement methods. In the context of the study, the CellTiter-Glo® assay was employed three times to assess the PHGDH inhibitor NCT-503.

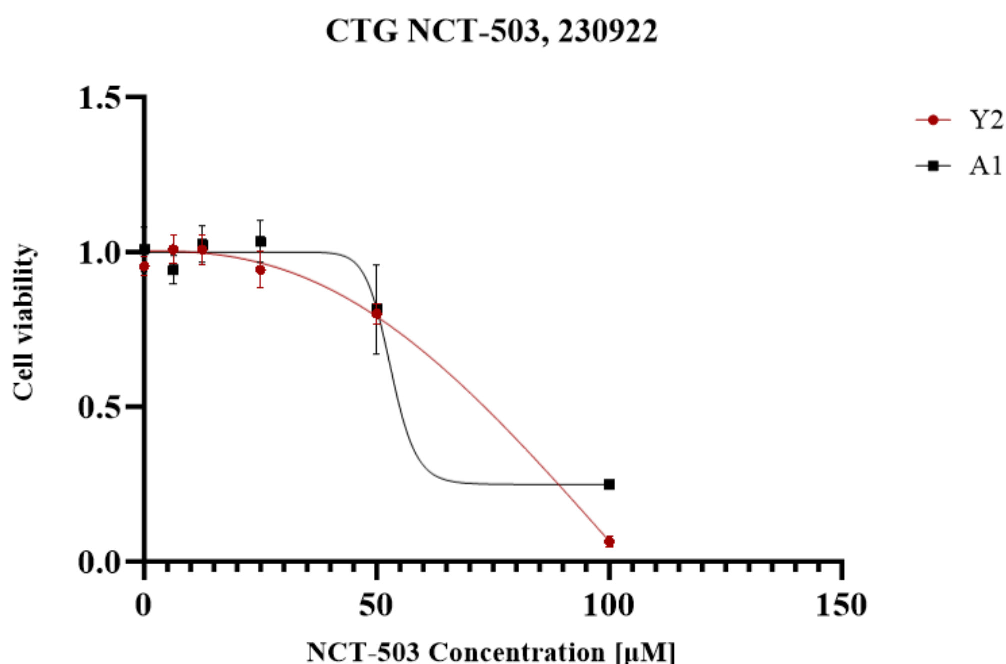

Fig. 7. Treated Y2 and A1 cells with drug NCT-503 for 72 hours and then measured the cell viability via ctg assay. The data was fit to a sigmoidal curve, calculated by the GraphPad Prism software based off the original data points and plotted as shown (Y2 red color and A1 black color lines respectively). The sigmoidal shape is used since it is used for earlier studies for drug response curve at Sayin lab. Error bars are shown as SEM. ATP levels for Y2 and A1 are normalized to the solvent DMSO.

The initial CTG experiment (Fig. 7) yielded valuable insights into the effect of NCT-503 treatment on the Y2 and A1 cell lines. Notably, the 100 µM dose of NCT-503 demonstrated a substantial impact on both cell lines, leading to a significant reduction in viability. This aligns with the expectations of targeting PHGDH, a critical enzyme in the serine synthesis pathway known to be upregulated in cancer cells.

Based on the data collected in fig. 7, we observe a gradual decrease in cell viability for both Y2 and A1 cell lines as the drug concentration falls below 50 µM, with a more pronounced

decline occurring within the range of 50 to 100 µM. At the 100 µM concentration, we note a more substantial presence of non-viable cells in the young mouse cell line compared to the older one.

Particularly for the Y2 cell line, there was one specific concentration where all values, except for the 6.25 µM concentration, exhibited significantly lower values in comparison to the other replicates at the same concentration. This suggests a potential anomaly or an irregular response to the drug treatment in this specific case.

Overall, the data in fig. 7 indicates a concentration-dependent effect on cell viability, with higher drug concentrations leading to a more substantial reduction in viability.

However, it's crucial to address the observed anomaly in the Y2 cell line, where a pipetting error in one column led to lower mean values. This underscores the importance of meticulous laboratory techniques and quality control to ensure accurate and reliable results. To further validate these findings and explore higher drug doses with the potential to eradicate all cells, a follow-up experiment will be conducted with concentrations ranging from 0 to 200 µM.

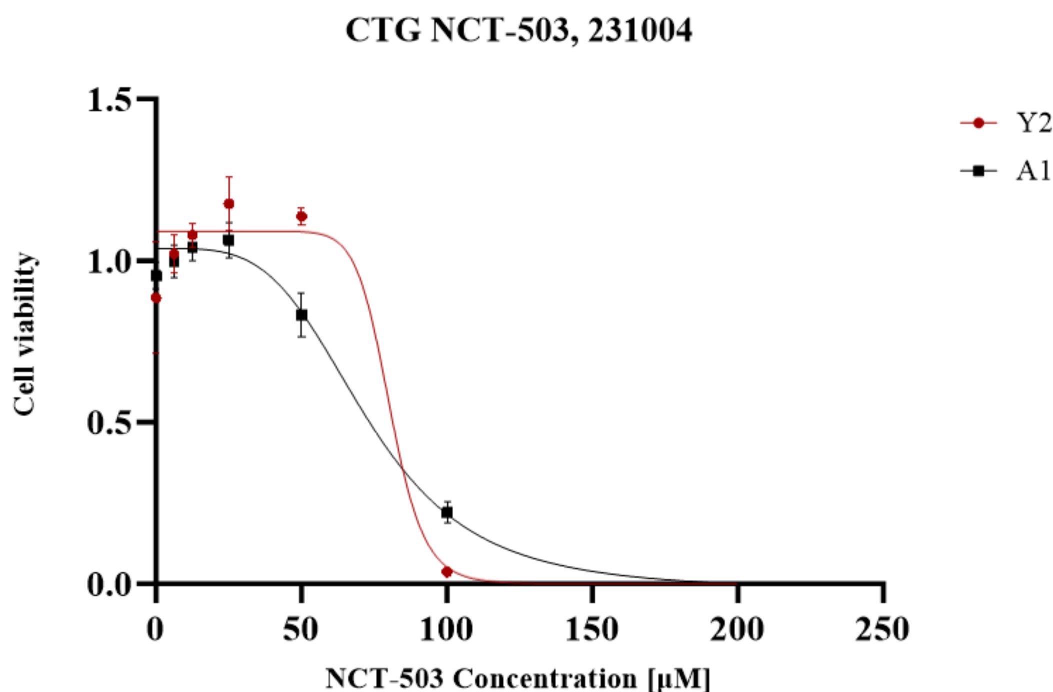

Fig. 8. Treated Y2 and A1 cells with drug NCT-503 for 72 hours and then measured the cell viability via ctg assay. The data was fit to a sigmoidal curve, calculated by the GraphPad Prism software based off the original data points and plotted as shown (Y2 red color and A1 black color lines respectively). The sigmoidal shape is used since it is used for earlier studies for drug response curve at Sayin lab. Error bars are shown as SEM. ATP levels for Y2 and A1 normalized to the solvent DMSO.

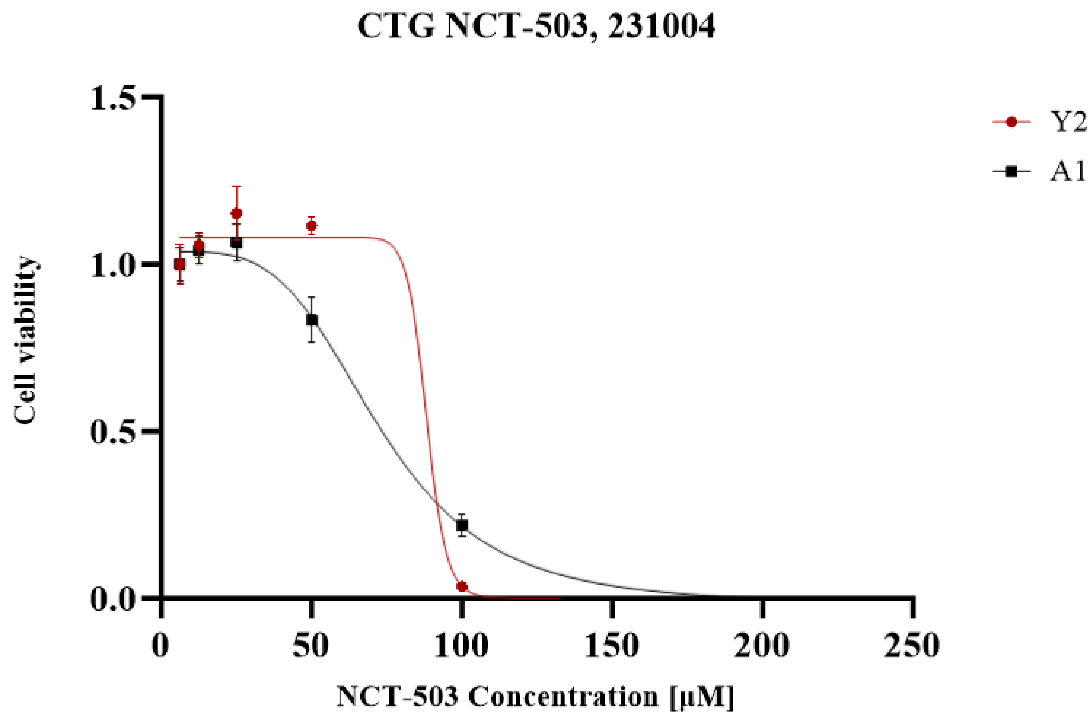

Fig. 9. Treated Y2 and A1 cells with drug NCT-503 for 72 hours and then measured the cell viability via ctg assay. The data was fit to a sigmoidal curve, calculated by the GraphPad Prism software based off the original data points and plotted as shown (Y2 red color and A1 black color lines respectively). The sigmoidal shape is used since it is used for earlier studies for drug response curve at Sayin lab.. Error bars are shown as SEM. ATP levels for Y2 and A1 normalized to the lowest concentration.

Based on the provided ATP values, we can discern a particular pattern in cell viability (fig. 7-9). Initially, there is an increase in viability at lower drug concentrations. However, this trend takes a turn beyond the 25 µM concentration, where we observe a noticeable decrease in cell viability.

The second CTG experiment, as seen in fig. 8 and fig. 9, while reinforcing the potential of NCT-503 as an anti-cancer agent, introduced new questions. The initial increase in viability at lower drug concentrations, followed by a perplexing decline, raises concerns about the reproducibility of the results.

A noteworthy observation is that the aged mice cells exhibit greater sensitivity to the drug compared to the young mice cells, particularly evident at concentrations up to 25 µM. Strikingly, at higher concentrations, this sensitivity trend seems to reverse, with the older cells displaying a less pronounced response to the drug, which may have been due to plating or pipetting error.

This data, showed in both fig. 8 and fig. 9 underscores the intricate interplay between drug concentration and cell viability, highlighting the age-dependent dynamics in the response to the drug treatment.

Possible sources of error during cell plating or serial dilutions need to be meticulously investigated to ensure the reliability of the data. The initial increase in viability at lower drug concentrations, followed by a decline, may be linked to the complexities of one-carbon metabolism. Altering the balance of metabolites, such as serine and glycine, can have a profound impact on cellular functions, including nucleotide synthesis and redox balance [22].

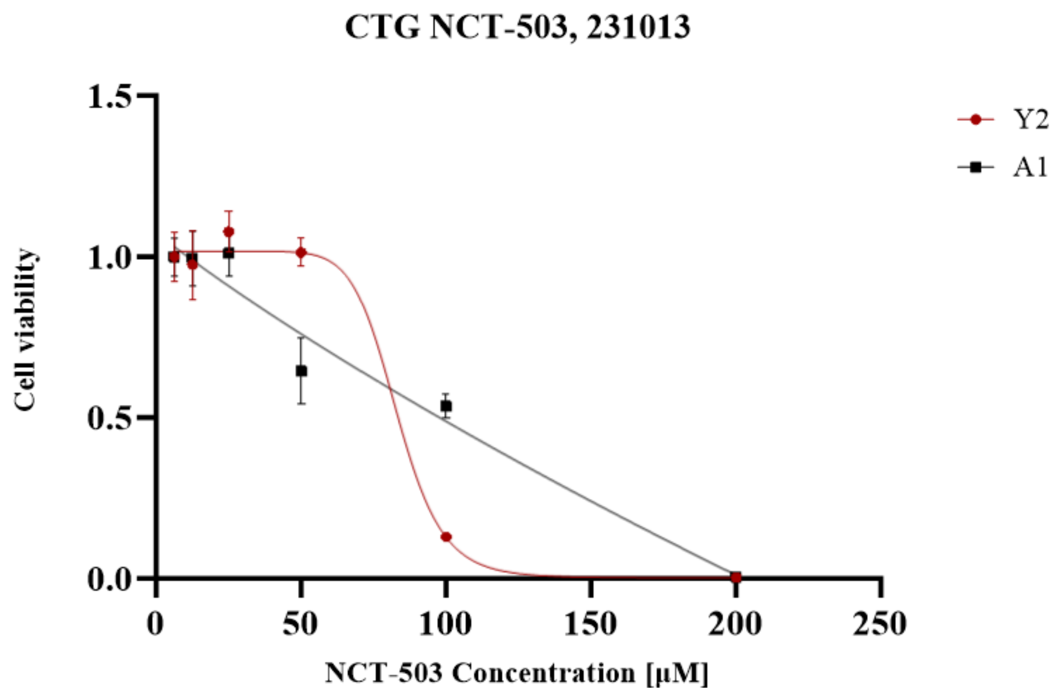

Fig. 10. Treated Y2 and A1 cells with drug NCT-503 for 72 hours and then measured the cell viability via ctg assay. The data was fit to a sigmoidal curve, calculated by the GraphPad Prism software based off the original data points and plotted as shown Y2 red color and A1 black color lines respectively). The sigmoidal shape is used since it is used for earlier studies for drug response curve at Sayin lab. Error bars are shown as SEM. ATP levels for Y2 and A1 normalized to the lowest concentration.

For both the Y2 and A1 cell lines for the third ctg, the ATP levels in the DMSO and no drug conditions were notably low, likely attributed to laboratory errors, potentially involving a too-low volume of reagent, possibly due to its depletion during the experiment.

To enhance the comparability of the data with the second CTG (fig. 8-9), which was firstly normalized to the DMSO (fig. 8), the raw data was further normalized to the lowest concentration (fig. 9 for 2nd ctg and fig. 10 for 3rd ctg) for both second and third CTG because it showed zero effect on cell viability. This normalization procedure helps to mitigate potential errors and discrepancies arising from the variations in initial conditions.

Intriguingly, the first and second CTG experiments reveal different trends in terms of which cell line exhibits greater sensitivity to the drug treatment. However, looking at fig. 10, the

third CTG experiment supports the findings of the second CTG, demonstrating that the A1 cell line is indeed more sensitive to the drug, at 50µM concentration. Notably, at the highest concentration of 200 µM, it is evident that both cell lines experience a significant decrease in viability, with a substantial presence of non-viable cells in both cases.

The third CTG experiment, which normalized data to the lowest concentration, provides additional insights. Notably, the results underscore the importance of proper laboratory techniques and controls. The initial low ATP levels in the DMSO and no drug conditions likely resulted from laboratory errors, emphasizing the need for meticulous handling of reagents and resources.

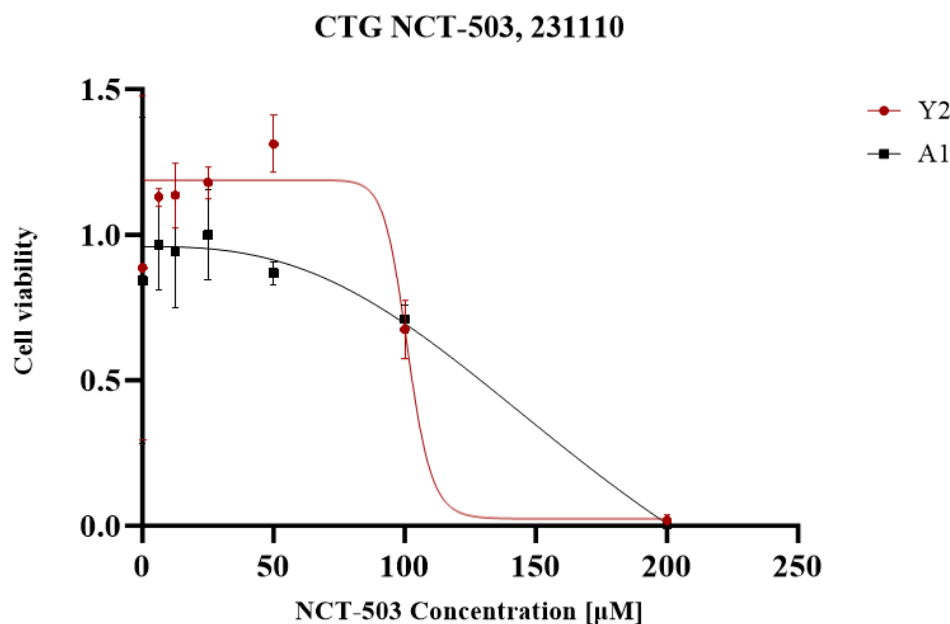

Fig. 11. Treated Y2 and A1 cells with drug NCT-503 for 72 hours and then measured the cell viability via ctg assay. The data was fit to a sigmoidal curve, calculated by the GraphPad Prism software based off the original data points and plotted as shown (Y2 red color and A1 black color lines respectively). The sigmoidal shape is used since it is used for earlier studies for drug response curve at Sayin lab.. Error bars are shown as SEM. ATP levels for Y2 and A1 normalized to the solvent DMSO.

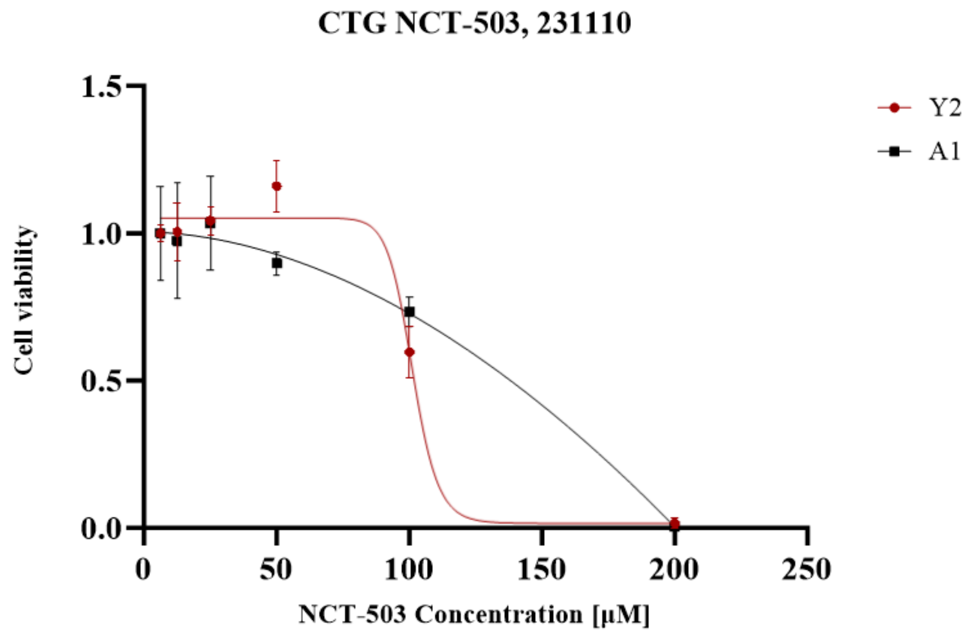

Fig. 12. Treated Y2 and A1 cells with drug NCT-503 for 72 hours and then measured the cell viability via ctg assay. The data was fit to a sigmoidal curve, calculated by the GraphPad Prism software based off the original data points and plotted as shown (Y2 red color and A1 black color lines respectively). The sigmoidal shape is used since it is used for earlier studies for drug response curve at Sayin lab.. Error bars are shown as SEM. ATP levels for Y2 and A1 normalized to the lowest concentration.

For both the Y2 and A1 cell lines, the ATP levels in the DMSO conditions were notably low for the fourth CTG, likely attributed to laboratory errors.

To enhance the comparability of the data with the second and third CTG, it was both normalized to the DMSO (fig. 11) and to the lowest concentration (fig. 12). This normalization procedure helps to mitigate potential errors and discrepancies arising from the variations in initial conditions.

The fourth CTG experiment supports the findings of the second and third CTG, demonstrating that the A1 cell line is more sensitive to the drug at 50µM of NCT-503 and then Y2 also becomes sensitive at 100µM. A difference from the other ones is that the viability is the same at 100 µM but for the other ones Y2 is significantly more sensitive at that point, as we can see on the t-test in table 1.

The results of the fourth CTG experiment, as seen in both fig. 11 and fig. 12, provide further insights into the sensitivity of the Y2 and A1 cell lines to NCT-503 treatment. Notably, the ATP levels in the DMSO conditions for both cell lines were unexpectedly low, indicating potential laboratory errors. Despite this, the normalization of data to both the DMSO and the lowest concentration was crucial for ensuring comparability with previous CTG experiments and mitigating errors arising from variations in initial conditions.

Interestingly, the fourth CTG experiment aligns with the findings of the second and third CTGs, reinforcing the notion that the Y2 cell line is more sensitive to the drug, particularly

after 100 µM of NCT-503. A unique aspect of the fourth CTG is the observation that the viability at 100 µM is comparable between the Y2 and A1 cell lines. However, detailed analysis, including t-tests, reveals that Y2 is significantly more sensitive at this concentration in the earlier experiments, highlighting the importance of statistical rigor in drawing conclusions.

Conc. (µM)	CTG 1 (DMSO)	CTG 2 (DMSO)	CTG 4 (DMSO)	CTG 2 (Lowest conc.)	CTG 3 (Lowest conc.)	CTG 4 (Lowest conc.)
200	-	0,10132271	0,2446667	0,099790532	0,518543	0,263781695
100	3,3717E-06	0,000595423	0,552260739	0,000607365	6,48602E-05	0,043257993
50	0,494084919	0,00132073	0,00125226	0,001836395	0,002526712	0,004760545
25	0,149066984	0,075746985	0,099006258	0,138469193	0,225631323	0,925019113
12,5	0,344328516	0,218638561	0,139820041	0,593492696	0,781569815	0,793503764
6,25	0,087133078	0,601269112	0,121112136	1	1	1
0	0,229782861	0,56415347	0,917420894	-	-	-

Table 1. With help of Excels t-test function, t-test comparing cell line Y2 to A1 with the different concentrations was done. The t-test is based on normalized CTG values to either DMSO or the lowest concentration. A value lower than 0.05 is counted as significant in this thesis.

According to the t-test there is only a significant difference between the cell lines at 100µM at all CTGs done except CTG 4, and at 50µM on all CTGs except for CTG 1, for the lower concentrations there is no significant difference and for the highest concentration there is no significant difference because everything is dead.

In summary, the data highlights the intricacies of drug concentration and cell sensitivity, with the experiments shedding light on the contrasting responses of the two cell lines to the drug treatment, particularly under varying concentration levels.

4.2. Results from Extracellular uptake assay using targeted metabolomics

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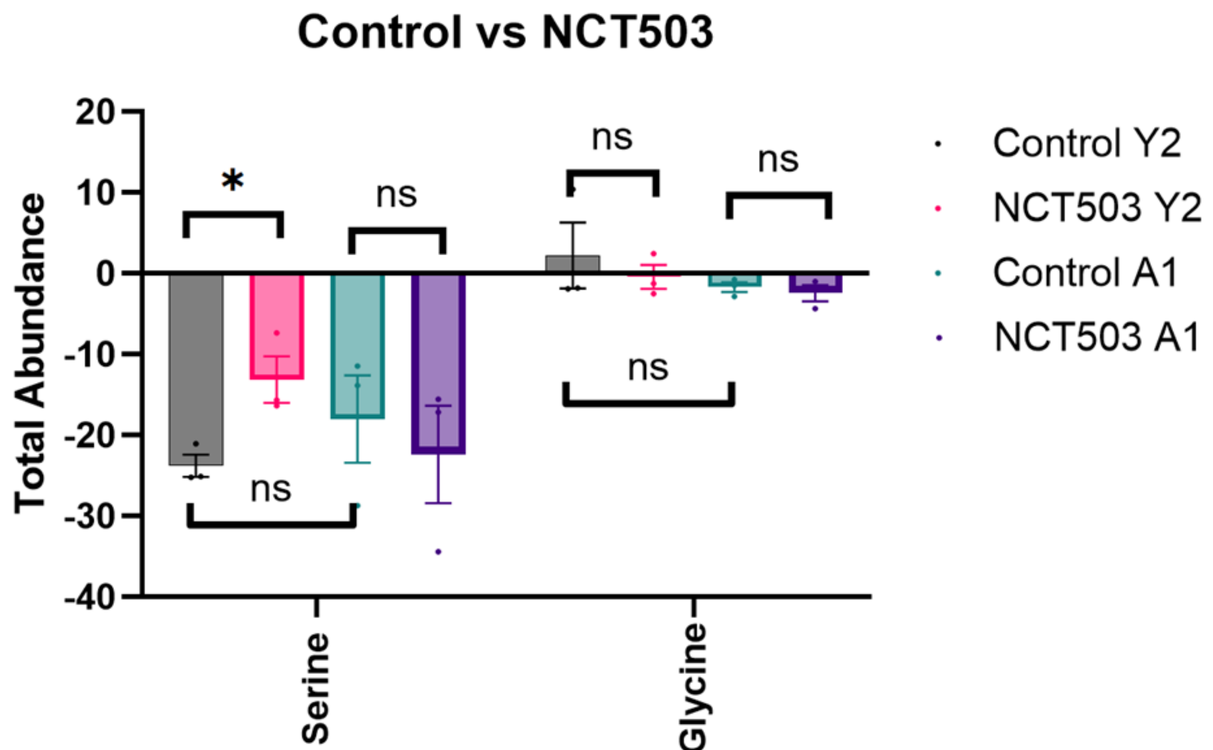

*Fig. 13. Treated Y2 and A1 cells with 25µM drug NCT-503 for 24 hours and then measured the total abundance of serine and glycine, comparing with controls of Y2 and A1 in an extracellular uptake assay. The data is the total ions detected and counted for that metabolite by the GCMS, normalized in Excel and plotted in the GraphPad Prism. Grey is representing control Y2, pink is representing treated Y2, turquoise is representing control A1 and purple treated A1. The negative value means the metabolite is secreted and positive value shows uptake. The graph includes significant differences: Ns= not significantly different, *= value lower than 0.05[23].*

According to figure 13, we can see that the treated cell cultures for Y2 is less negative than the control for the metabolite serine and less positive for glycine. For A1 the treated cell cultures are more negative for both serine and glycine. The Y2 cell cultures seems to secrete serine and after treatment it secretes less which indicates that an inhibition of serine production is made. It is taking up glycine from the media, but with the treatment it takes up less.

The study discussed in the article [24] provides insights into the relationship between serine and glycine metabolism in cancer cells under different nutrient conditions. While the specific mechanism behind the decrease in glycine uptake when serine secretion is decreased is not explicitly addressed in the provided excerpts, there is a known interconnection between serine and glycine metabolism.

Serine and glycine are interconvertible amino acids in cells through the action of serine hydroxymethyltransferase (SHMT) enzymes. Serine can be converted to glycine via the enzyme serine hydroxymethyltransferase, and glycine can be converted back to serine through the glycine cleavage system. This interconversion is crucial for one-carbon metabolism, nucleotide synthesis, and maintenance of redox balance in cells.

In the context of the study, the decrease in serine uptake and the decrease in glycine uptake observed in cells grown in Plasmax compared to DMEM may suggest a coordinated regulation of serine and glycine metabolism to adapt to different nutrient conditions. In the context of the study discussed in the article, where cancer cells grown in Plasmax showed decreased serine uptake without a compensatory increase in glycine uptake compared to cells in DMEM, it suggests a specific metabolic adaptation in response to the nutrient conditions provided by the different media. However, in a scenario where serine secretion is decreased, cells may respond by increasing glycine uptake to ensure the availability of both amino acids for metabolic processes.

Therefore, the expected outcome for glycine uptake after a decrease in serine secretion would likely involve an increase in glycine uptake by the cells to maintain the balance between serine and glycine metabolism and support essential cellular functions such as nucleotide synthesis, one-carbon metabolism, and redox balance [24]. This means that our results may be affected by unknown factors and include errors.

According to fig. 13, the A1 cell cultures secrete more of both serine and glycine after treatment.

The observed increase in serine and glycine secretion after treatment in aged cells suggests complex cellular responses to stress and metabolic alterations. This response may involve compensatory mechanisms to maintain cellular homeostasis, metabolic reprogramming to adapt to changes in the cellular environment, and increased nutrient demands due to aging-related factors. Additionally, dysregulated metabolism and cellular senescence in aged cells could contribute to altered secretion patterns of serine and glycine. Overall, these findings highlight the intricate interplay of cellular processes in aged cells and emphasize the need for further research to understand the underlying mechanisms and their implications for cellular function in aging [25].

Also, there is only a significant difference between control vs treated Y2, so the other results can't be trusted. Therefor a higher concentration of NCT-503 was tested to see if there is significant difference for both cell lines when increasing the dose.

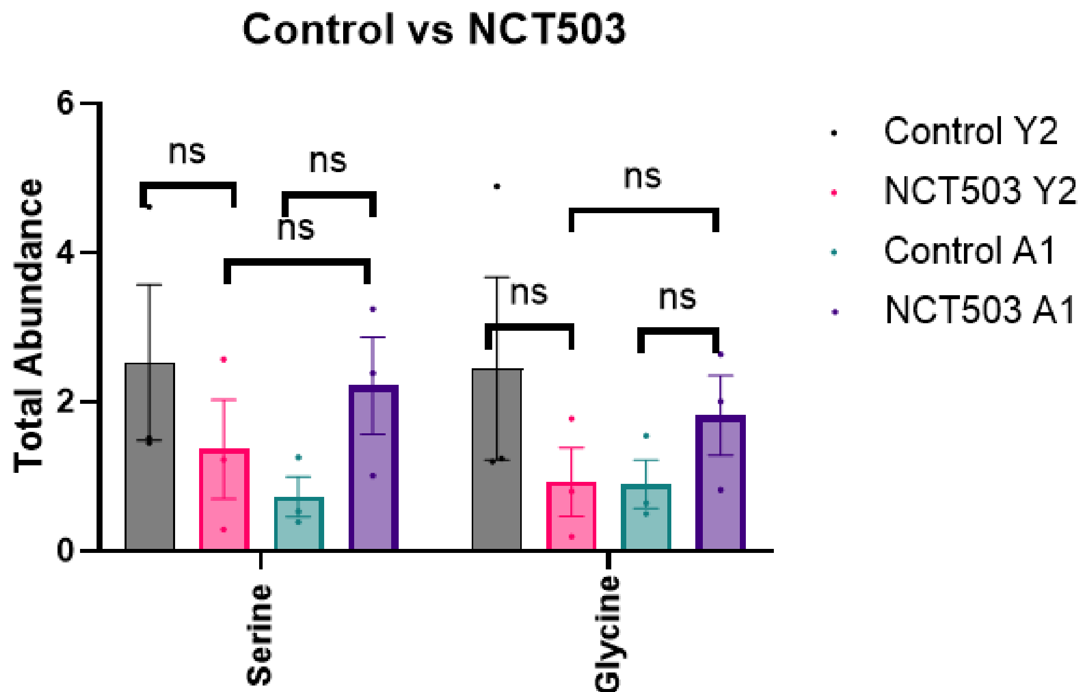

*Fig. 14. Treated Y2 and A1 cells with 50µM drug NCT-503 for 24 hours and then measured the total abundance of serine and glycine, comparing with controls of Y2 and A1 in an extracellular uptake assay. The data is the total ions detected and counted for that metabolite by the GCMS, normalized in Excel and plotted in the GraphPad Prism. Grey is representing control Y2, pink is representing treated Y2, turquoise is representing control A1 and purple treated A1. The negative value means the metabolite is secreted and positive value shows uptake. The graph includes significant differences: Ns= not significantly different, *= value lower than 0.05[23].*

According to figure 14 the Y2 cell cultures seems to take up both serine and glycine from the media before treatment, but with the treatment it takes up less. For A1 cell cultures the results are opposite to Y2, serine and glycine is taken up from the media before treatment, but the uptake is increasing with the treatment.

A decrease in uptake of both serine and glycine means that the treatment affected the cells' ability to absorb those metabolites. This could be due to several factors such as that the treatment inhibits the activity of enzymes in serine/glycine metabolism. The inhibition of the enzyme's activity could cause cellular stress responses that are triggering adaptive mechanisms for cell viability or downregulate the uptake of serine and glycine from the media [26].

An increase in uptake of both serine and glycine means that the treatment stimulates the uptake of the metabolites. A common cause for this to happen is a compensatory response to restore cellular homeostasis or a protective response to enhance availability of serine and glycine for cellular functions and survival.

An increase indicates that the result from the treatment is negative since more serine and glycine provide fuel for cell growth and could help the tumors grow and it could mean that the

aged cell line is resistant to the treatment. A decrease on the other hand could be positive because of the opposite reasons [27].

Comparing with the results in figure 13, it is only glycine for Y2 cell line that show a similar result, the other ones have totally different results.

Also, none of the results are statistically significantly different.

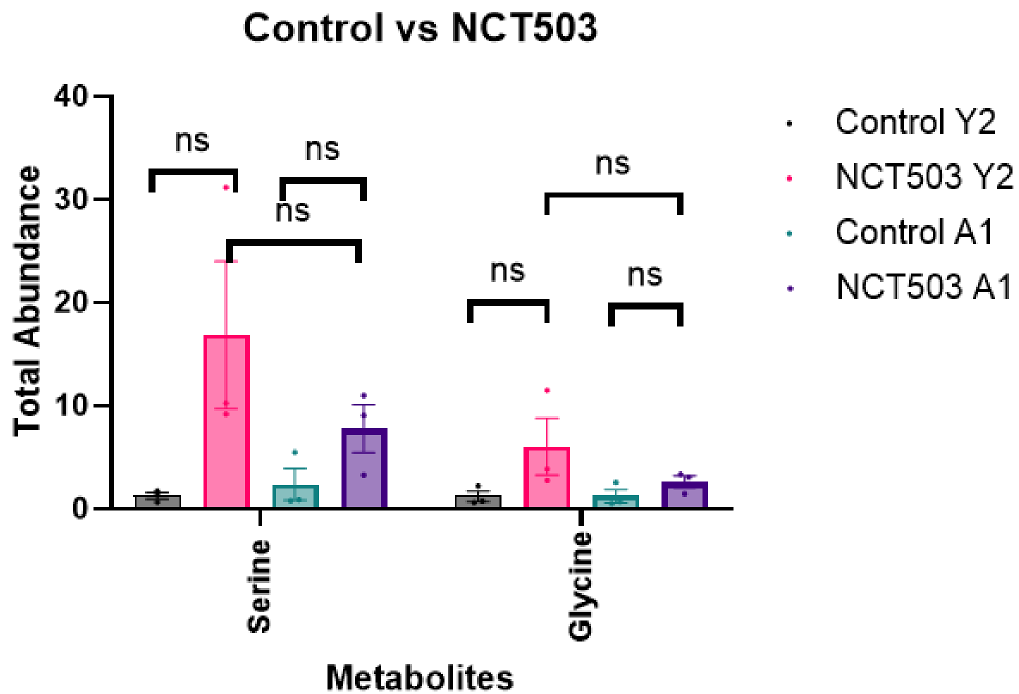

*Fig. 15. Treated Y2 and A1 cells with 50µM drug NCT-503 for 24 hours and then measured the total abundance of serine and glycine, comparing with controls of Y2 and A1 in an extracellular uptake assay. The data is the total ions detected and counted for that metabolite by the GCMS, normalized in Excel and plotted in the GraphPad Prism. Grey is representing control Y2, pink is representing treated Y2, turquoise is representing control A1 and purple treated A1. The negative value means the metabolite is secreted and positive value shows uptake. The graph includes significant differences: Ns= not significantly different, *= value lower than 0.05[23].*

According to figure 15 the result is the opposite from what is seen in figure 14 when looking at the young cell line. After treatment Y2 cell cultures has increased the uptake of both serine and glycine.

The treated A1 cell cultures has also an increased uptake from the media, as seen before in figure 14 as well.

An increased uptake as mentioned above means that the treatment stimulates the uptake of the metabolites and a common cause for this to happen is a compensatory response to restore cellular homeostasis or a protective response to enhance availability of serine and glycine for

cellular functions and survival. It is also said above that it is a negative result for the treatment.

5. Discussion

5.1. CTG assays suggests that NCT-503 effectively inhibits cancer cell viability

The serine and glycine biosynthesis pathways are crucial for cancer cell proliferation and survival. PHGDH, a key enzyme in the serine synthesis pathway, is often overexpressed in various cancer types, including lung adenocarcinoma (LUAD), and represents a potential target for cancer therapy [28]. The results confirm that inhibiting PHGDH can effectively hinder the growth and viability of cancer cells, a promising finding for potential therapeutic interventions.

The initial increase in viability at lower drug concentrations, followed by a decline, may be linked to the complexities of one-carbon metabolism. Altering the balance of metabolites, such as serine and glycine, can have a profound impact on cellular functions, including nucleotide synthesis and redox balance [29].

Moreover, the reversal in the sensitivity of the Y2 and A1 cell lines compared to the first CTG experiment is intriguing. This discrepancy suggests that the response to the drug may be more complex and context-dependent than initially anticipated. For example, the differential response could be influenced by factors such as the age of the cells, their specific genetic makeup, or other environmental conditions [30].

Of particular interest is the observation that at the highest drug concentrations (200 µM), both cell lines experienced significant cell death. This aligns with the goal of developing a drug that can effectively eliminate cancer cells, but it also raises questions about potential side effects or collateral damage to healthy cells. Further experiments are needed to confirm these results and assess the sensitivity of both cell lines across a wider range of drug concentrations.

Determining the optimal drug concentrations for effective cancer treatment while minimizing potential side effects is a critical challenge in the development of targeted anti-metabolic therapies. The balance between cancer cell targeting and minimizing harm to normal cells is a key consideration in drug development. [31]

Comparing the results across all CTG experiments reveals a consistent pattern. The A1 cell line consistently demonstrates higher sensitivity to NCT-503 treatment, especially at lower concentrations than 100 µM. But at higher concentrations where cell death occurs is Y2 more sensitive. This consistency supports the robustness of the observed effects and strengthens the conclusion that A1 is more responsive to the drug compared to Y2. Since the majority of patients with lung cancer of the subtype Adenocarcinoma is elders, the results are promising. A1 being more sensitive means it is more likely to be able to treat elders, especially with lower dose which would be positive to minimize the risks of harming other cells or organs in their body.

The consistently low ATP levels in the DMSO conditions across multiple experiments raise concerns about potential laboratory errors. While efforts were made to normalize data and enhance comparability, the presence of low ATP levels in control conditions underscores the need for meticulous attention to laboratory procedures. Further investigations and refinements in experimental techniques will be crucial for ensuring the reliability of results. Also, more of

the CTG reagent could be used in each well to increase the detection of ATP levels or use more cells.

Despite the promising findings regarding NCT-503 as an anti-cancer agent, the complexity of drug responses and the potential for laboratory errors highlight the need for ongoing research. Future experiments, both in vitro and in vivo, will be essential to validate these results and explore the translational potential of NCT-503 as a therapeutic intervention.

One compelling question arising from our results is why older cells could be more sensitive than younger cells at lower drug concentrations. Several factors could contribute to this observation. Firstly, age-related changes in cellular metabolism and homeostasis may render older cells more susceptible to alterations in one-carbon metabolism. For instance, older cells may exhibit dysregulated metabolic pathways or decreased adaptive capacity compared to younger cells, making them more vulnerable to perturbations induced by PHGDH inhibition. PHGDH expression levels have been shown to change with age. Research indicates that PHGDH mRNA and protein levels increase in Alzheimer's disease pathology, as demonstrated in both mouse models and human cohorts. This increase correlates with disease progression and symptomatic development, suggesting that PHGDH expression is influenced by age and disease state [32].

Additionally, aging is associated with cellular senescence and alterations in signaling pathways that regulate cell growth and survival. These age-related changes may sensitize older cells to the cytotoxic effects of the drug, leading to increased sensitivity at lower concentrations. Furthermore, differences in the expression or activity of drug transporters and metabolic enzymes between young and old cells could contribute to variations in drug uptake, metabolism, and response.

In conclusion, these CTG experiments collectively contribute to our understanding of NCT-503's potential as an anti-cancer agent. While the results are promising and consistently demonstrate the sensitivity of the A1 cell line, challenges related to potential laboratory errors necessitate further investigation.

5.2. Serine and Glycine Uptake Patterns Reflect Varied Cellular Responses in Lung Cancer

The data shown in figures 7, 8, and 9 provide fascinating new information on how therapy affects cell cultures, especially with regard to serine and glycine uptake and secretion patterns. But as pointed out, there are differences in the outcomes, which begs the question of the accuracy and interpretation of the information. Let's examine these results in more detail and talk about possible explanations for the observed variations.

Figure 7 illustrates how the treated Y2 cell cultures exhibit decreased serine secretion and glycine absorption, which suggests that serine production has been inhibited and that glycine use has decreased. Conversely, there is an increase in serine and glycine secretion in the treated A1 cell cultures. These findings imply that the two cell lines' respective metabolic reactions to the therapy were different. This could imply a potential disruption of metabolic pathways involved in serine and glycine metabolism for Y2 cell line. Depending on the context, such alterations might be desirable if they lead to inhibition of cancer cell

proliferation or survival. For A1 cell line the increase in serine and glycine secretion could indicate that it adapts to the changes to survive, this means that the drug initially works, but the cells activate survival pathways to promote tumor growth and cell survival. This does not mean that the treatment does not work, but that the A1 cell line could be in need of a longer treatment-time or higher concentration of the NCT-503 [33].

Comparable outcomes are shown in Figure 8, where treated A1 cell cultures exhibit higher serine and glycine uptake while treated Y2 cell cultures exhibit decreased uptake of these metabolites. There are concerns regarding the reliability and reproducibility of the experimental settings and measurements in light of this difference from figure 7.

While increased nutrient uptake for A1 cell line could be interpreted as a mechanism to support cell survival or proliferation, it's important to note that the ultimate impact on cell viability or tumor growth cannot be determined solely based on uptake assays. The decrease in serine and glycine uptake observed in the treated Y2 cell cultures may suggest inhibition or disruption of metabolic pathways involved in nutrient uptake. This could indicate a potential effect of the treatment on reducing cell proliferation or viability. However, similar to the A1 cell line, further assessments beyond uptake assays are necessary to validate these findings and assess overall treatment efficacy.

Moreover, figure 9 presents an additional variation in the data, demonstrating enhanced serine and glycine uptake by both Y2 and A1 cell cultures following treatment This highlights the complex nature of cellular responses to therapy and the need for additional research, as it contrasts with the results of figure 8 for the Y2 cell line.

The desired outcome is to observe indications of a successful treatment reflected in the results of the uptake assays. As mentioned earlier, this is evident in certain graphs, where we anticipate observing either a reduction in the uptake of serine and glycine or a decline in the secretion of these metabolites. Therefore, conducting additional repeated runs is essential to yield some of the results akin to those depicted in figures 7 or 8.

There is a particular interest in observing these effects primarily within the aged cell line, as it is intended to simulate the cellular characteristics of older individuals, who constitute our target demographic. By focusing on the aged cell line, we aim to ascertain the treatment's efficacy in a population more representative of the patients who would benefit most from this therapeutic intervention.

5.3. Discrepancies Between CTG and Uptake Assays in Evaluating NCT-503 Efficacy

After looking at the results from both Cell Titer Glow Assay and Uptake Assay it is hard to tell whether the treatment with NCT-503 is effective or not, mostly because of the variety within the results of the Uptake assay.

In two out of three trials the results showed that treatment of Y2 cell-line made the cells take up less glycine from the media, and for A1 cell-line more glycine and serine is taken up from the media afterwards. This shows that there is a possibility of a positive response of the

treatment for the younger cell-line, but a negative response of the old cell-line. The lack of a corresponding positive effect in uptake assays suggests that NCT-503 may not directly affect the cellular uptake of specific nutrients, such as serine and glycine, despite its efficacy in inhibiting cell viability. This could indicate that the mechanism of action of NCT-503 primarily targets other aspects of cellular metabolism or signaling pathways.

On the other hand, the CTG showed a positive response, that the viability of the cells decreased a lot. The positive effect observed in CTG assays suggests that NCT-503 effectively inhibits cancer cell viability or proliferation, as evidenced by reduced ATP levels. This indicates that the drug can exert cytotoxic effects on the cancer cells, which is a desirable outcome in cancer therapy [34]. The positive response in CTG assays indicates that NCT-503 has a significant impact on overall cellular health and function, leading to reduced cell viability. This could reflect the drug's ability to disrupt critical cellular processes or metabolic pathways essential for cancer cell survival. There is a need of looking in to side effects to make sure that there is no damage on the healthy cells.

The CTG assay, relying on ATP levels, provides a measure of overall cell viability but offers limited insights into cellular metabolism. In contrast, uptake assays delve deeper into cellular processes by assessing metabolite release and utilization. By focusing on serine and glycine uptake, these assays can evaluate the inhibition of serine synthesis and transporter activity. This comparative analysis offers a more comprehensive understanding of the treatment's impact on cellular metabolism, particularly in the context of cancer cell proliferation and survival. The discrepancy between CTG and uptake assays highlights the complexity of cellular responses to drug treatment. It suggests that while NCT-503 effectively inhibits overall cell viability, its effects on specific metabolic pathways or nutrient uptake mechanisms may be nuanced or context-dependent.

The paper "Inhibiting PHGDH with NCT-503 reroutes glucose-derived carbons into the TCA cycle, independently of its on-target effect" can strengthen the results by providing additional evidence and mechanistic insights into the effects of NCT-503 treatment on cell viability and serine metabolism. The study demonstrated that NCT-503 treatment reduced the viability of neuroblastoma cells expressing both high and low levels of PHGDH [5]. This finding aligns with the thesis testing NCT-503 on lung cancer cells, where a decrease in cell viability could be a crucial outcome to assess the efficacy of the treatment.

Moreover, the study confirmed the inhibition of serine synthesis in neuroblastoma cells treated with NCT-503. This result supports the thesis by highlighting the impact of NCT-503 on serine metabolism, which is essential for cell proliferation and survival. The inhibition of serine synthesis could contribute to the observed decrease in cell viability, further strengthening the thesis's hypothesis regarding the potential anti-cancer effects of NCT-503 in lung cancer cells.

5.4. Lack of significant results

There is also a lack of significant results which leaves us with the question if our results are trustworthy or if the outcome is because of something other than the added drug. When confronted with nonsignificant results, it's crucial to approach them with care. Statistical

significance indicates the likelihood that observed differences are not due to chance. However, when results aren't statistically significant, it suggests that observed variations could be due to random chance or factors not adequately accounted for. This means that even if there are some promising findings there is a high need of more research.

The only significant results in this study is shown at the concentrations 50 µM and 100 µM for the CTG, meaning that at those concentrations, the aged vs the young cell lines responded different to the treatment. It also means that all other results could be by chance and not because of the treatment. And we also have one significant result in figure 7 on serine secretion for the Y2 cell line, which is a positive sign since it indicates that the treatment worked.

5.5. Sources of error

Numerous forms of error can have a substantial impact on how well experimental results are interpreted and how reliable they are. Numerous factors could have affected the results of this research, which could have resulted in errors or discrepancies.

One important aspect is the disruption brought about by the GC-MS instrument's malfunction, which required maintenance and downtime in between experimental runs. The accuracy of metabolite measurements may have been impacted by the variability in equipment performance and data quality brought forth by this outage. The reproducibility of data may have been impacted by residual effects or changes in instrument behavior that remained despite efforts to recalibrate and validate the instrument after repair.

Additionally, the collaborative nature of the experimental procedures, with multiple individuals handling cell cultures and conducting assays, introduces the possibility of human error. In some cases, we were two helping each other since we both ran samples at the same time on the GC-MS. Differences in technique, handling, or interpretation among personnel may have led to inconsistencies in experimental execution, sample handling, or data recording. Moreover, the involvement of multiple individuals increases the complexity of quality control and standardization efforts, heightening the risk of procedural errors or oversights.

Another issue encountered during the lab work involved the preparation of diFBS. A few times, the diFBS leaked from the tubing, likely due to the high speed of the shaker. To address this, we reduced the mixing speed. This could also happen if the clips are not tightened or if there are holes in the tubing film. This problem might have affected the consistency of the final media, potentially impacting the experimental results. To mitigate this problem in the future, it is recommended to maintain a moderate speed, check if there are any holes in the film and that the clips securing the film is correctly closed.

Furthermore, the reliance on data processing and analysis using software tools like Excel introduces another potential source of error. Mistakes in data entry, formula application, or data manipulation within spreadsheets can propagate errors throughout the analysis pipeline, leading to erroneous conclusions or misinterpretations of results. These errors may be compounded by the complexity of the data and the intricacies of the analytical techniques employed.

5.6. Further investigation

This thesis on its own could not answer the question fully if the treatment with NCT-503 is a useful method to treat Adenocarcinoma. There is a need to further investigate the question to make sure the promising results is not caused by chance.

Repeat uptake assay:

To improve the existing results from uptake assay, the same exact experiment would need to be repeated a couple of times, to find a pattern or matching results, hopefully significant ones. A goal would be to have at least three matching results to make a fair conclusion. It could also be of interest to look at a wider range of concentrations.

Look at intracellular metabolites:

The next thing would then be to look into intracellular metabolites to give a broader understanding of the cellular response to the treatment. Intracellular metabolite analysis provides direct insight into metabolic pathways affected by the treatment. By quantifying metabolite levels within the cell, researchers can identify specific alterations in metabolic fluxes, enzyme activity, and pathway dynamics, offering a deeper understanding of how the treatment impacts cellular metabolism.

Key metabolic intermediates can be measured using intracellular metabolite analysis, which sheds light on the state of metabolic pathways. Metabolite level changes can reveal possible targets for therapeutic intervention or biomarkers of therapy response by indicating disturbances or changes in metabolic processes.

To begin it would be of most interest to measure serine and glycine. Serine is directly synthesized by the enzyme PHGDH (phosphoglycerate dehydrogenase), which is targeted by NCT-503. Measuring serine levels will help determine the on-target effect of NCT-503. Glycine is closely related to serine metabolism. PHGDH inhibition may also affect glycine levels since serine can be converted to glycine.

A significant decrease in intracellular serine levels upon NCT-503 treatment is expected, indicating effective inhibition of PHGDH. Potential decrease in glycine levels due to reduced serine availability [35].

When time is available it would also be of interest to measure metabolites like 3-Phosphoglycerate (3-PG) since 3-PG is the substrate for PHGDH in the serine biosynthesis pathway. Accumulation of 3-PG can indicate the inhibition of PHGDH.

Key metabolites in glycolysis could also be of interest to measure because cancer cells frequently rely on glycolysis for energy production (the Warburg effect). Moreover, modifications to these metabolites can reveal the efficacy and metabolic effects of treatments such as NCT-503. By identifying compensatory mechanisms and metabolic reprogramming in cancer cells, measuring these metabolites offers insights into the cells' energy status and nutrition usage. This knowledge is critical for creating tailored therapies and comprehending drug reactions, which will ultimately lead to the development of more potent cancer

treatments.

The discovery of metabolic fingerprints linked to therapeutic response or resistance is made possible through the profiling of intracellular metabolites. Metabolite abundance or depletion patterns can be used as biomarkers to track illness development, stratify patient populations, or predict treatment efficacy, hence improving personalized medicine techniques.

A thorough evaluation of the effects of treatment can be obtained by combining intracellular metabolite analysis with functional tests, such as cell viability or proliferation assays. Mechanistic insights into how modifications in cellular metabolism affect overall cell activity can be obtained by linking changes in metabolite levels with cellular phenotypes.

Understanding the mechanisms of action behind therapy efficacy or resistance is made easier by analyzing intracellular metabolites. Through the establishment of connections between alterations in metabolite levels and particular physiological processes or signaling pathways, scientists might discover new avenues for drug discovery or combine complementary treatments to improve therapeutic efficacy.

To summarize, intracellular metabolite measurement is a useful tool that enhances uptake assays by offering comprehensive understanding of the metabolic changes brought about by a treatment. By improving our knowledge of how treatments affect cellular metabolism, this integrative approach presents prospects for precision medicine and therapeutic development in the study and treatment of cancer.

Use human cells instead of mouse cells:

The thesis could be improved by using human cells. Although mice have long been useful models for studying cancer, they might not accurately represent the subtleties of human illness, especially when it comes to aging. Adenocarcinoma research using human cell models provides a more physiologically appropriate platform for understanding age-related variations in tumor biology and treatment responses.

To close the gap between preclinical research and therapeutic outcomes, the research could be moving from mouse cell models to human cell models. Understanding adenocarcinoma through human cell model research can yield important details on the effectiveness, security, and possible adverse effects of NCT503 in elderly people.

Switching from mouse cell models to human cell models in cancer research poses several challenges. Firstly, human cell lines may be harder to obtain and maintain, requiring specialized culture conditions. Ethical considerations also come into play, as obtaining human cells necessitates adherence to stringent ethical guidelines. Human cell lines exhibit greater genetic heterogeneity compared to mouse models, which can complicate experimental interpretations. Additionally, technical variability may be higher in human cell lines due to differences in culture conditions and genetic drift. Despite these challenges, human cell models offer enhanced clinical relevance and translational potential, better reflecting human disease biology.

***In vivo* trials:**

The thesis might be made much more insightful and relevant by including *in vivo* tests, which shed light on how the drug under test, NCT503, acts in living things and more closely resembles physiological responses seen in the real world. Before moving on to clinical trials, researchers can have a more thorough grasp of the drug's efficacy, safety profile, and potential adverse effects by examining its effects on animal models.

Furthermore, the study of intricate relationships between the medication and different organ systems, as well as how it affects general health and survival, is made possible by *in vivo* trials. The medication's pharmacokinetics, biodistribution, and metabolic destiny within the body are all important factors to take into account during the drug development and regulatory approval processes, and this holistic approach offers useful information on these areas.

6. Conclusion

In revisiting the aims of our study, we set out to investigate the efficacy of NCT-503 as a potential anti-cancer drug targeting the serine synthesis pathway, particularly in the context of lung adenocarcinoma. Through a series of experiments utilizing CTG assays and uptake assays, we aimed to assess the drug's impact on cancer cell viability, proliferation, and cellular metabolism.

The findings obtained from these studies provide a preliminary assessment of NCT-503's potential as an anti-cancer agent. In our CTG assays, we consistently observed a significant reduction in cancer cell viability upon treatment with NCT-503, particularly at concentrations above 50 µM. This indicates the drug's efficacy in inhibiting cancer cell proliferation and highlights the relevance of targeting metabolic pathways, such as serine synthesis, in cancer therapy. Furthermore, the uptake assays revealed insights into the drug's impact on cellular metabolism, with varying responses observed among different cell lines.

This approach will prove useful in expanding our understanding of how targeting PHGDH with NCT-503 can disrupt cancer cell metabolism and inhibit tumor growth. The relevance of targeting PHGDH is undoubtedly supported by the consistent reduction in cancer cell viability observed across multiple experiments, underscoring the potential of NCT-503 as a therapeutic intervention in cancer treatment.

However, it is important to acknowledge the limitations encountered during the course of this study. These include potential errors in laboratory techniques, variability in experimental conditions, and challenges associated with interpreting complex data. Despite these limitations, the findings gained from these studies indicate may assist in guiding future research efforts and informing the development of targeted anti-metabolic therapies.

Moving forward, further research is warranted to validate these findings and explore the translational potential of NCT-503 as a therapeutic intervention in cancer treatment. This may involve conducting additional experiments to confirm the observed effects, investigating potential mechanisms of action, and assessing the drug's efficacy in preclinical models and clinical trials. Additionally, efforts to optimize experimental techniques, standardize protocols, and enhance data analysis methods will be crucial in ensuring the reliability and reproducibility of results.

In conclusion, the findings obtained from these studies provide valuable insights into the potential of NCT-503 as an effective anti-cancer agent targeting the serine synthesis pathway. Despite the challenges encountered, the results obtained contribute to our understanding of cancer cell metabolism and highlight the importance of targeted metabolic therapies in cancer treatment.

7. References

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