From Department of Laboratory Medicine, Division of Pathology

Karolinska Institutet, Stockholm, Sweden

SELENIUM COMPOUNDS AS A NOVEL CLASS OF EXPERIMENTAL CANCER CHEMOTHERAPEUTICS

Arun Kumar Selvam

Karolinska Institutet

Stockholm 2021

Published by Karolinska Institutet.

Printed by Universitetsservice US-AB, 2021

ISBN 978-91-8016-136-7

Cover illustration: Selenium compounds from nutrient supplements to chemotherapeutic agents. Art by Sougat Misra.

Selenium compounds as a novel class of experimental cancer chemotherapeutics

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Arun Kumar Selvam

The thesis will be defended in public at 4X, Alfred Nobels Alle 8, level 4, Huddinge, 19th March 2021, 10.00AM

Principal Supervisor:
Professor Mikael Björnstedt
Karolinska Institutet
Department of Laboratory Medicine
Division of Pathology

Co-supervisor(s):
Assistant Research Professor Sougat Misra
The Pennsylvania State University
Department of Veterinary and Biomedical Sciences

Opponent:
Professor Michael Jonathan Davies
University of Copenhagen
Department of Biomedical Sciences

Examination Board:
Professor Richard Palmqvist
Umeå universitet
Department of Medical Biosciences

Professor Göran Andersson
Karolinska Institutet
Department of Laboratory Medicine
Division of Pathology

Professor Magnus Björkholm
Karolinska Institutet
Department of Medicine

To my family and everyone who supported me

திருக்குறள் (Thirukural) – 396
அதிகாரம்/Chapter: கல்வி / Learning

தொட்டனைத் தூறும் மணற்கேணி மாந்தர்க்குக்
கற்றனைத் தூறும் அறிவு.

Meaning: *Water will flow from a well in the sand in proportion to the depth to which it is dug, and knowledge will flow from a person in proportion to his/her learning*

- ***Thiruvalluvar***

POPULAR SCIENCE SUMMARY OF THE THESIS

Selenium is an essential micronutrient for human health, selenium deficiency or overdose leads to health problems, so optimal intake (50-200µg/day) is necessary to maintain proper health. Selenium at high doses is more deadly to cancer cells than to normal cells because cancer cells can easily absorb selenium. Too much accumulation of selenium compounds triggers several metabolic pathways that cannot be controlled by cancer cells, which becomes a disadvantage for cancer cell growth and leads to cancer cell death. In this project, we are investigating different ways to target cancer cells with different selenium compounds.

In the first project, we used selenium compounds for acute promyelocytic leukemia (APL). APL is a cancer of the white blood cells, the standard treatment for this disease is ATRA (all-trans retinoic acid) in combination with arsenic trioxide (ATO). This treatment leads to many side effects after treatment. When sodium selenite was used instead of ATO, we observed a significant reduction in diseased cells. We propose that ATRA in combination with selenite provides a better treatment with minimal or no side effects.

Selenium toxicity may be enhanced in cancer cells by increasing selenium uptake capacity. In my second project, we investigated several natural and synthetic chemical compounds that can increase the expression of an important protein that can directly or indirectly enhance selenium uptake in cancer cells. Our results showed that selenium in combination with a diphenyl diselenide compound increased selenium uptake by cancer cells several-fold, thereby inducing cancer cell death.

A major problem with chemotherapy is that cancer-killing compounds not only kill the cancer cells but also affect the surrounding normal cells, which is considered a side effect of cancer treatment. The only way to reduce such harmful effects is to find a way to target only the cancer cells and leave the normal cells unharmed. In the fourth project, we specifically targeted liver cancer cells using mRNA coupled with microRNA techniques in combination with selenium compounds. To increase the uptake of this mRNA into the target cells, we used a lipid nanoparticle carrier as a vehicle. Our results showed that selenium compounds are highly metabolized and induce cancer cell-specific (liver) toxicity using this system.

In the fifth project, we investigated the effects of selenium directly in tissue sections from patients with pancreatic cancer. Resected tumor tissue from pancreatic cancer patients was sliced and incubated with selenium compounds for two days. Our results showed that selenium compounds induced cell death in cancer cells while preserving the surrounding normal pancreatic cells.

Our experimental findings showed the usefulness of selenium compounds in treating cancer and their potential application in cancer chemotherapeutics. This simple drug can be given to cancer patients by carefully evaluating their response to selenium compounds using our tissue culture model.

ABSTRACT

Selenium is an essential micronutrient for humans, it has a narrow margin between antioxidant and pro-oxidant effects. Redox-active selenium compounds have the potency to increase ROS levels in cancer cells, providing a plausible window for therapeutic intervention. Redox-active selenium compounds such as sodium selenite (Se), selenocystine (SeC), and Se-methylselenocysteine (MSC) have been shown to inhibit growth, angiogenesis, and induce apoptosis by altering the redox potential (oxidative stress) in various tumor cells *in vitro*. Different selenium compounds produce different metabolites that act on tumor cells through multiple pathways. Sodium selenite is readily reduced to hydrogen selenide (HSe-) by extracellular cysteine, whereas selenocysteine is reduced to HSe^- by enzymatic conversion by selenocysteine lyase. Another important selenium compound, MSC, is a prodrug metabolized to methylselenol by kynurenine aminotransferase 1 (KYAT1 or CCBL1). Hydrogen selenide (HSe^-) and methylselenol (MS) are two important intermediate metabolites that are highly redox-active by inducing the production of ROS and initiating cell death via redox-regulated signaling pathways. Hydrogen selenide is more readily taken up by the cell compared to selenite. These intermediate molecules can effectively redox cycle with oxygen in the presence of NADPH and thiols, thus enhancing oxidative stress in malignant cells. Nevertheless, the anti-cancer properties of selenium compounds have not been fully characterized. In this work, our objective was to describe the anti-cancer properties of various selenium compounds using different methods and experimental models that are easily translatable from *in vitro* to *in vivo*.

Selenite at physiological concentrations in combination with ATRA completely abolished the expression of the PML/RARα oncoprotein and increased the expression of the transcription factors RAR, PU.1 and FOXO3A, providing a plausible basis for the increased differentiation in cells of acute promyelocytic leukemia (APL). The extracellular milieu is important for selenite cytotoxicity, i.e. selenite is readily reduced to hydrogen selenide (HSe^-) by extracellular cysteine, the xCT (cystine/glutamate transporter) antiporter is very important for HSe^- turnover. Diphenyl diselenide, a small- molecule compound, increases the expression of xCT and its key regulatory genes such as NRF2 and ATF4 in vitro. When diphenyl diselenide was co-incubated with selenite or selenocysteine, we observed multiple sensitizing effects in almost all cancer cell lines tested. This provides a strong correlation between extracellular thiols and the cytotoxicity of selenite and selenocysteine.

Kynurenine aminotransferase 1 (KYAT1 or CCBL1) is a PLP-dependent enzyme and plays an important role in MSC metabolism. KYAT1 has dual enzyme activity, transamination and β-elimination towards the single substrate. MSC is considered a prodrug that is not toxic as long as it is not metabolized by KYAT1. MSC is reduced by transamination to β-methylselenopyruvate (MSP) and by β-elimination to monomethylselenol. Several assays exist to determine the transamination activity of KYAT1, but very few simple assays exist to determine the β-elimination activity of KYAT1, which is not reliable because it is not a direct measure of MS. We introduced a simple novel coupled assay to determine the β-elimination

activity of KYAT1. This assay method combines two enzyme systems, i.e. thioredoxin reductase1 (TrxR1) and KYAT1. MS is an excellent substrate for thioredoxin reductase1. MSC is metabolized to MS by β-elimination activity, and this can be used as a substrate for TrxR1, which is monitored spectrophotometrically by the oxidation of NADPH.

Overexpression of KYAT1 may be an advantage in exploring the anti-tumor property of MSC, as it plays an important role in MSC metabolism. Both metabolites of MSC (MSP and MS) play critical roles in anti-tumor activity. MSP is known to inhibit HDAC activity, while MS has been shown to increase the formation of ROS and induce redox imbalance in the tumor. We used therapeutic mRNA techniques to induce KYAT1 expression using a lipid nanoparticle (LNPs)-based delivery system in hepatocellular carcinoma (HCC) cells. the addition of antisense microRNA122 (HCC-specific) with KYAT1mRNA showed precise targeting of HCC cells. Our results demonstrate successful targeted therapy in HCC cells with MSC.

The choice of the model system is very important in drug screening. Cell culture, 2D and 3D models are widely used, but the reproducibility is very low when transferred to *in vivo*. Our group has established an *ex vivo* slice culture model for pancreatic ductal adenocarcinoma (PDAC). We used this *ex vivo* model to test the anti-cancer properties of sodium selenite and MSC. Our results, both by histology and transcriptomics data, show that sodium selenite at a concentration of 15 μM (concentration below MTD in humans) exhibited pronounced anti-tumor activity by targeting multiple hallmark genes that support cancer growth and progression.

In this work, we have shown that redox-active selenium compounds as potential anti-cancer agents by (1) mechanisms to facilitate uptake by altering the expression of SLC7A11 (xCT) through small-molecule pharmacological compounds, (2) increasing the metabolizing enzymes (KYAT1) using different methods and targeted therapy (3) used different model (*ex vivo*) to mimic the *in vivo* settings.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications and manuscripts.

I. Sougat Misra*[1], **Arun Kumar Selvam**, Marita Wallenberg, Aditya Ambati, András Matolcsy, Isabelle Magalhaes, Gilbert Lauter and Mikael Björnstedt*.
Selenite promotes all-trans retinoic acid-induced maturation of acute promyelocytic leukemia cells. *Oncotarget*. 7(46), (2016), 74686–74700.

II. **Arun Kumar Selvam[1]**, Isabelle Magalhaes[2], Roberto Gramignoli[1], Christina Hebert[3], Mikael Björnstedt[1], Sougat Misra[1,4#]
Microenvironment redox control by SLC7A11 is a druggable target in cancer. *Manuscript.*

III. **Arun Kumar Selvam** and Mikael Björnstedt *. A Novel Assay Method to Determine the β-Elimination of Se-Methylselenocysteine to Monomethylselenol by Kynurenine Aminotransferase 1. *Antioxidants, 9 (*2020), 139.

IV. **Arun Kumar Selvam[1]**, Rim Jawad[1,2], Roberto Gramignoli[1], Adnane Achour[4], Hugh Salter[1,3] and Mikael Björnstedt[1,*].
Targeted enzyme assisted chemotherapy (TEAC) – a novel microRNA-guided and selenium-based regimen to specifically eradicate hepatocellular carcinoma. *Manuscript*.

V. Carlos Fernandez Moro[1,2*], **Arun Kumar Selvam[1*]**, Mehran Ghaderi[2*], Béla Bozóky[2], Soledad Pouso Elduayen[2], Joakim Dillner[1] and Mikael Björnstedt[1#]
Redox-active selenium compounds are superior chemotherapeutics to pancreas cancer in an *ex vivo* model of human surgical specimens. *Maunscript*.
*authors contributed equally

Related research article, review paper and a book chapter published by the defendant not included in the thesis.

Gábor Lendvai[1], Tímea Szekerczés[1], Endre Kontsek[1], **Arun Selvam[2]**, Attila Szakos[2], Zsuzsa Schaff[1], Mikael Björnstedt[2] and András Kiss[1#]. The Effect of Methylselenocysteine and Sodium Selenite Treatment on microRNA Expression in Liver Cancer Cell Lines. *Pathol Oncol Res*, 26(4), 2020, 2669–2681.

Sougat Misra[1], Mallory Boylan[2], **Arun Selvam[1]**, Julian E. Spallholz[2] and Mikael Björnstedt[1*]. Redox-Active Selenium Compounds - From Toxicity and Cell Death to Cancer Treatment. *Nutrients 7* (2015), 3536-3556.

Arun Kumar Selvam, Mikael Björnstedt and Sougat Misra. (2018). Selenium compounds as potent chemotherapeutic agents in cancer. B.Michalke (Ed.), Molecular & integrative toxicity, *Springer* (2018), 251-269.

CONTENTS

LIST OF ABBREVIATIONS

APL	Acute promyelocytic leukemia
AP1	Activator protein 1
BAX	BCL2-associated X protein
CC	Cholangiocarcinoma
CCBL	Cysteine-S-Conjugate Beta-Lyase
ERK	Extracellular signal-regulated kinase
GCL	Glutamate-Cysteine ligase
GLRX	Glutaredoxin
GPX	Glutathione peroxidases
GR	Glutathione reductase
GS	Glutamine synthetase
GSH	Glutathione
GS-Se-SG	Selenodiglutathione
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylases
HIF1-α	Hypoxia-induced factor 1-alpha
HO1	Heme oxygenase1
JNK	JUN N-terminal kinase
KRAP1	Kelch like ECH associated protein 1
KYAT	Kynurenine aminotransferase
LNP	Lipid nanoparticles
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSC	Se-methylselenocysteine
MSP	β-methylselenopyruvate
MTD	Maximum tolerated dose

NADPH	Nicotinamide adenine dinucleotide phosphate
NASH	Nonalcoholic fatty liver disease
NF-κB	Nuclear factor κB
NOX	NADPH oxidase
NRF2	Nuclear factor erythroid 2-related factor 2
PDAC	Pancreatic ductal adenocarcinoma
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLP	Pyridoxal 5'-phosphate
PMP	Pyridoxamine 5'-phosphate
PRDX	Peroxiredoxin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCLY	Selenocystine lyase
SeC	Selenocystine
SLC7A11	Solute carrier family 7 membrane 11
TRX	Thioredoxin
TXNRD1	Thioredoxin reductases 1
VEGF	Vascular Endothelial Growth Factor

1 BACKGROUND

1.1 CANCER

Cancer is the second cause of mortality responsible for several millions of deaths every year around the world. It is not a single disease, but it is a group of diseases characterized by uncontrolled/unregulated growth of transformed cells (1). There exist multiple causes for cancer cell initiation such as unrepairable mutations during cell division, heredity, chemical exposures, physical agents, radiations, infections, autoimmune disorders and diet (2-5). Normal cells acquire neoplastic behaviors by acquiring eight hallmarks of cancer such as sustaining proliferative signaling, evading growth suppression, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, deregulating cellular energetics and avoiding immune destruction (6, 7).

1.1.1 Cancer incidence

According to the World Health Organization in 2012, 14.1 million new cancer cases were diagnosed and 32.6 million people living with cancer around the world within 5 years of diagnosis. An estimated 8.2 million people died in 2012 and 8.8 million in 2015 due to cancer (excluding non-melanoma skin cancer). In Europe, it was estimated that 3.9 million new cases with 1.9 million deaths (excluding non-melanoma skin cancer) occurred in 2018 (8, 9). In the United States, 1.7 million new cases with 0.6 million deaths were estimated in 2018 (10). Among visceral cancers, liver and pancreatic cancers are among the most important causes of cancer-related death (11). They both have a poor prognosis with limited treatment possibilities. There exist multiple treatment modalities such as chemotherapy, surgery, radiation therapy, immunotherapy, hormonal therapy, and laser therapy. Personalized medicine or targeted therapies are relatively new entrants in the field of cancer treatment (12-15), but so far with marginal or no effect in visceral cancer. Successful treatment of cancer remains to be a challenging task despite remarkable progress in therapeutic options.

1.1.2 Liver Cancer

Liver cancer is the fifth most common type of cancer worldwide and ranked second in cancer-related mortality. Most liver cancer arises from the hepatocytes (hepatocellular carcinoma (HCC), >70%) and the intrahepatic bile ducts (cholangiocarcinoma (CC), >15%) (16). HCC is one of the most common liver cancers and the prognosis remains poor for this disease. There

are several known risk factors for the development of HCCs, the common characteristics among them were cirrhosis, viral hepatitis, alcohol use, tobacco use, metabolic factors, nonalcoholic fatty liver disease (NASH) and genetic susceptibility (16-18). Due to late diagnosis, only 5 to 15% of patients are eligible for surgical resections, late-stage patients are treated with sorafenib (a multikinase inhibitor). Prolonged use of sorafenib results in mutable side-effects (19, 20) with an average survival time of only 3-5 months. Further research is necessary to improve the patient's survival and early diagnosis of the disease.

1.1.3 Pancreatic cancer

Pancreatic cancer is an increasing health problem with 460,000 new cases diagnosed worldwide in 2018 with 331,000 deaths per year. Pancreatic ductal adenocarcinoma (PDAC) is one of the most common forms of pancreatic cancer (~85%), which originates from the exocrine component, and it is the most common and lethal form of pancreatic cancer. It is the 12th most common type of cancer with a poor prognosis and the seventh leading cause of cancer-related mortality (9, 11). The major risk factor for the progression of this disease is exposure to carcinogens, chronic pancreatitis, tobacco use, type 2 diabetes mellitus, obesity, and inherent mutations (21-23). It is one of the most aggressive types of cancer with a 5-year survival rate of between 6 and 9 % in the USA (22). Surgery is the most effective treatment in PDAC but only 20% of patients are eligible for surgery because of late diagnosis. However, surgery is never curative but prolong survival by one to three years (24), in the remaining 80% of patient's tumor is locally too advanced and spread to the peritoneal cavity or distant parts of the body such as the liver and lungs (22, 24). These patients are subjected to chemotherapy. Gemcitabine has been used as a first-line of chemotherapy to treat PDAC (25), recent development in adjuvant therapy such as FOLFIRINOX (folinic acid, fluorouracil, irinotecan and oxaliplatin) regimen demonstrated a significantly longer survival among patients with resected pancreatic cancer, with the expense of higher prevalence of side-effects (26, 27). This illustrates the critical need for more effective medical treatments both for first- and second-line therapy to increase the patient's survival. So far, no treatments have been proven efficient for pancreatic cancer.

1.2 DIVERGENCE OF METABOLIC PATHWAYS IN CANCER

1.2.1 Energy metabolism in cancer cells

Typically, normal cells generate ATP (energy) by utilizing glucose via glycolysis (2 ATP), Krebs's cycle (2 ATP) followed by oxidative phosphorylation (32 ATP) in the presence of oxygen to generate 36 molecules of ATP at the expense of 2 ATP (1 glucose = 36 ATP). Glycolysis occurs in the cytoplasm and the latter two cycles in mitochondria. In cancer cells, however, the primary source of energy is from glycolysis, and this process is termed the Warburg effect (28). Therefore, this results in the consumption of relatively higher amounts of glucose to generate ATP (1 glucose = 2ATP) for their energy source (29) as aerobic glycolysis. Even though it is an energy-consuming process, cancer cells use the end product (lactic acid and bicarbonic acid) as an advantage for their growth and survival i.e. eluding from the host immune defense system (29). These abnormal metabolic pathways also activate several oncogenes (e.g., *AKT*, *RAS*, *MYC*) (30, 31) and often suppress the tumor suppressor genes *P53* (32) and the anti-apoptotic protein BCL2. During tumor progression, the tumor microenvironment becomes more acidic, making it stressful for the normal cells around the tumor. Tumor cells promote angiogenesis to fulfill their nutrient and oxygen needs, but the newly formed blood vessels are usually immature and abnormal. This in turn makes the oxygen supply limited to the cancer cells creating the hypoxic condition (32). This condition leads to the overexpression of several proteins such as hypoxia-induced factor 1-alpha (HIF1-α), TWIST (protein favors cancer metastasis and EMT) (33, 34) and VEGF (for new blood vessel formations) (35). Such metabolic reprogramming leads cancer cells to rely on the glycolysis pathway, reduced oxygen requirements, and high consumption of lactate to meet their energy needs (36). Pharmacological targeting of such key adaptive metabolic pathways has been explored in cancer drug development and currently, few candidate drugs are in clinical trials for their efficacy evaluation (37, 38).

1.2.2 Cellular redox homeostasis

The equilibrium between the oxidation/reduction (redox) reactions in the biological system is termed redox homeostasis. Redox homeostasis inside the cell mainly depends on the production and clearance of reactive oxygen and nitrogen species (ROS and RNS, respectively), which are generated during mitochondrial respiration, in peroxisomes and via the endoplasmic reticulum (38, 39). ROS is an important component for the normal cellular functions and modulation of intracellular ROS level is vital for many cellular signaling

pathways such as proliferation, differentiation, cell cycle and anti- and pro-apoptotic signaling (40). In contrast, elevated levels of ROS are deleterious and reduced levels of ROS are detrimental (6). The basal level of ROS production during cell metabolism decides the fate of the cells (41). ROS play crucial roles in stimulating multiple kinases such as PKC (protein kinase C), p38 MAPK (p38 mitogen-activated protein kinase), ERK (extracellular signal-regulated kinase ½), PI3K/Akt (phosphoinositide 3-kinase/serine-threonine kinase), PKB (protein kinase B) and JNK (JUN N-terminal kinase) (42-44). ROS modulate the expression of transcription factors such as NRF2 (nuclear factor erythroid 2-related factor 2), AP1 (activator protein 1), NF-κB (nuclear factor κB), HIF-1α (hypoxia-inducible transcription factor 1α) and P53, all of which are involved in regulating the expression of several antioxidant genes (44-46). The above explains the importance of ROS in both normal and pathophysiological functions of cells. Under normal physiological conditions, ROS homeostasis is maintained by the ROS scavenging antioxidant systems such as vitamins (A, C and E), enzymes (SOD, catalase, GPx, GR, peroxidase, TRX and HO-1) and antioxidant molecules (GSH, coenzyme Q, ferritin, bilirubin and free thiols). When intracellular ROS production is in excess, be it either endogenous due to pathological conditions or by external stimuli, it often leads to DNA damage and the malfunctions of proteins. Malignancies are such pathological conditions in which uncontrolled cells proliferation fuels higher metabolic turnover and alters cellular redox balance.

1.2.3 Redox imbalances in cancer cells

Recent studies suggest that cancer cells of multiple origins acquire redox imbalance (mostly oxidative environment) when compared to benign primary cells. The degree of oxidation or reduction is associated with the aggressive behavior of a tumor (47, 48). ROS at elevated levels can be deleterious to cellular proteins, lipids and DNA, leading to malfunction of cells and has been associated with tumorigenesis. Elevated ROS levels induce genomic instability either directly or by inducing the overexpression of NOX via PKC. The latter is also responsible for high cell proliferation and invasiveness in several tumors (49). It has also been shown that NOX is responsible for the generation of ROS by a feedback loop mechanism (50).

Due to high metabolic turnover, cancer cells generate higher levels of ROS but the deleterious effects of ROS are often effectively evaded by adaptive mechanisms including overexpression of several antioxidant enzymes (SOD, catalase, GPX, GR, PRDXs, TRX, TXNRD1 and HO-

1) (51-54), transcription factors (NRF-2, AP1, NF-κB and HIF-1α) that regulate the expression of many antioxidant genes (55-57) and genes (*GCL* and *GS*) for the production of cellular thiols (GSH) (58). To escape from cell death, cancer cells downregulate the proteins responsible for pro-apoptotic and apoptotic signals (P53, ERK, JNK, MAPK, c-Raf, caspase 9 and BAX) (59-61) and overexpress anti-apoptotic proteins (BCL-2 family) (62). Moreover, ROS induces the expression of PI3K/Akt which promotes cancer metastasis via inducing MMP-9 (matrix metalloproteinase-9) (63). The above-mentioned reprogramming of key cell-signaling pathways explain the adaptive response to oxidative stress and partly explain the mechanism of resistance to cancer chemotherapeutic drugs.

1.3 CONVENTIONAL CANCER TREATMENT

Several regimens exist for cancer treatment including chemotherapy, immunotherapy, radiation therapy, hormone therapy and surgery. The treatment regime depends on the stage and aggressive behavior of the tumor. Most solid tumors are treated with surgery and/or in combination with chemo/radiotherapy, while non-solid tumors are treated with chemotherapy or immunotherapy or radiation therapy or a combination of all three or any two regimes. Over 200 standard chemotherapeutic agents are approved in the US to treat cancer. The advancement in chemotherapeutic regimen is rapidly increasing but the major limitation is that non-tumor cells are also affected and development of chemoresistant (64). Further combination with radiation therapy sensitizes these cells to chemotherapy but side effects are again the major problems. To reduce the side-effects, now immunotherapy was employed such as Pembrolizumab (immune checkpoint inhibitor), thanks to the advances in the knowledge of cancer pathogenesis. By combining these therapies, the patient's survival rate has increased, but the quality of patient's life is still questionable, so new innovative treatment regimens such as personalized medicine and targeted therapy are in need to increase the patient's survival rate and increase the quality of life of the patients.

1.4 THE NOVEL THERAPEUTIC REGIME IN CANCER TREATMENT

1.4.1 mRNA based treatments

Synthetic mRNA has emerged as an effective gene transfection tool in a wide range of therapeutic applications such as a vaccine, cancer treatment and various genetic disorders (65). It can be either silencing disease (RNAi-mediated) causing genes or overexpressing therapeutic proteins into the target organ exogenously. mRNA-based therapy has various advantages over

DNA-based delivery. mRNA does not integrate into the host genome, smaller than DNA, protein translation occurs directly in the cytoplasm, less immunogenic response, less time for translation into protein hence it results in rapid and efficient protein expression in target tissues with a longer half-life and less prone to enzyme degradation (66, 67). One major challenge in this regime is the cell membrane and its complex structure, so it is important to select a suitable vehicle system to deliver the therapeutic mRNA into the target organ or tissue (68).

1.4.2 Lipid nanoparticle-based delivery system

Lipid nanoparticles (LNPs) are one of the most advanced platforms for delivering synthetic mRNA into humans safely and recently LNPs are evaluated in several clinical trials in delivering therapeutic mRNA/siRNAs (68, 69). Encapsulating therapeutic targets (drug or mRNA/siRNAs) into LNPs can protect them during transport and facilitate delivery into the cell membrane via a fusion-based pathway (70, 71). Disease-specific antibodies can also be incorporated into LNPs to target the pathogenic cells or tissues (72)

1.4.3 MicroRNA based targeted therapy

MicroRNAs (miRNAs) are small non-coding RNAs that function as guide molecules in RNA silencing. These RNAs are involved in many physiological and pathological processes by targeting most protein-coding transcripts (73). However, these are often dysregulated in cancer in which they are either over-expressed or under-expressed (74, 75). In conditions, when these are under-expressed in tumors compared to normal counterparts, it provides a novel strategy to target cancer cells by increasing the expression of certain proteins that can inflict cell death. This could be achieved by delivering a synthetic mRNA containing the antisense miRNA-binding sequence into the target organ or tissues (76). While the mRNA will be silenced in normal cells by the presence of the target microRNA.

Delivery of synthetic therapeutic mRNA using LNPs along with disease-specific antibodies and microRNA tagging strategy might be a novel strategy to target cancer cells with high specificity and accuracy without intervening in normal cell functions.

1.5 IS REDOX IMBALANCE IN CANCER CELLS AN AMENABLE THERAPEUTIC TARGET?

Several studies have conclusively shown that cancer cells exhibit a higher basal level of ROS than normal cells (38, 47, 77, 78) (**Fig. 1**). But cancer cells escape from ROS-induced cell

damage and death by activating different antioxidant defense mechanisms and other signaling pathways. When these redox regulatory mechanisms of tumor adaptation are targeted for anticancer therapy, adaptive ROS homeostasis is perturbed and leads to cell death by apoptosis, necrosis, or necroptosis (79). The underlying concept relies on the hypothesis that further elevation in ROS levels will damage cancer cells, while a similar increase in ROS levels in normal cells is within their tolerance levels (38). In several studies, it has been shown that several redox-active selenium compounds induce oxidative damages in cancer cells, thereby presenting these as candidate molecules with potential applications in cancer chemotherapy.

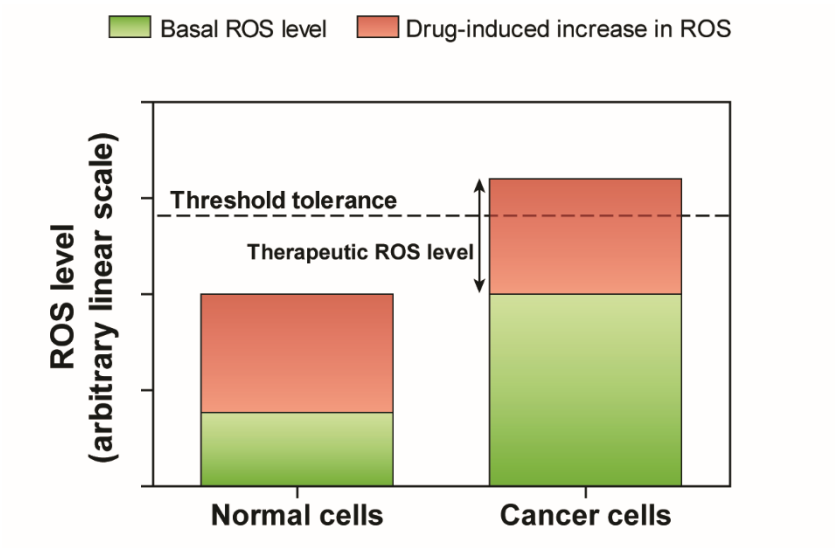

Figure 1: A conceptual schematic showing how modulation of ROS levels may be used for cancer therapy. Adapted from *Misra et al 2015 (77).*

1.5.1 Redox-active selenium compounds

Selenium is an essential micronutrient that acts as an antioxidant and pro-oxidant. Its biological effects strictly depend on chemical speciation, applied dose and the exposure duration and properties of the target cell. The window of requirement and toxicity is quite narrow for selenium (80, 81). The biological activity of selenium is known to be mediated through selenoproteins, which contain the 21st amino acid, selenocysteine (82). Several selenoproteins catalyze the reduction of disulfide bonds in proteins and peptides and thus play critical roles in protein folding (82, 83). The selenoprotein, glutathione peroxidases (GPx) is the key enzyme involved in the detoxification of hydrogen peroxide to water molecules at the expense of 2GSH molecules (84, 85). Other important selenoproteins like mammalian

thioredoxin reductases (TrxR) regulate cell growth and apoptosis, among other functions (86). It is also important in activating several low molecular weight antioxidants (87-89).

Inorganic selenium compounds such as selenite are shown to be oxidants of reduced glutathione (GSH) and they could oxidize a large quantity of GSH in the presence of oxygen (90). It is readily reduced non-enzymatically by cysteine and enzymatically by the Trx system or GLRX systems. Selenite and selenodiglutathione (GS-Se-SG) are highly redox-active and have a high reactivity towards thiols (91). However, some selenium compounds such as Se-methylselenocysteine and selenomethionine are not redox-active. Metabolic transformation converts these compounds into monomethylselenol which is highly redox-active, cytotoxic to cancer cells and implicated in anticancer properties. Such redox-active selenium compounds and their applications as experimental chemotherapeutic agents will be elaborated in detail in the following sections.

1.5.2 Selenium compounds in cancer treatments

In the past few decades, there has been increased interest in using redox-active selenium compounds in cancer prevention and cancer treatment. Our research group was the first to conduct a human phase I clinical trial in terminally ill cancer patients (92) to find the MTD (maximum tolerated dose) of selenite. This phase I clinical trial from our group showed that the tolerable dose (MTD) for selenite is 10.2 mg/m^2 body surface with a short half-life (18.25h) (92).

1.6 DIFFERENT REDOX-ACTIVE SELENIUM COMPOUNDS AND THEIR METABOLIC TRANSFORMATIONS

There exist several organic and inorganic selenium compounds. Among these, sodium selenite has been extensively investigated as an experimental cancer therapeutic agent. Several studies indicate that selenocystine, is also an interesting candidate with potent anticancer properties. Other compounds such as selenomethionine and Se-methylselenocysteine require enzymatic cleavage to generate redox-active metabolites. The following section describes the metabolism of sodium selenite, selenocystine and Se-methylselenocysteine and their tumor cytotoxic effects in preclinical studies.

1.6.1 Sodium selenite

Sodium selenite (Na_2SeO_3) is a redox-active selenium compound (93). Intracellularly selenite reacts with GSH (major intracellular thiol) to form selenodiglutathione (GS-Se-SG), which is further metabolized into selenide (94, 95) by GSH, cysteine, thioredoxin (TRX), thioredoxin reductases (TrxR) (96), or the glutaredoxin (GLRX) system. Thioredoxin, thioredoxin reductases and GSH pathways rely on NADPH production for sustaining their activity and they are essential to prevent the redox imbalance through the reduction of protein disulfides and glutathione disulfide (94, 96, 97). During the reaction, hydrogen selenide is generated as an intermediate metabolite which can be further metabolized and used for selenoprotein biosynthesis (98). Excess hydrogen selenide redox cycle in the presence of oxygen and thiols leading to a non-stoichiometric consumption of thiols, resulting in the generation of superoxide anion (96, 99, 100) (**Fig. 2**). Superoxide anion is highly reactive and spontaneously oxidizes different biomolecules including proteins and lipids.

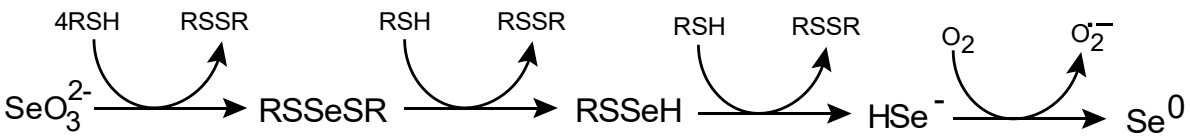

Figure 2: Scheme for the selenite reaction with thiols to generate hydrogen selenide and superoxide anion. Adapted from *Kumar et al., 1992 (96, 101).*

Like any other chemical agents, cellular uptake determines the cytotoxicity of sodium selenite. The influx of selenite anion is really poor (102). However, the reduction of selenite to selenide greatly accelerates the uptake of selenium from selenite (102, 103). Therefore, the extracellular reductive milieu is a key determinant of selenite cytotoxicity (103). While GSH: GSSG redox couple plays a critical role in maintaining the intracellular redox potential, and the extracellular redox potential is mainly regulated by the cysteine:cystine redox couple. In this context, cystine-glutamate exchanger (Gene: *SLC7A11*, Protein name: xCT) plays a critical role by taking up extracellular cystine in the exchange of intracellular glutamate (104). Cystine is reduced intracellularly to cysteine and excess cysteine effluxes out of the cells by multiple transport systems, including MRP (**Fig. 4**). It has been shown that malignant cells overexpress the xCT antiporter and MRP transporters as an adaptive mechanism to survive and detoxify xenobiotics. Their expression is associated with multidrug resistance and poor prognosis in cancer patients (105). If cysteine is in excess in the extracellular milieu, selenite is reduced to selenide by extracellular thiols (103). Selenide is highly permeable and cytotoxic to cancer cells (105). The fraction of selenide that enters the cells reacts with GSH and

follows a similar reaction to generate superoxide, eventually leading to apoptosis or necrosis depending on the strength of the stimuli (96, 100).

Findings from several studies have shown that selenite exerts higher cytotoxicity to cancer cells than normal healthy cells (103, 106) It has also been shown that several drug-resistant cancer cell lines are highly sensitive to selenite treatment compared to their non-sensitive counterparts. It has been shown that sodium selenite exhibit higher cytotoxicity to human malignant mesothelioma, glioma and osteosarcoma cells (107) compared to their nonmalignant counterparts (108, 109). Selenite treatment induces apoptotic cell death in various cancer cell lines such as ovarian cancer (110), lung (111), acute promyelocytic leukemia (APL) (112), breast (113), colorectal (114), mesothelioma (107), liver (115) and prostate cancer (84, 116, 117).

Selenite targets several cellular signaling pathways and triggers oxidative stress (84, 110, 111, 113) as mentioned earlier. Few of the studies are highlighted here. Selenite was shown to induce apoptosis in leukemic cell lines (NB4 cells) via inducing caspase-3 cleavage by activating pro-apoptotic protein BCL2 (112). Selenite at pharmacological concentration induced cytotoxicity by increasing the expression of *cyclin B1, Cdc2, p34* and *p21* and downregulated *PCNA* and *cyclin D1* and cleavage of *PARP* and induce cell cycle arrest in p53 wild-type colorectal cancer cell lines (114) and prostate cancer cell lines (116, 117), while these effects were limited in mutated or p53 null cell lines (114, 116, 117). In another study, prolonged exposure (72 h) of selenite treatment elevated the expression of SODs and modulated the expression of important genes such as AP1 (113), NF-kB and HIF*1α* and leads to apoptotic cell death in a prostate cancer cell line (LNCaP) (84). In the drug-resistant lung cancer cell line (A549), selenite treatment resulted in upregulation of different kinases such as *MAPKs* and *(PI3K)/AKT* pathway, pro-apoptotic protein Bax, death receptor such as Fas*, DR4* and suppressed the expression of anti-apoptotic protein such as Bcl-2, Bcl-xL and pro-caspases -3, -8 and -9. Increased generation of ROS elicited collapse in mitochondrial membrane potential and resulted in the loss of mitochondrial membrane protein (MMP) (111). It was also shown that selenite downregulated the genes responsible for angiogenesis such as HIF and VEGF (118) which are key for cancer metastasis, among others.

1.6.2 Se-methylselenocysteine

Se-methylselenocysteine (MSC) itself is not an active cytotoxic compound. Its metabolic transformation into methyselenol (CH_3SeH) and β-methylselenopyruvate (MSP) was reported

to have cancer-preventive and anti-tumor properties (98, 119-122). MSC is enzymatically metabolized by several enzymes, but KYAT1 (Kynurenine aminotransferase 1) and KYAT3 (Kynurenine aminotransferase 3) play major roles, among others. These enzymes are also known as CCBL1 (Cysteine-S-Conjugate Beta-Lyase 1) (123, 124) and CCBL2 (Cysteine-S-Conjugate Beta-Lyase 2) (125), respectively, because of their dual functionality in transamination and beta-elimination reactions with single substrates (126). KYAT1 metabolizes MSC into CH_3SeH (MS) or β-methylselenopyruvate *via* beta-elimination or transamination pathways, respectively. The present discussion is focused on the metabolic transformation of MSC by the KYAT1 enzyme as there are very few published studies on KYAT3, and the mechanism of action is not yet completely delineated.

KYAT1 is a multifunctional PLP-dependent (pyridoxal 5'-phosphate) enzyme involved in cleaving carbon-sulfur bonds, catalyzing the amino acid substrates into corresponding α-keto acids (127-129). KYAT1 is also shown to cleave carbon-selenium bonds because of the structural similarities between sulfur and selenium (123). In general, the KYAT1 enzyme favors transamination over a beta-elimination reaction. When MSC is used as a substrate, it favors beta-elimination over transamination (123, 124, 130). This might be because of a weaker C-Se bond as compared to the C-S bond (124, 131, 132). KYAT1-mediated cleavage of MSC is shown in **Figure 3**. During transamination reaction, PLP is converted into PMP (pyridoxamine 5'-phosphate). Moreover, the presence of PMP and PLP plays a crucial role in determining the reaction (123, 127, 133). To increase the rate of beta-elimination reaction, the enzyme requires PLP as a co-factor. As a consequence, the addition of α-ketoacid as co-substrates ensures to maintain the PLP-form of the enzyme for an effective and continuous beta-elimination reaction (130), thereby facilitating the generation of methylselenol from MSC (**Fig. 3B)**, which has important implications on cancer cell survival as outlined below.

Methylselenol is known to be highly redox-reactive and cytotoxic to the cells at higher concentrations and antagonizes the growth and survival of cancer cells (120, 134). It is involved in redox signaling, alters the cell signaling pathways and exhibits anti-proliferative and pro-apoptotic properties as shown in different malignant cells (122, 128, 132).

The transamination product of MSC, β-methylselenopyruvate (MSP) structurally resembles butyrate which is a known HDAC inhibitor (119, 135-137). HDAC inhibition triggers apoptosis and cell cycle arrest in cancer cells through chromatin remodeling (137). MSP was also reported to be involved in several biological regulations such as downregulating important genes including *HIF-1α, VEGF,* and *GLUT1*, abrogate transcription of androgen

receptor proteins and inhibit HDAC (128, 138). MSC has been shown to induce apoptosis in several cancer cell lines such as colon cancer (137, 139), prostate (119, 140), osteosarcoma (141), renal cancer (142), fibrosarcoma (122) and head and neck carcinoma (143, 144).

The beta-elimination product of MSC, methylselenol, is highly redox-active and reduces the invasiveness of tumors by inhibiting MMP-2 and induce cell cycle arrest and apoptosis via multiple cell signaling pathways in fibrosarcoma cells (145). It has been shown to upregulate tumor suppressor genes such as *CDKN1C/p57KIP2, HMOX1*, cell adhesion and signaling molecule genes *PECAM1* (inducing Bax-mediated apoptosis), *PPARG* (increases growth inhibition, apoptosis and differentiation of tumor cell populations) in fibrosarcoma cells (145). Methylselenol was also shown to downregulate important tumor progression genes such as *BCL2A1*, *PI3K/AKT*, *HHIP* protein involved in the hedgehog signaling pathway, *Wig1* zinc finger protein which is a p53 target protein, phosphorylate *ERK1/2* signaling and c-Myc oncoprotein in fibrosarcoma, colon and prostate cancer cell lines (122, 145, 146).

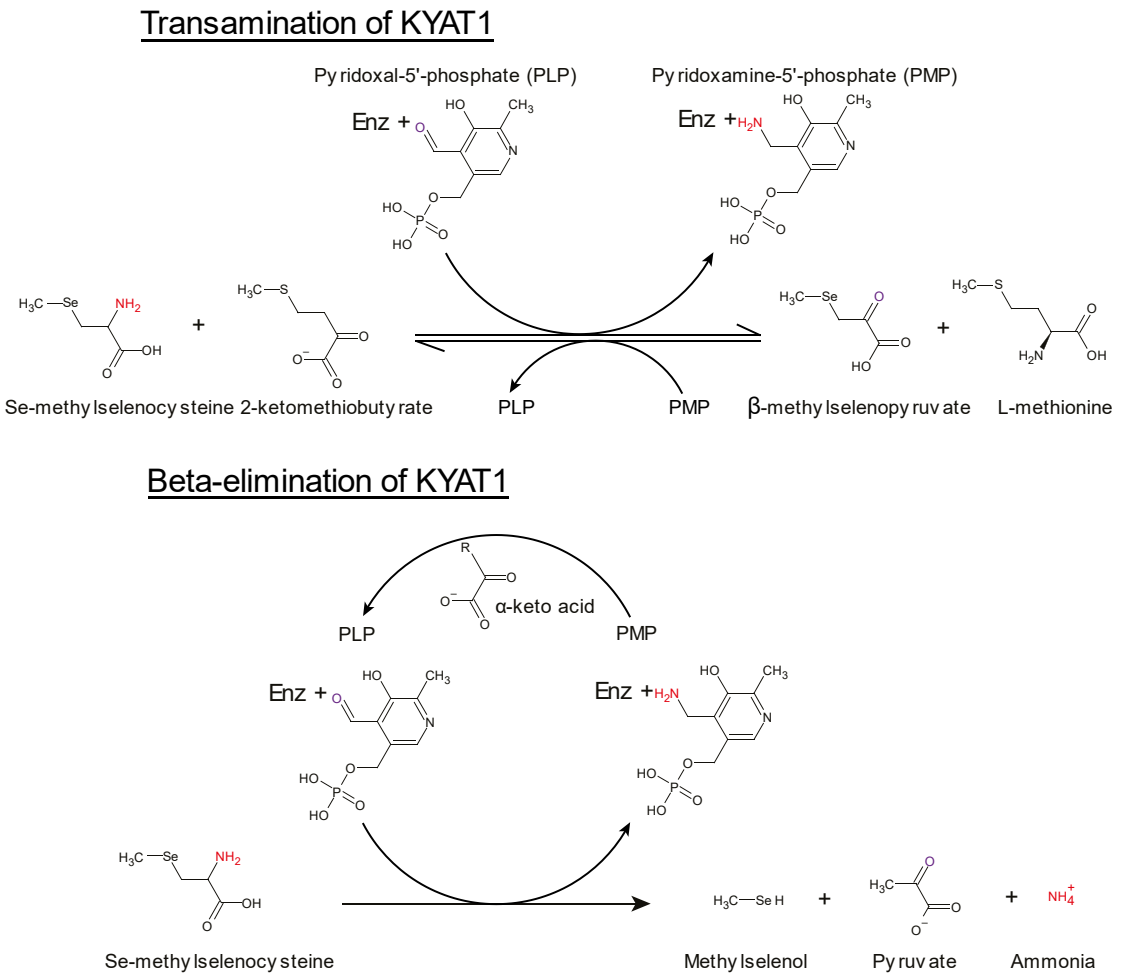

Figure 3: The proposed mechanisms of KYAT1 enzyme in metabolizing (**Top**) se-methylselenocysteine via transamination into β-methylselenopyruvate and L-methionine in the

presence of 2-keto-methiobutyrate as a co-substrate and (**Bottom**) The beta-elimination of se-methylselenocysteine into methylselenol, pyruvate and ammonium. The conversion of PLP to PMP is shown in both the reaction, the addition of α-keto acid enhances the conversion of PMP to PLP for the continuous beta-elimination reaction to happen. Adopted from Selvam *et al.*, 2018 (101).

Whereas MSP treatment resulted in cleavage of caspase -3, -6, -7 and -9 and PARP in a dose-dependent manner and induced the expression of *p21*, which depend on *Sp1/Sp3* site. Deletion of *Sp1/Sp3* abrogated *p21* expression in colorectal cancer and it also altered the expression of *p53* gene (137). Similar effects were also seen in a prostate cancer cell line (119). In the follow-up study, the same group investigated a renal cancer cell line and reported that MSC treatment sensitized the *TRAIL* (Tumor necrosis factor-related apoptosis-inducing ligand)-resistant cancer cell lines by increasing the apoptotic cell death and cell cycle arrest at sub-G1 phase by downregulating *BcL-2*, increased *DEVDase* activity, cleavage of procaspase-3 and *PARP* (142). MSC increased the expression of *Connexin-43* in a dose-dependent manner and induced apoptosis in prostate cancer cell lines (140).

1.6.3 Selenocystine

The selenium analog of cystine, selenocystine (SeC) has potent anti-cancer properties. It is an analog of cystine in which sulfur is replaced with selenium. It is also a redox-active selenium compound that has the potency to oxidize cellular thiols such as cysteine, GSH and homocysteine in the presence of hydroperoxides (147).

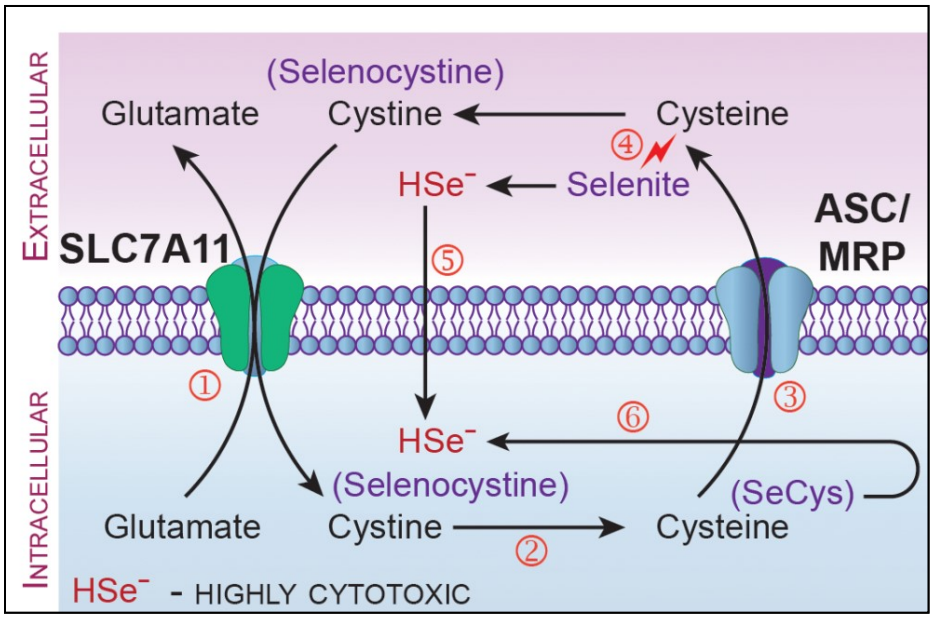

Figure 4: The function of xCT (SLC7A11) antiporter and MRPs in selenite and selenocystine uptake across the cell membrane (Art: Sougat Misra who owns the copyrights of this image).

SeC is believed to be taken up by the same transporters for cystine namely SLC7A11 (103, 148), SLC7A9 (149) and SLC7A13 (150). Among these SLC7A11 is thought to have a major role in SeC transport across the cell membrane (151). Inside the cytoplasm, SeC is reduced by enzymes such as TrxR (152), GR (153) and GLRX1 (100) in the presence of excess NADPH to form selenocysteine. Selenocysteine spontaneously reacts with GSH to form selenocysteine-glutathione selenenyl sulfide (CysSeSG) (154). Further CysSeSG is metabolized into hydrogen selenide by selenocysteine beta-lyase enzyme (SCLY) (154, 155) or by GR in the presence of GSH and NADPH (153, 154). As described earlier, the intermediary metabolite HSe- is highly cytotoxic (96, 100). A schematic representation of selenocystine uptake and metabolism is shown in **figure 4**.

1.7 SELENIUM COMPOUNDS AS ANTI-TUMOR AGENTS: STUDIES ON ANIMAL MODELS

Selenium compounds have been widely used as experimental chemotherapeutics either as single agents or in combinations with commonly used cytostatic drugs to increase the efficacy of the treatment and/or to reduce the side effects caused by the cytostatic drugs. In 1949, Clayton and Baumann showed the anti-proliferative effect of sodium selenite in liver tumors induced by an azo dye in rats (132). Rats fed with 5 ppm of sodium selenite for 4 weeks reduced the tumor burden by 50% in the liver. Another study in 2005 by Björkhem-Bergman et al., showed similar results in a chemically induced hepatocellular carcinoma model in rats (156). Milner and Hsu in 1981 showed the prolonged life span of sodium selenite treated cerebellar deficient folia (CDF) mice transplanted with L1210 leukemic cells into the peritoneal cavity. This study showed that the mice which received 40 µg of selenite/day i.p. for 7 days resulted in 90% cure (157). Watrach et al., 1984 showed the dose-dependent effect of sodium selenite in growth inhibition of human breast cancer cells i.e. MCF7 and MDA-MB 231 cell lines. When these were transplanted into nude mice, treatment with 0,8 µg of Se/g body weight resulted in 80-93% of tumor reduction without any effects to the normal cells (158). Following this, Baldew et al., 1989 showed the chemo-protective role of sodium selenite (2 mg of Se/kg body weight, 1h before cisplatin treatment) in cisplatin-induced nephrotoxicity in BALB/c mice and Wister rats with Prima breast carcinoma and MPC 11 plasmacytoma without reducing the antitumor activity (159).

MSC has been shown to inhibit the growth of prostate cancer cells in xenograft models (160). MSC is efficiently metabolized and excreted as methylated selenium compounds even at a low dose (64 µg/kg in rats) and it was shown that it can be readily accumulated in the pancreas, liver and kidney (161). In a later study, it was shown that 2 ppm of MSC

supplement in rats caused an inhibitory effect on the cancer cell population (98). This study confers that MSC has higher antitumor activity than selenite and selenocysteine (98). Cao et al., 2004 investigated the effect of MSC in combination with different cytostatic drugs *i.e.,* Irinotecan, FU, Oxaliplatin, Taxol, Cisplatin, and Doxorubicin in human colon and head and neck tumor xenograft models in mice. This study comprised both drug-resistant and drug-sensitive, colon (HCT-8 and HT-29) and head and neck (FaDu and A253) cancer models. The cure rate in the combination treatment with MSC was 100% in animals bearing xenografts of the drug-sensitive tumor (FaDu and HCT-8), whereas, in the resistant tumor (A253 and HT-29), the cure rate was 60% and 20% respectively (162). Furthermore, MSC protected against cytostatic drug-induced side effects. In another study, MSC co-treatment with irinotecan in head and neck tumor (FaDu) bearing xenograft mice model unveiled the inhibitory potential of MSC towards HIF-1α and its transcriptional targets VEGF and CAIX, thereby reducing anti-angiogenic activity (143). MSC in combination with cyclophosphamide (CTX), cisplatin, oxaliplatin, and irinotecan reduced the side effects caused by these cytostatic drugs and increased the therapeutic index in synergy in xenograft athymic nude mice and Fischer rat model (163).

As discussed earlier and from the above studies, it is evident that the redox-active selenium compounds target multiple signaling pathways in multiple malignant cells. This appeals to further investigations on these redox-active selenium compounds for further clinical evaluation as potent cancer chemotherapeutic agents either alone or in combination with commonly used cytostatic drugs.

1.8 A SUITABLE MODEL SYSTEM FOR DRUG TESTING

The preference of the experimental model plays a vital role in the field of drug testing to investigate the drug response and efficacy of the drug in killing or attenuating cancer cells. The standard approach for drug testing involves mainly the use of commercially available 2D or 3D cancer cell lines, which is easier for high-throughput drug screening (164). Even though it is easy to work, one major problem in the cell culture model is the uncertainty about the origin of the cell lines, mycoplasma contamination, absence of tumor microenvironment and in some cases changes in phenotype characteristics of the cells which makes the results unreliable or difficult to translate into *in vivo* settings (165).

Animal models in cancer research mainly comprise immune-suppressed mice xenograft models transplanted with human-derived tumor cells or tissues and use of genetically engineered mouse

models such as knockout and knock-in studies (166, 167). However, interspecies discrepancies render these results difficult to translate to humans. Besides mouse is a mouse, not a human, and thus the metabolism and tolerance of study drugs could vary greatly among different species (168). Developing new experimental models that closely resemble a human *in vivo* setting is of utmost importance to overcome these limitations.

1.8.1 *Ex vivo* organotypic tissue slice model

The advantage of the organotypic slice culture model is that it depicts the *in vivo* complexity of the tumor environment with minimal manipulation of the tissue. This technique involves the thin slicing of tissues using krumdieck tissue slicer or vibratome and culturing in optimized culture conditions (164, 169). Short-term exposure of study drugs (24-96h) in the tissues should be sufficient to determine the efficacy of the drug and to select the choice of treatments (170). This technique will help to screen the drug that responds to individual patients. *Ex vivo* slice culture model was successfully used to study the drug response in various cancer types such as breast, liver, lung and prostate (171-173). Our group has recently established an *ex vivo* organotypic slice culture model for human PDAC. We have successfully cultured the sliced tissue (350 µM thickness) for up to four days with good preservation of tissue integrity and viability (174). These features represent the slice culture a state of the art with a low-throughput but high-content experimental platform for drug testing, screening and investigations on drugs that respond to individual PDAC patients (**Fig. 5**). Such personalized treatment methods need to extensively be studied to increase the cancer patient's survival.

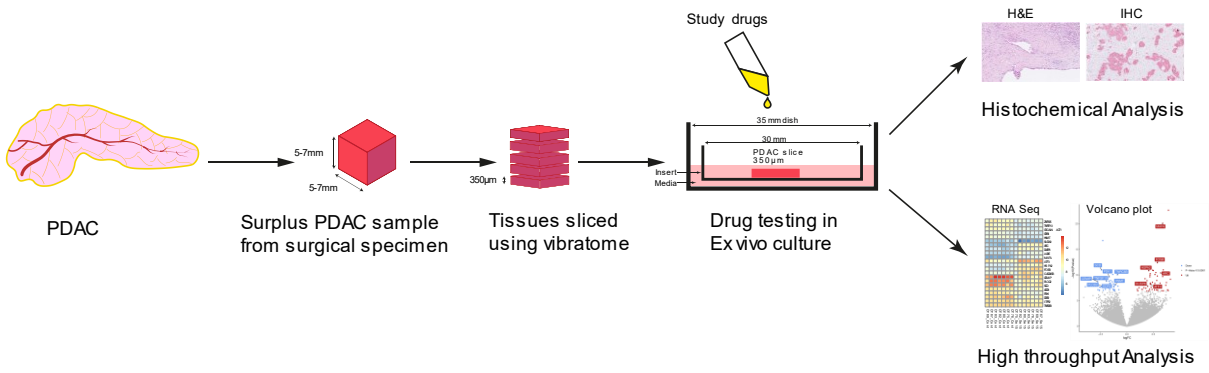

Figure 5 Schematic representation of workflow in PDAC *ex vivo* model of human surgical specimens.

2 RESEARCH AIMS

The overall aim of this thesis was to explore the use of redox-active selenium compounds as a potential anti-cancer agent and to decipher its mechanisms to facilitate its uptake by various methods and models.

The specific aims of each project were as follows:

I. The study aimed to use sodium selenite in combination with ATRA in the treatment of acute promyelocytic leukemia (APL)

II. The study aimed to investigate the cytotoxicity of different selenium compounds alone or in combination with other synthetic and natural compounds that could modulate cellular redox homeostasis and to evaluate if these redox-modulatory compounds could enhance the cytotoxicity of selenium compounds.

III. The study aimed to establish a simple, reliable and reproducible assay to detect the KYAT1 enzyme's beta-elimination product (methylselenol) of MSC.

IV. The study aimed to investigate the cytotoxic effects of MSC in HCC cell lines by selectively inducing KYAT1 expressions via therapeutic mRNA-microRNA techniques and regulate the expression of KYAT enzymes to increase the metabolism of MSC.

V. The study aimed to investigate the feasibility and reproducibility of the *ex vivo* tissue slice culture model for drug sensitivity testing and to test redox-active selenium compounds in human PDAC surgical specimens.

3 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATION

In project II, IV, & V we used human surgical samples. In project II & IV we used human liver hepatocytes cells isolated from Dr. Ewa Ellis's lab, permit nr. 2010/678-31/3, 2017/269-31 and 2016/1309-31, 2019-04866. In project V the tissues samples were collected from surgical specimens of primary PDAC patients resected at Karolinska University Hospital. Written informed consent was obtained from all patients before surgery. The study was approved by the Regional Ethical Review Board, Stockholm (diary number 2012/1657-31/4, 2013-044, 2018/2654-32 and 2019-04866). All study procedures were performed following the relevant guidelines and regulations with the declaration of Helsinki.

3.2 VIABILITY ASSAY

The WST1-proliferation assay kit was used in project I and II, additionally, ATP based CellTiter-Glo® Cell Viability Assay was also adopted to check the variability between two different methods. Other proliferation/cytotoxicity assays were also carried out *i.e.,* Quant™ -iT PicoGreen ds DNA assay kit following manufacturer's instructions, Trypan blue and acid phosphatase assay for validation of cytotoxicity/proliferation data from other assays. We have used ATP based CellTiter-Glo® Cell Viability Assay as a standard method for project IV, because the WST1-proliferation assay interferes with MSC metabolism and we observe an unreliable absorbance.

3.3 COUPLED BETA-ELIMINATION ACTIVITY ASSAY FOR KYAT1

The enzyme KYAT1 metabolizes MSC into methylselenol via β-elimination activity. By a coupled TrxR1-KYAT1 assay, the generation of methylselenol was monitored. This method was optimized for both pure proteins and crude cell extracts. Briefly, 100 µL of reaction mixture contained 100 mM potassium phosphate buffer pH 7.4, 5 mM of Se-methylselenocysteine, 100 µM of dimethyl-2-oxoglutarate, 100 µM of α-keto-γ-(methylthio) butyric acid sodium salt, 10 µM of pyridoxal 5'-phosphate hydrate, 0.4 µg mammalian thioredoxin reductase1 (TrxR1), 400 µM of NADPH and 50 ng of pure KYAT1 protein or 20 µg of protein from whole cell lysate. The reaction mixture was pre-incubated for 5 min at 37 °C before adding TrxR1 and NADPH. Continuous measurement of NADPH consumption was recorded at 340 nm for every 30s using a spectrophotometer. The assay mixture without TrxR1 was used as blank. Under this condition, the extinction coefficient was 6220 M^{-1} cm^{-1} for NADPH.

3.4 LIPID NANOPARTICLE TRANSFECTION

Cells were seeded at a different seeding density for each cell line (depending on their doubling time). The next day LNP encapsulated mRNA+/-miRNA were diluted with EMEM

medium with required concentration and equilibrated for 30 min. Media from cell culture were aspirated and replaced with media containing LNPs and incubated for 16-24 h and treated with respective compounds by changing the media containing the study compounds.

3.5 *EX VIVO* ORGANOTYPIC SLICE CULTURE MODEL

Fresh surgical PDAC primary samples were sliced into 350µM thick slices using a vibratome. The first cut slice was immediately fixed and embedded in paraffin for 0 h control. Subsequently sliced PDAC rested on an insert placed in a culture dish containing optimized culture media. The next day the culture media was replaced with media containing the study drug. Tissues slices were harvested after 48 h of treatment for further evaluation.

Most of the methods used in this thesis were systematically explained in the published papers and manuscripts. For detailed information about the specific methods, please read the method section in each paper.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Selenite promotes all-trans retinoic acid-induced maturation of acute promyelocytic leukemia cells.

Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML), which is identified by a specific t (15;17) chromosomal translocation that encodes a fusion of promyelocytic leukemia (PML) and retinoic acid receptor α (RARα) protein. PML/ RARα fusion results in abnormalities in the proliferation/ differentiation coupling of hematopoietic stem cells at early developmental stages and prevent apoptosis of promyelocytes and block the normal granulocytic differentiation (175, 176). PML/ RARα shares common DNA binding sites with RARα and PU.1, both of which are important transcription factors for myeloid and lymphoid cell differentiation (177, 178).

In recent years, APL has become a highly curable disease with complete remission (CR) in almost all patients (175, 176, 179). In the late 80's, the efficacy of all-*trans* retinoic acid (ATRA) in the treatment of APL patients was published. ATRA alone resulted in 85% CR (180). Further in the 90's, arsenic trioxide (ATO) was added to the treatment regime. This combination had a major effect on APL treatment *i.e.,* it accounts for about 80% to 95% CR (175, 180, 181). ATRA induces differentiation of APL cells and arrests the growth of leukemia-initiating cells (LIC) (182) while ATO leads to the degradation of RARα by binding to PML/ RAR α oncoprotein and induce apoptosis to leukemic promyelocytes. It also down-regulates the Bcl-2 gene, which is an integral intercellular membrane protein that suppresses programmed cell death (176, 181). The major problem in using this combination is that ATO is a carcinogen that mainly causes skin cancer, increased activity of cytochrome P-450, upregulation of multidrug resistance protein 1 (MDR1) and inhibition of thioredoxin reductase. A high dosage of ATRA often leads to ATRA syndrome associated with elevated WBC count. Our results demonstrate that replacing selenite instead of ATO in combination with ATRA increased the differentiation of NB4 cells (APL). This was evident by high levels of CD11b expression at both mRNA and protein levels. Flow cytometry data revealed that the combination treatment significantly increased the level of HLA-DR and CD68 (monocytes/macrophage marker), CD62L and CD16 (neutrophil lineage marker) in CD11b+ cells. This data strongly indicates that the NB4 cells underwent differentiation in the combination treatments. Targeted degradation of PML-RARα represents an established mechanism of complete remission in APL. Herein, we have shown a similar mechanism of action by sodium selenite, a redox-active selenium compound with inherent catalytic capacity to participate in thiol/disulfide interaction reactions involved in the removal of zinc from zinc/thiolated coordination sites (183). Experimental evidence on selenite-mediated inhibition of DNA binding activity of zinc finger (184) transcription factor SP1 and release of zinc are congruent with our data. Selenite in combination with ATRA induced important transcription

factors PU.1 and FOXO3A, and the redox-regulatory enzymes such as Grx1, Grx2, TrxR1, Trx, TrxR2 and Trx2.

Taken together with the above results, it is evident that selenium at physiological concentration showed a potent cytotoxic effect in APL cells when combined with a low dose of ATRA it increased the differentiation potential of APL cells.

4.2 PAPER II

Microenvironment redox control by SLC7A11 is a druggable target in cancer.

Selenite is known to be toxic to various types of cancer cells (112, 185), with autophagy/apoptosis being the major cell death pathways (186). Selenite induces pro-apoptotic activity by its ability to consume cellular GSH and induce oxidative stress (187). Selenite-induced oxidative stress is the result of converting GSH to GSSG (oxidized glutathione) accomplished by generating selenodiglutathione and superoxide anions i.e. ROS. Cancer cells that are resistant to certain cytostatic drugs are often sensitive to selenite. Previous investigations from our research group have shown that the levels of extracellular thiols correlate with susceptibility to selenite treatment (91). In 2009 our group showed that the cystine/glutamate transporter (xCT) renders cancer cells to be highly susceptible for selenite cytotoxicity due to the facilitation of extracellular reduction by cysteine (103). Increased cellular uptake and cytotoxicity of redox-active selenium compounds, such as selenite and selenocystine, is regulated by the xCT antiporter and ASC/MRP transport systems. The activation of the cystine/glutamate transporter (xCT) renders cancer cells to be highly susceptible to selenite toxicity (103). xCT transporter expression has been increasingly connected to tumor growth and drug resistance and is regulated by the antioxidant response element (ARE). This transporter is essential for the uptake of cystine required for the synthesis of intracellular GSH (188). NFE2L2 controls different antioxidant pathways such as production, regeneration and utilization of GSH, the thioredoxin pathway, NADPH production and quinone detoxification and it is a direct regulator of xCT (97). The uptake of hydrogen selenide is more rapid than that of selenite itself. Thereby, if the expression of xCT (cystine/glutamate exchange) can be enhanced, it facilitates the production of more HSe- from sodium selenite. Concomitantly, the uptake of HSe- by the cancer cells can be dramatically increased and induce cell death.

This study focuses on two key selenium compounds, sodium selenite and selenocystine. The cytotoxicity of these compounds was evaluated in more than 20 different cancer cell lines. Our results showed that sodium selenite was more cytotoxic than selenocystine. Based on the earlier findings that extracellular redox potential imparts a key role in the cytotoxicity and the plausible roles of xCT antiporter in maintaining extracellular redox potential, we screened different natural and synthetic compounds that could alter the extracellular redox potential into a more reductive milieu. Diphenyl diselenide is highly effective in altering the extracellular redox potential by increasing the expression of *SLC7A11*, the gene encoding for xCT. Selenite and selenocystine in combination with diphenyl diselenide, showed a several-

fold cytotoxic effect in most of the tested cancer cell lines. When we interrogated the mechanism behind the observed effect, we found out that diphenyl diselenide increased the activities of transcription factors NRF2 and ATF4, both of which directly regulate the expression of *SLC7A11*. Several redox regulatory genes were also overexpressed following treatment with diphenyl diselenide. When we tested the cytotoxicity of these selenium compounds as a single agent or in combination with diphenyl diselenide in normal cells (primary human hepatocytes and peripheral blood mononuclear cells), potentiation of cytotoxicity was minimal or absent.

4.3 PROJECT III

A Novel Assay Method to Determine the β-Elimination of Se-Methylselenocysteine to Monomethylselenol by Kynurenine Aminotransferase 1.

Kynurenine aminotransferase 1 (KYAT1) is a multifunctional PLP-dependent enzyme involved in cleaving carbon-sulfur bonds, which transforms the amino acid substrates into corresponding alpha-keto acids and vice-versa. It is involved in transamination and beta-elimination activity with a single substrate. The presence of pyridoxamine-5'-phosphate (PMP) and pyridoxal 5'-phosphate (PLP) determine the nature of its pathway either transamination or beta-elimination (123, 127, 133). KYAT1 is also shown to cleave the carbon-selenium bond, because of its weaker bond compared to the C-S bond (131, 132). KYAT1 plays a major role in Se-methylselenocysteine (MSC) metabolism. KYAT1 converts MSC into methylselenol (MS) via β-elimination activity. MS is highly volatile, redox-active, and has great potency in inducing ROS in cancer cells, their use in cancer chemotherapeutics is being extensively investigated by several research groups. MS is highly unstable, so it is difficult to detect during the reaction. Only a few assays are described, but they are not reliable and reproducible, because they measure pyruvate a metabolite that is produced during the β-elimination reaction. We developed a novel and simple assay that can monitor the production of MS generation in a continuous assay. MS is an excellent substrate for thioredoxin reductase 1 (TrxR1). KYAT1 generates MS and this can be utilized as a substrate for TrxR1, why we combined these two enzymes and detected the β-elimination product using NADPH oxidation spectrophotometrically. The calculated apparent K_m and V_{max} were 5.84 ± 0.95 mM and 1.12 ± 0.08 nmol/min respectively, the turnover number (K_{cat}/K_m) was 27.4 mM^{-1} min^{-1} for the above enzyme reaction. We used KYAT1 overexpressed cells, several inducers and inhibitors of the KYAT1 enzyme to validate the reproducibility of this enzyme assay.

4.4 PROJECT IV

Targeted enzyme assisted chemotherapy (TEAC) – a novel microRNA-guided and selenium-based regimen to specifically eradicate hepatocellular carcinoma

Se-methylselenocysteine (MSC) is a pro-drug which is relatively non-toxic to cancer cells at low concentrations compared to other selenium compounds e.g. selenite. MSC cytotoxicity in

cancer cells is based on the metabolic enzyme that cleaves MSC into its active products either β-methylselenopyruvate or methylselenol. Both the metabolites of MSC have chemotherapeutic and chemopreventive properties. β-methylselenopyruvate act as an HDAC inhibitor and induce apoptosis in cancer cells. The other metabolite MS is highly cytotoxic and has the potency to generate an ample amount of ROS by oxidizing thiols. We initially investigated the sensitivity of MSC towards different hepatocellular carcinoma cell lines. Results from the cytotoxicity experiments showed similar cytotoxicity in all the tested HCC cell lines and human primary hepatocytes. The concentrations of MSC required to achieve half-maximal growth inhibition in these cells were too high. We posited that the overexpression of KYAT1, an enzyme that converts MSC into its toxic intermediate methylselenol, could further potentiate the cytotoxicity of MSC. To achieve this, we used a KYAT1 overexpression plasmid and LNP (lipid nanoparticle)-encapsulated KYAT1mRNA, as two different approaches to increase the expression of KYAT1 in HCC cell lines. Both these approaches successfully increased the expression of active KYAT1 as shown by enzyme assay. When HEPG2 and Hep3B cells were transfected with KYAT1-LNP, we found MSC cytotoxicity was potentiated in both the cell lines. However, the required dose level to induce cytotoxic effects was still too high. Since KYAT1 possesses both transamination and beta-elimination activity, we hypothesized that selective inhibition of transamination activity may increase the beta-elimination activity, and thereby MSC would be more cytotoxic due to increased generation of methylselenol. We screened several KYAT1 inducers and inhibitors using transamination and β-elimination activity assays. When these compounds were used along with MSC in KYAT1-overexpressed HCC cells, we observed a different cytotoxicity profile with different modifiers. AOAA, an inhibitor of KYAT1, apparently inhibits the enzyme, which was reflected by complete protection from MSC cytotoxicity in HCC cell lines. All α-keto acid analogs that we have used showed a certain degree of sensitization, but individual compounds behaved differently between the cell lines. Of those, PPA, KMB and IPA showed sensitization towards MSC in all three HCC cell lines.

When KYAT1mRNA was tagged with HCC specific microRNA (miR122) we observed a cancer cell-specific MSC cytotoxicity. MicroRNA122 is known to be widely expressed in normal hepatocytes whilst downregulated in HCC. The liver-specific microRNA miR122 accounts for about 70% of total microRNAs in the liver and plays an important role in liver metabolism and hepatocyte differentiation. Multiple (3X) sequence of antisense miR122 was added at the 3'end of KYAT1mRNA and transfected into HCC cell lines. The cell with high miR122 expression (Huh7) had a protective effect with MSC cytotoxicity, on the other hand, cells with minimal miR122 expression became more sensitive to MSC treatment. The overall concept is that miR122 antisense elements bind endogenous miR122 and prevents the translation of KYAT1mRNA into protein, while cells with minimal or no miR122 translate KYATmRNA into protein thereby sensitizing the cells to MSC cytotoxicity. Our results indicate that miRNA antisense targets are efficient in achieving tumor-specific cytotoxicity in cell types where miR122 are modulated.

4.5 PROJECT V

Redox-active selenium compounds are superior chemotherapeutics to pancreas cancer in an *ex vivo* model of human surgical specimens

Redox-active selenium compounds were shown to have potent anti-tumor and chemopreventive properties by several preclinical studies. These studies are mostly based on cell culture, 2D or 3D models, Knock-out or Knock-in mouse models and patient-derived xenograft models. The major limitations in these models are tumor microenvironment and species differences. New drug testing methods that mimic the *in vivo* settings are in need to test the study drug with all the cancer tissue components including the tumor microenvironment. In 2019 our group published a unique *ex vivo* organotypic culture model for PDAC (174). In this study, we adopted an *ex vivo* organotypic slice culture model to test the efficacy of sodium selenite and se-methylselenocysteine (MSC) in pancreatic adenocarcinoma surgical specimens.

Pancreatic cancer (mainly PDAC) has a poor prognosis because of late diagnosis. The difficult part in treating pancreatic cancer is because of the stroma which can scavenge the cytotoxic drug and protect cancer cells from drug-induced effects. Our results showed that selenite was more potent than MSC in PDAC culture. Selenite showed prominent cancer clearance at the dose lower than the published MTD for humans (92) with intact stromal components. Selenite and MSC showed a dose-dependent response in all nine investigated PDAC cases. MSC in combination with α-ketoacid (IPA) increased the efficacy of MSC cytotoxicity. Our transcriptomic data for selenite revealed that it targeted multiple pathways that are important for tumor cell growth, invasion and progression. This *ex vivo* model creates a new regime of personalized medicine that mimics *in vivo* system to evaluate drug response for individual PDAC patients in a short time. This kind of clinical setting is important to the prognosis for patients with PDAC.

5 CONCLUSIONS

In this thesis work, we focused on highlighting the usefulness of redox-active selenium compounds in cancer chemotherapeutics. Our preclinical studies with different approaches helped us to understand the metabolism and nature of different redox-active selenium compounds. In my first study, we showed that selenite is a potent redox-active compound in destabilizing zinc-finger oncogenic PML/RARα protein thereby increases the oncoprotein degradation, in combination with ATRA it induced transcription factors responsible for the cell differentiation. In my second project, we have identified several small-molecule pharmacological compounds that increase the metabolism and uptake of selenium compounds, thereby increasing cancer eliminating efficacy. A compound such as diphenyl diselenide in combination with redox-active selenium compounds showed a remarkable cytotoxic effect in most of the cancer cell lines without affecting normal cells *in vitro*. Our vision to introduce a simple and reliable assay to detect methylselenol was also accomplished in this thesis. In my third study, We have coupled two enzymes (TrxR1 and KYAT1) system to detect methylselenol (MS) generation via spectrophotometry. TrxR1 uses MS as a substrate and these were monitored by the oxidation of NADPH to $NADP^+$.

In my fourth project, we have employed an LNP-encapsulated therapeutic mRNA-microRNA-based genetic regulation approach for targeted-therapy. Our result demonstrated the precise targeting of MSC cytotoxicity to liver cancer cells without affecting normal hepatocytes *in vitro*. Another advantage in using this system with MSC is that KYAT1 is a harmless metabolic enzyme so any off-target effects would be less serious, while induction of cell death protein might cause deleterious effect even with negligible leakage in the systems. The addition of α-ketoacid in combination with MSC increased the efficacy of MSC metabolism and related cytotoxicity in HCC cell lines. Finally, we showed the usefulness and effectiveness of the *ex vivo* PDAC slice culture organotypic model in drug testing, mainly selenium compounds. Our transcriptomic data showed substantial evidence to support the multi-pathway targeting potential of selenium compounds in the pancreatic cancer *ex vivo* model. Selenite at a concentration below published MTD showed remarkable efficacy in eradicating cancer cells with the highly preserved stromal component.

Our experimental data highlighted different aspects of redox-active selenium compounds and, to bring this simple and inexpensive drug into clinical practice. Further systematic investigations are needed to understand the pharmacology and clinical applicability of these compounds in cancer treatments.

6 FUTURE PERSPECTIVE

My thesis work tried to answer several research questions in using redox-active selenium compounds in cancer chemotherapeutics with targeted and personalized therapy using various methods and a unique *ex vivo* PDAC model. Project IV can be extended to various cancer types by identifying cancer-specific microRNAs and antigens. Cancer-specific antibodies can be incorporated on the surface of the LNPs to target only cancer cells without any off-target effect on normal cells, furthermore, the inclusion of disease-specific microRNA can increase the efficacy of targeted therapy. A site-directed mutagenesis approach in KYAT1 will be applied to target the sequence responsible for enhancing the β-elimination activity and suppress the transamination activity. This will benefit the production of highly cytotoxic metabolite methylselenol. By combining the above microRNA and antibody techniques along with mutated KYAT1, we can achieve high precision in targeting cancer cells with no or minimal effect to normal cells with MSC.

In project V, our readout mainly focuses on the tissue outgrowth, but there are PDAC samples that did not show any outgrowth, but the tissue slice is viable and preserved during incubation. So, further advanced techniques such as tumor-specific markers or some other high throughput techniques should be combined to increase the effectiveness of this model for routine robust evaluation of drug responses. The concept from project IV will be employed in the PDAC *ex vivo* model to validate our targeted therapy strategy. We are also investigating the feasibility to extend our *ex vivo* slice culture model to another cancer type such as hepatocellular carcinoma (HCC). Finally, comparing the drug response from the *ex vivo* model to *in vivo* model is also necessary to understand the effectiveness of this model and for further clinical trial evaluation. Our group has already done a clinical phase I trial "SECAR" to evaluate the cytotoxicity and pharmacokinetics of sodium selenite in end-stage cancer patients. Our experience from a previous clinical trial and the above results encourage us to follow-up with our second clinical trial "SEACAT", to evaluate the MSC cytotoxicity and the feasibility of delivering therapeutic mRNA-microRNA (Wt or mutant KYAT1 with miR122) via LNPs in liver compromised cancer patients. This approach might open up a new venture for personalized medicine with redox-active selenium compounds alone or in combination with small-molecule pharmacological compounds.

7 FUNDING SOURCES

This thesis work was financially supported by;

Cancerfonden (The Swedish Cancer Society), Cancer och Allergifonden, Radiumhemmets forskningsfonder, Moderna Therapeutics, The Jochnick Foundation and Stockholm Country Council (ALF).

8 ACKNOWLEDGEMENTS

After moving to Sweden for my studies, I went through several changes in my life, and most of it happened during my PhD journey at **Karolinska Institutet**. With each step it has made me stronger and stronger, I would like to thank everyone who has shaped my life from a spoiled child to a responsible person. When I was young, I wanted to be a space scientist and told my parents that I wanted to be like **Dr. A.P.J Abdul Kalam**. But when I grew up, my focus changed to biological sciences because of the motivation I received from my biology teacher. And now I am so grateful to my biology teachers **Uma** and **Alamelu** mam. In my PhD journey, I have learned many things from each and everyone I have met, and I am so grateful to everyone who has supported me in my scientific development and to some who have taught me a life lesson that has made it all worthwhile for me.

I am grateful to my main supervisor **Mikael Björnstedt** for giving me the opportunity to perform my PhD studies in your research group. You supported and motivated me in all difficult situations. Your commitment towards the selenium field and your family always inspired me, and your decision-making ability in all challenging times was an opportunity for me to learn for life while working with you. Thank you for the many fruitful scientific discussions and the many new innovative instant ideas during our group meetings. I am very grateful for your great support towards the end of my PhD career, without you it would have been impossible for me. Your openness towards the group members is amazing and commendable.

To my co-supervisor **Sougat Misra,** I am grateful for your support throughout my career and for teaching me most things from scratch. I still remember the first day I met you and the in-depth scientific discussion we had with a beer at your apartment, you are always full of new ideas and enthusiasm. Your dedication to science is always inspiring. I thank you for your valuable advice, intense scientific discussions and for introducing me to tennis. You are always an inspiring person to those around you.

Lena Hernberg, thank you for helping us order things for our group. What would we do without you. Thank you for all the kindness, help and support.

Ali Razaghi, thank you for your help and for sharing your knowledge during our group meetings. I would like to thank our former group members **Rim Jawad** and **Antje Zickler**

for their kindness, **Marita Wallenberg Lundgren** for your wonderful smile and positivity, **Lisa Arodin Selenius** for sharing knowledge and interesting discussions.

I would like to thank **Carlos Fernández Moro** from clinical pathology for sharing his valuable knowledge during our group meetings. Thank you **Soledad Pouso Elduayen** for your help and caring towards everyone, you are an amazing person.

I would like to thank my collaborators **Gilbert Lauter**, **Julian E. Spallholz**, **Mallory Boylan** for the productive collaboration, **Mehran Ghaderi** for helping me with transcriptomics, **Christina Hebert** for HPLC analysis **Isabelle Magalhaes** and **Aditya Ambati** for teaching me flow cytometry, **Gábor Lendvai** and **András Kiss** for sharing your knowledge about microRNA and for the fruitful collaboration, **Adnane Achour, Hugh Salter** and **Tatiana Sandalova** for the intense scientific discussion and collaboration.

I am also very grateful to the head of the pathology department, **Göran Andersson**, for making the pathology department a pleasant working environment for everyone. Department administrator **Mia Bjerke** for helping anyone in need. I would like to thank all the project leaders in the pathology department for their role in my PhD career: **Stephen Strom**, **Roberto Gramignoli**, **Katalin Dobra**, **Jonas Fuxe, Thomas Näreoja, Birgitta Sander, Jaakko Patrakka and Joakim Dillner.**

I would like to thank the former and present PhD student administrator **Arja Kramsu** and **Ann Mellquist** for their help during my PhD journey, **Matti Sällberg** and **Anthony Wright**, who have supported me throughout my PhD studies, and the service team **Rasmus Larsson** and **Mikael Hammarstedt**, who have made ANA Futura a pleasant working environment for all and have taken care of the delivery.

I would like to thank all the past and present members of the Pathology Division who have made the environment very competitive and enjoyable: **Anja, Anh, Agata, Martin, Christina. P, Laia, Ashish, Francesco, Loffe, Ghazal, Tunde, Rita, Kristina, Zurab** and **Massoud** for your scientific discussions during the pathology seminars, and the current members **Magali**, a new Dr from our department, **Mihaela** who is always gentle and kind, **Suchita** who is always cheerful and spreads happiness, **Joman** who is always friendly and helpful, **Christina Hammarstedt**, who is always helpful in the animal house and enjoyed our little chats during the breaks. **Gosia, Maria H, Nikolina, Dina, Emmanuella, Gizem,**

Mohammad Morsy, Janne and **Tahira** for their support and great scientific discussions during the seminars. I would like to thank all the teachers at Pathology Division **Annica, Anders R, Barbro, Gareth, Jolly, Rosita, Ulla, Pernilla and Sara W.**

I would like to thank my German friends, prof. Dr. **Volkmar Braun,** Dr. **Avijit Pramanik, Uwe, Louis,** Dr. **Barabara Di Ventura** and **Anjum Ara** for your contribution to my scientific career.

I am very happy to have met this wonderful Swedish family in Stockholm **Sara Yoon** and **Hans Ove Larsson**. Thank you for being positive, motivating, supportive and taking care of me like your own son. When I am with you, I have never missed my family and whenever I am down my feet always find their way to your home. Without both of you, it would have been very hard for me. We made many memories together; the ski trip and the Calabria trip were some of the best trips of my life. The food you make with love is always the best, especially the kimchi, it's just wow. **Henrik Persson** another family from Mariestad, my first teacher in Sweden since 2011, you taught me everything from cleaning to building the own house like a Swede, except Swedish (I was a lazy guy to learn the language but you tried hard and still trying). You are one of the most important people in my life because you turned me from a spoiled kid to a mature and responsible guy. Thank you so much for showing love and taking care of me like your own son.

To my dear friend and partner in crime **Raghu (Raghuram Chittoor Srinivasan),** without you, I would have been super bored, and I feel that very strongly now in your absence. You as a person are very positive and charming but get drunk very quickly. We have known each other for more than fourteen years but the best time was from 2014 to 2018. We did all the good and bad things together because we always had a similar ideology and future perspective. And that's what made us best friends, thank you for making all the times memorable. **Hanna**, another sweet person I have met. I love the fact that you have not changed even a little bit since we first met. Thank you for being the same cheerful and caring friend. I can never forget Raghu's bachelor party at your house which will remain one of the funniest memories. Thanks for sharing your knowledge, also for introducing me to different cuisines and for your books and gifts. **Micheal Kann**, it's always nice to meet you and Hats-off to you for bearing with Hanna.

I am very happy and grateful to have met my Flemingsberg friends **Ram, Kala** and their adorable son **Rakshan**, you are like an extended family away from home, always helpful

and friendly. **Sharan** and **Sujatha**, thank you for the fun game nights and the great outings we had together. **Roshan** and **Pooja** (newest member-welcome) thank you for the good times, North Indian food and your bicycle riding skills. **Madhurya**, thank you for being such a sweet little happy person.

I am thankful to the Almighty for so many things in life, but I have never thanked you for all that you both have done for me, **A.R Selvam (Appa)** and **Vijayalakshmi (Amma)**. The support and faith you both have put in me have brought me this far. Thank you to my very first friend, my elder brother **Manivasagam** for loving and motivating me and **Kavitha (Anni)** for the care. Also, I am very happy to welcome the newest member of Selvam Clan **Dharrshan**, my nephew. A very special thank you to my late grandfather **Arunachalam Nadar**, whose footsteps I have followed, who was a man of his words, you will always live in me. I would also like to thank my relatives and extended family for their unconditional love and support.

Finally, I would like to thank the love of life, my wife **Anjali Bhaskar**, for your motivation and trust in me, just thanks are not enough for you my dear. You always stayed by my side through the hard times (which mostly happened because of you) and pampered me, and you always trusted my decision and supported me till here. Even though we have only lived together for a short time, we have learned so many things together and enjoyed most of them, even the arguments we have are always a pleasant moment for me. We have traveled a lot in this short time and expect a lot more in the future. You are always the best. I could not get anyone better than you, thank you for everything you have done for me

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