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SELENOCOMPOUNDS IN LEUKEMIA TREATMENT: ADVANTAGES AND PITFALLS

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Front cover shows selenocompound (the moon) attacking leukemia cells.
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Selenocompounds in Leukemia Treatment: Advantages and Pitfalls

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To all faithful researchers

To my beloved families

献予所有诚挚的研究者以及我的家人

ABSTRACT

In 2018, cancer was reported to be the second top cause of morbidity and mortality globally with over 9 million deaths. Hematological malignancies including leukemia constitute about 7% of the total cancer cases. Substantial developments in treatment modalities and strategies have increased the 5-year survival rate in leukemia to around 60% in developed countries. However, complete remission and long-term disease control are not yet achieved. Oxidative stress is imprinted in many types of cancer including leukemia and represents a valuable trait for achieving leukemia-selective cytotoxicity. The present thesis represents a systematic study of the role of redox-active selenocompounds (SeCs) in leukemia treatment.

Six diverse selenocompounds representing different compound classes were studied. Among them, *p*-xyelenselenocyanate (*p*-XSC) was shown to have the most potent cytotoxic activity against several leukemia cell lines carrying distinct oncogenes. *p*-XSC exerted its cytotoxicity in a concentration- and time-dependent manner. Mechanistic studies revealed that the cytotoxicity of *p*-XSC was mediated by upregulation of oxidative stress and accompanied with massive mitochondria damages. Importantly, the cytotoxicity of SeCs was antagonized by albumin which is ubiquitously present in biological conditions. By the combination of two distinct, but complementary selenium speciation methods including liquid chromatography-mass spectrometry and X-ray absorption spectroscopy, we showed that cytotoxic SeCs were capable of transforming into selenol intermediates that subsequently bound to albumin via selenium-sulfur bond. Furthermore, we found that the macromolecular selenocompound-albumin conjugate was also internalized and able to kill leukemia cells.

In addition to interfering with cytotoxicity, binding of SeCs to albumin also hindered the quantification of these compounds in biological matrix e.g. plasma. To elucidate the pharmacokinetics properties of SeCs for *in vivo* applications, we developed a novel REductive Cleavage and Instant Derivatization (RECID) method, by which we were able to measure both free and albumin-bound SeCs. In the leukemia mouse model, intravenous administration of *p*-XSC was shown to reduce the disease burden in whole body as well as in bone marrow.

In conclusion, the results obtained in the present thesis provide substantial experimental evidences that redox-active SeCs, in particular *p*-XSC, possess high therapeutic potential as treatment for leukemia. Further investigations to optimize treatment regimen and to design an appropriate drug carrier are needed to achieve successful clinical trials.

LIST OF SCIENTIFIC PAPERS

I. **Wenyi Zheng#**, Ying Zhao#, Rui He, Dhanu Gupta, Samir El-Andaloussi, Manuchehr Abedi-Valugerdi, Manuel Valiente, Moustapha Hassan. Utilization of redox-active selenocompound for leukemia treatment in vitro and in vivo. (Manuscript)

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II. **Wenyi Zheng**, Fadwa Benkessou, Brigitte Twelkmeyer, Siyao Wang, Tobias Ginman, Håkan Ottosson, Manuchehr Abedi-Valugerdi, Maria Angels Subirana, Ying Zhao, and Moustapha Hassan. Rapid and robust quantification of *p*-xyleneselenocyanate in plasma via derivatization. *Analytical Chemistry*. 2017, 89: 7586-7592.

III. **Wenyi Zheng**, Rui He, Roberto Boada, Maria Angels Subirana, Tobias Ginman, Håkan Ottosson, Manuel Valiente, Moustapha Hassan. A general covalent binding model between cytotoxic selenocompounds and albumin revealed by mass spectrometry and X-ray absorption spectroscopy. *Scientific Reports*. (Accepted).

IV. **Wenyi Zheng**, Roberto Boada, Rui He, Tingting Xiao, Fei Ye, Laura Simonelli, Manuel Valiente, Ying Zhao Moustapha Hassan. Extracellular albumin covalently sequesters selenocompounds and determines cytotoxicity. *International Journal of Molecular Sciences*. 2019, 20: 4734-4746.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. Amin R, He R, Gupta D, **Zheng W**, Burmakin M, Mohammad DK, DePierre JW, Sadeghi B, Olauson H, Wernerson A, El-Andaloussi S, Hassan M, Abedi-Valugerdi M.. The kidney injury caused by the onset of acute graft-versus-host disease is associated with down-regulation of αKlotho. *Int Immunopharmacol*. 2020, 78: 106042.

II. He R, **Zheng W**, Ginman T, Ottosson H, Norgren S, Zhao Y, Hassan M. Pharmacokinetic profile of N-acetylcysteine amide and its main metabolite in mice using new analytical method. *Eur J Pharm Sci.* 2019,143: 105158.

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IV. Nakamura M, Zhang Y, Yang Y, Sonmez C, **Zheng W**, Huang G, Seki T, Iwamoto H, Ding B, Yin L, Foukakis T, Hatschek T, Li X, Hosaka K, Li J, Yu G, Wang X, Liu Y, Cao Y. Off-tumor targets compromise antiangiogenic drug sensitivity by inducing kidney erythropoietin production. *Proc Natl Acad Sci U S A*. 2017, 114(45): E9635-E9644.

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VI. Abedi-Valugerdi M, **Zheng W**, Benkessou F, Zhao Y, Hassan M. Differential effects of low-dose fludarabine or 5-fluorouracil on the tumor growth and myeloid derived immunosuppression status of tumor-bearing mice. *Int Immunopharmacol*. 2017, 47: 173-181.

VII. Abedi-Valugerdi M, Wolfsberger J, Pillai PR, **Zheng W**, Sadeghi B, Zhao Y, Hassan M. Suppressive effects of low-dose 5-fluorouracil, busulfan or treosulfan on the expansion of circulatory neutrophils and myeloid derived immunosuppressor cells in tumor-bearing mice. *Int Immunopharmacol*. 2016, 40: 41-49.

CONTENTS

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
BLI	Bioluminescence imaging
Cys	Cysteine
$CysSe_2$	Selenocystine
DMEM	Dulbecco's modified eagle medium
ESI-MS	Electrospray ionization-mass spectrometry
EXAFS	Extended X-ray absorption fine structure
FBS	Fetal bovine serum
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HP	Human plasma
LC-MS	Liquid chromatography-mass spectrometry
MeSeA	Methylseleninic acid
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NMR	Nuclear magnetic resonance
p-XSC	*p*-Xyleneselenocyanate
p-XSC-FBS	Mixture between *p*-xyleneselenocyanate and fetal bovine serum
p-XSC-SM	Conjugate between *p*-xyleneselenocyanate and small molecule thiol
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
S	Sulfur
Se	Selenium
SeC	Selenocompound
SeCys	Selenocysteine
SeC-albumin	Conjugate between selenocompound and albumin
SeC-BSA	Conjugate between selenocompound and bovine serum albumin
SeC-FBS	Mixture between selenocompound and fetal bovine serum
SeC-HP	Mixture between selenocompound and human plasma
SeC-HSA	Conjugate between selenocompound and human serum albumin
SeC-NEM	Selenol derivative of selenocompound
TCEP	Tris(2-carboxyethyl)phosphine
WBC	White blood cells

1 INTRODUCTION

1.1 Leukemia

1.1.1 Definition of leukemia

Cancer is a collective name given to a number of related diseases in which abnormal cells divide without control and can invade neighboring tissues. The malignant cells can spread to other parts of the body through the blood and lymphatic systems. Cancer can be classified according to two systems, either according to the tissue from where the cancer originates (histologically) or by primary site from where cancer starts. According to the histological classification, cancer could be classified into carcinoma, sarcoma, melanoma, myeloma, lymphoma, and leukemia. Leukemia is popularly known as "blood cancer" and arises from the outgrowth of abnormal white blood cell (WBC, also called leukocyte; Figure 1A).[1] The large amounts of abnormal WBC do not form solid mass but primarily occupy bone marrow and blood. According to the rate of disease progression, leukemia can be divided into acute and chronic forms; while according to the cell lineage leukemia can be lymphoid or myeloid. Leukemia in general is further divided into four major categories: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML). Integration of cytogenetic information on the top of morphological diagnosis has enabled finer classification of leukemia, particularly AML, and informed better clinical practices.[1]

1.1.2 Leukemia epidemiology and prognosis

According to the *GLOBOCAN* 2018, leukemia was newly diagnosed in 437 thousands (2.4% of all cancer cases) patients worldwide and resulted into 309 thousands (3.2% of all cases) deaths. The numbers are expected to increase till 656 and 491 thousands by 2040, respectively (Figure 1B).[2] In the United States, the overall 5-year survival rate for leukemia has almost quadrupled during the period of 1960-2014 (from 14% till 65%); however, that for AML remains as low as 28%.[3] The 5-year survival rate in Sweden is above 60% for all leukemia subtypes and around 25% for AML by 2016,[4] which is similar to that in the United States. In China, the estimated incidences and deaths in leukemia during 2015 were 75.3 and 53.4 thousands cases, respectively.[5] In sharp contrast, the 5-year survival rate for leukemia patients that were diagnosed by 2015 was as low as 25.4% (Figure 1C).[6]

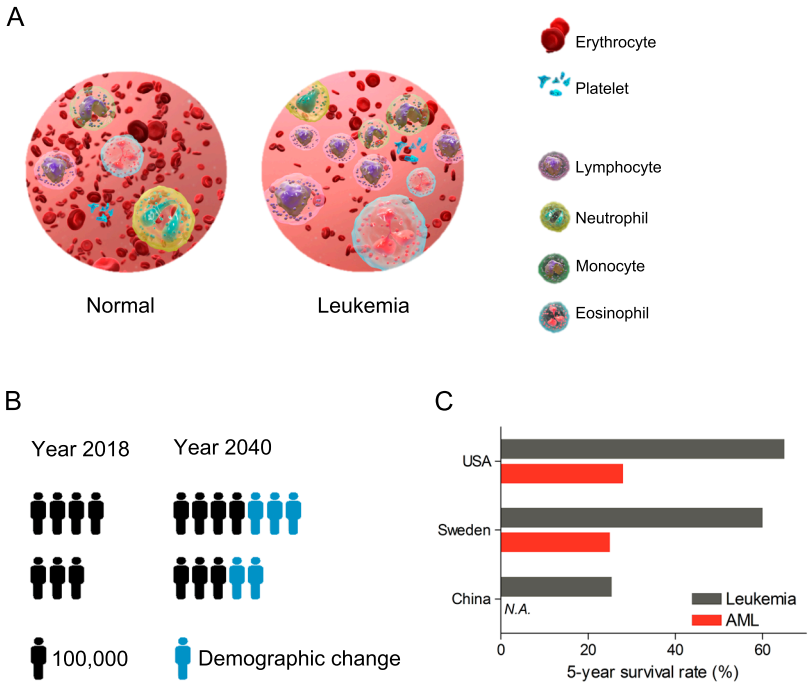

Figure 1. Etiology, epidemiology and prognosis of leukemia

(A) Normal blood cells contain erythrocyte, platelet, and leukocyte. The overgrowth of abnormal and immature leukocyte that comprises lymphocyte, neutrophil, monocyte and eosinophil leads to development of leukemia. (B) Incidence of leukemia in 2018 and 2040 worldwide.[2] (C) 5-Year survival rate of leukemia and AML in USA, Sweden and China.[3-6]

Panel A was reprinted with permission from Manu Sharma (https://www.scientificanimations.com/) and adapted.

1.2 Leukemia Treatment

1.2.1 Current treatments

Several options are available for leukemia treatment including chemotherapy, radiotherapy, targeted therapy, and adoptive cell therapy. Chemotherapy uses cytostatics to eradicate leukemia cells directly and is currently the major form of treatment. Commonly used cytostatics could be categorized into antimetabolites, alkylating agents, antitumor antibiotics, vinca alkaloids and anti-microtubule agents. Chemotherapy alone or in combination with radiotherapy is used as neoadjuvant-, adjuvant- and consolidation-therapy in hematological diseases. In contrast to the long history of using the chemotherapy, targeted therapy is an emerging but powerful option that originates from better understandings of the cytogenetic features of

leukemia. In principle, it mainly affects leukemia cells that express certain target molecules, therefore off-target toxicity and/or the adverse effects are minimized.[7] Adoptive cell therapy relies on intravenous infusion of functional immune cells for cancer regression and treatment, with the best-known procedure being hematopoietic stem cell transplantation. Interestingly, the last decade has witnessed tremendous advances in clinical use of chimeric antigen receptor-expressing T cells which are genetically engineered *ex vivo* to empower more functionality, like specificity and persistence, for certain subtype of cancer.[8] Cancer immunotherapy including checkpoint inhibitors and cytokines acts through mobilization of the patient's own immune system (usually under suppression) for identifying and destroying cancer cells. In spite of the tremendous and long-lasting efficacy against solid tumor, clinical practice of checkpoint inhibitors is still limited in leukemia.[9]

1.2.2 Limitations of chemotherapy

Chemotherapy e.g. cytostatics are non-specific drugs, and they act on not only leukemia cells but also fast-proliferating healthy cells like gastrointestinal tract epithelial cells and hair follicle cells. The toxicity of cytostatic drugs on healthy cells eventually manifests as side effects, e.g. mucositis, nausea, loss of weight and appetite, anemia, alopecia, infection and fatigue.

Apart from the side effects, resistance to chemotherapy develops on basis of intrinsic and adaptive machanisms.[10] Some leukemia subpopulation including leukemic stem cells have low proliferation rate but high self-renewing and adaptive ability, thereby being less vulnerable to chemotherapy.[11] The resistance is also endowed by the bone marrow niche which comprises leukemia cell-preserving endothelial cells and mesenchymal stromal cells.[12] Drug resistance indeed predisposes chemotherapy as a therapeutic rather than curative treatment and contributes to relapse later on.

Refractory and relapsed leukemia cells are reported to upregulate drug efflux transporters and detoxifying enzymes to reduce intracellular drug exposure.[10] It has been reported that upregulation of the efflux transporter protein, P-glycoprotein, could follow repeated treatment with antitumor antibiotics, and thus treatment failure and relapse were observed.[13] Furthermore, the first-line chemotherapy for AML, cytarabine, was found to be less effective in leukemia patients whose cells express high levels of SAMHD1 (SAM domain and HD domain-containing protein 1, an enzyme for inactivating cytarabine).[14] In addition, since several chemotherapeutic drugs (e.g. alkylating agents, platinum-based drugs, and tetracycline) induce inter- or intra-strand crosslinks and hence DNA damage, cells that have upregulated DNA repair mechanism are found to develop resistance.[15] In particular, leukemia cells with impaired DNA damage response were also reported to resist chemotherapy.[16,17]

1.2.3 Advances in chemotherapy

Following the Precision Medicine Initiative in combination with personalized treatment, there has been a strong tendency to shift the treatment paradigm from chemo-based therapy to targeted therapy. Due to the rapid developments in the medical researches, a great number of molecular targets have been identified and tested for leukemia treatment. Today several targeted therapies e.g. tyrosine kinase inhibitors and monoclonal antibodies are available on the clinic. Nevertheless, targeted therapy is applicable to a limited population of leukemia patients and subjected to quick development of resistance.[18,19] It is thus rationale to combine the wide applicability of chemotherapy and high specificity of targeted therapy.

Reduce side effect

In clinical practice, prophylactic or palliative medications are adopted to manage the side effects caused by chemotherapy, like administration of the antioxidant N-acetylcysteine (NAC) for liver protection.[20] Pharmacogenetics studies are paving the way to elucidate the mechanism underlying organ-specific toxicity caused by chemotherapeutic agents. For example, on basis of the causative role of CYP2J2 in paclitaxel-derived neuropathy, administration of telmisartan (a CYP2J inhibitor) was shown to reduce the severity of neuropathy.[21]

During the last decade, researches are more focused on tuning drug biodistribution as a possibility to reduce the off-target effect of chemotherapy. One strategy is to design and equip nanocarrier as targeted drug delivery system to leukemia lesions and further leukemia cells.[22,23] In the case of doxorubicin, adoption of liposomal formulation was shown to reduce cardiotoxicity and myelosuppression without compromising the clinical outcome.[24] Antibody-drug conjugate (ADC) is a complex composed of an antibody targeting surface marker of cancer cell and a small cytostatic molecule, thereby featuring the specificity of antibody and toxicity of cytostatics. Dozens of ADCs are undergoing clinical trials and two ADCs are already approved for leukemia treatment including gemtuzumab ozogamicin and inotuzumab ozogamicin.[25]

Reverse drug resistance

Leukemia cells are persistently evolving to survive from treatments including chemotherapy. Several mechanisms of resistance have been revealed by many groups as discussed above. Depending on the drug resistance mechanism, corresponding inhibitor has been implemented to enhance the sensitivity to chemotherapy.[26] For instance, in context of chemotherapy resistance caused by impaired DNA damage recognition, the small molecule JIB-04 (an inhibitor of multiple histone demethylases) could reverse DNA damage recognition in leukemia cells and improve the response to cytarabine *in vitro* and *in vivo*.[16]

Explore novel function of chemotherapy

Immunological cell death (ICD) refers to any kinds of cell death that could initiate effective anticancer immune response. The underlying principles include enhancement of the antigenicity (release of neoantigen) and adjuvanticity (activation of damage-associated molecular patterns).[27] Recognition of ICD induced by chemotherapy represents a novel perspective to prolong disease-free survival for cancer patients. Rational combination of chemotherapy with immunotherapy, like checkpoint inhibitor, could improve the treatment efficacy and increase the survival rate. The dose of cytostatics required to induce ICD is significantly lower compared to the conventional chemotherapeutic dose in order to avoid excess immunosuppression.[28] Cyclophosphamide, anthracycline and platinum derivatives are among the most reported cytostatics to be used as ICD inducers. In AML, translocation of calreticulin to the cell surface was implicated in initiation of immune response upon chemotherapy.[29,30]

Develop new chemotherapeutics

Along with optimization of available chemotherapeutics and development of novel treatment modalities, there have been continuous efforts to search for new chemotherapeutic entities from plants, microorganisms and chemical libraries,[31-35] and synthesize new compounds[36]. Outstanding examples are the ruthenium complexes[37] and selenocompounds (SeCs)[38].

1.3 Targeting Oxidative Stress for Leukemia Treatment

1.3.1 Oxidative stress

Cells mainly utilize oxidative phosphorylation to generate energy, in which each oxygen molecule (O_2) is eventually converted into harmless water. However, an estimation of 2% of O_2 consumed receives one electron from the mitochondrial electron transport chain per molecule and transforms into superoxide anion (O_2^-). Superoxide is also produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, and metabolic enzymes. It can be further converted to peroxide and peroxynitrite with the help of superoxide dismutase and nitric oxide synthase. In proximity with ferrous, superoxide can undergo Fenton reaction to yield the highly reactive hydroxyl radicals. Reactive oxygen species (ROS) are the collective name describing oxygen-containing radicals as well as non-radical species, and the major source of them is mitochondria (Figure 2).[39]

ROS can modify cellular components and initiate signaling cascades. To regulate the biological effects of ROS, cells develop and upregulate the antioxidative system comprising both enzymes and non-enzymatic molecules (e.g. glutathione, flavonoids, and vitamins). Common antioxidative enzymes include superoxide dis-

mutase, catalase, thioredoxin, peroxiredoxin, etc. Glutathione (GSH) and NADPH are the major reducing equivalents by their own and also function as chaperone molecules of many antioxidative enzymes in glutathione and thioredoxin systems. The antioxidative system is orchestrated by transcriptional network. One of the key transcription factors is nuclear factor erythroid 2-related factor 2 (Nrf2) which can upregulate multiple antioxidative molecules.[40]

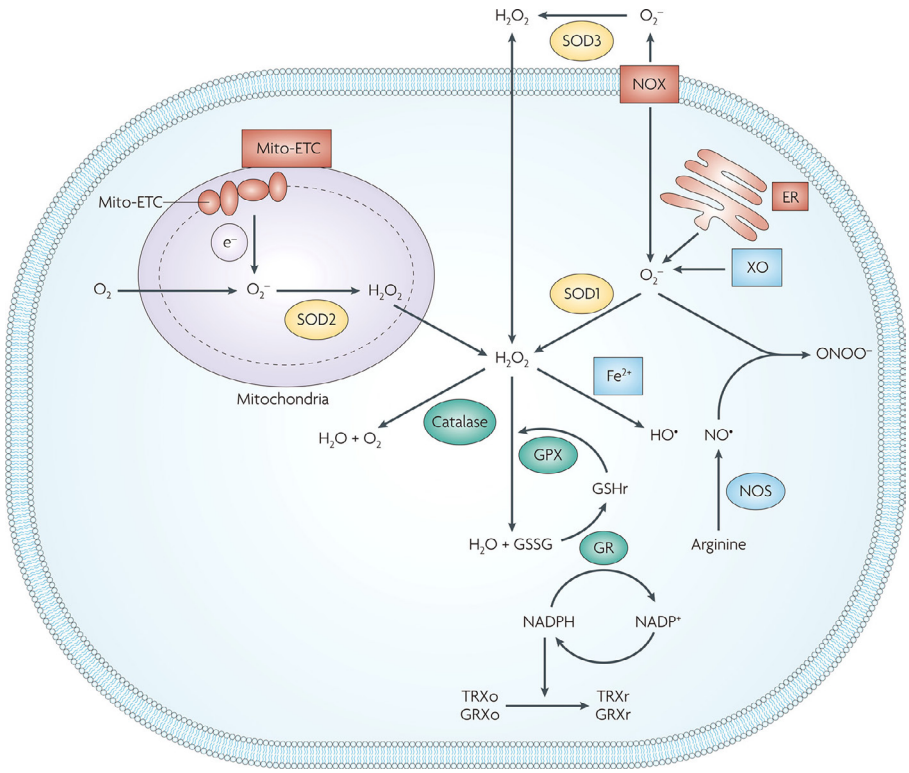

Figure 2. Redox regulation in cells

Superoxide anion (O_2^-) is produced by mitochondrial electron transport chain (Mito-ETC), endoplasmic reticulum (ER), and NADPH oxidase (NOX). Other forms of ROS, like peroxide, peroxynitrite and hydroxyl radical, could derive from superoxide through chemical or enzymatic processes. To maintain redox homeostasis, a range of enzymes are available to scavenge different forms of ROS, like superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX). The activity of GPX was replenished by glutathione reductase (GR) pathway.

Reprinted with permission from American Chemical Society.[41]

In healthy cells, the generation and removal of ROS are extremely regulated to maintain redox homeostasis; whereas the imbalance of them, referred as oxidative stress, has been noticed in an array of pathological cases including cancer. In laboratory test, oxidative stress is usually characterized by the elevation of oxidized glutathione (GSSG) in relative to reduced form.

1.3.2 Role of reactive oxygen species in cancer

The role of ROS in cancer can be categorized into two aspects: cancer-promoting and cancer-inhibiting (Figure 3). It's well established that free radicals could attack DNA, the damage of which can ultimately lead to mutations and genomic instability. This in turn could result in the development of several cancer types. In addition, accumulating lines of evidences support that cancer cells could take advantage of low to moderate level of ROS as signaling molecules to facilitate survival, proliferation, migration, angiogenesis, and etc.[42] These observations overwhelmingly underline the rationale behind reinforcing the antioxidative system, like daily supplementation of antioxidants, for cancer prevention.

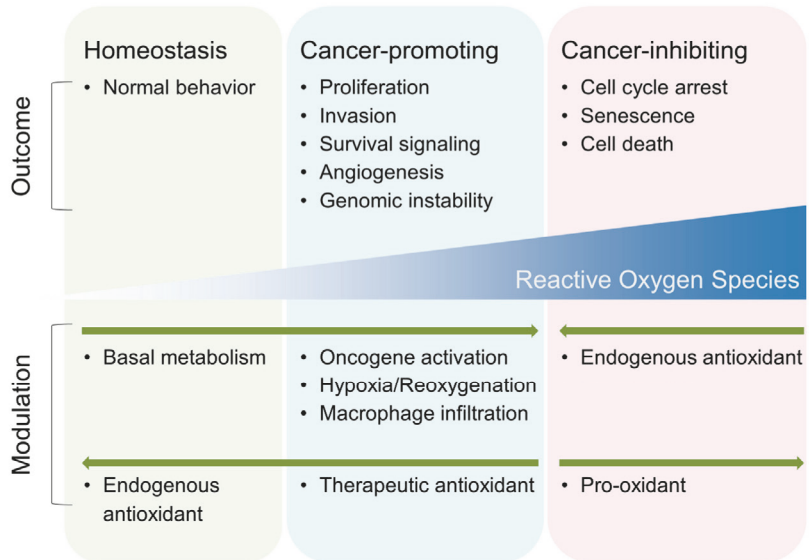

Figure 3. Generation, regulation and effects of ROS

ROS are byproducts of aerobic metabolism. In normal cells, their level is well controlled by endogenous antioxidant. Cancer cells feature high ROS generation as a result of oncogene activation, etc. Moderate level of ROS promotes cancer development and progression via activating many processes like proliferation, genomic instability, etc., while the cancer-promoting functions could be abolished by therapeutic antioxidant. Excessive ROS however arrest cell cycle and induce cell senescence and death. Therapeutic pro-oxidants can further exacerbate oxidative stress and kill cancer.

Reprinted with permission from Taylor & Francis and adapted.[45]

On the other hand, once the level is beyond the tolerance threshold, ROS cause irreversible damages to cellular components and jeopardize cell function. In fact, this is the mechanism behind the efficacy of radiotherapy for cancer treatment. Interestingly, cancer cells in metastasis were reported to suffer from even more oxidative stress than the resident counterparts. In consistence, disruption of the antioxidative system through inhibition of NADPH synthesis reduced distant metastasis of melanoma cells.[43] Recently, Wiel et al. have reported that administration of antioxidants facilitates cancer metastasis by reducing the level of oxidative stress.[44] The results are of high clinical relevance and public concern but have to be confirmed in other investigations.

Due to the fast metabolic cycles, cancer cells have increased ROS generation rate and thus higher level of oxidative stress. Oxidative stress in cancer cells is also indicated by upregulation of the antioxidative system in compensation to ROS generation. In leukemia, ROS are constitutively elevated. Such increment has been shown to promote disease progression and drug resistance by inducing genomic instability and activing growth signaling and survival pathways.[46-48] Persistent oxidative stress in leukemia cells predisposes higher vulnerability to further redox disruption compared to normal counterparts.[49,50] As an alternative to molecular targets, oxidative stress might be a promising target that could be exploited for leukemia treatment in a selective manner.

1.3.3 Strategies for induction of oxidative stress in leukemia cells

As oxidative stress reflects the imbalance between generation and scavenging of ROS, two different strategies are feasible for induction of oxidative stress: increasing ROS generation and undermining the antioxidative system (Table 1).

Table 1. Strategies for induction of oxidative stress in leukemia cells

Strategy	Method
ROS inducer	Radiotherapy Chemotherapy Novel redox-active compound Nanomedicine Photodynamic agent
Antioxidative system inhibitor	Inhibitor of antioxidative enzyme GSH-depleting agent GSH synthesis inhibitor

Radiotherapy is a good example showing that ROS generation could be reliably used for cancer treatment.[51] In addition, many standard-of-care chemotherapeutic agents could directly or indirectly upregulate ROS, like arsenic trioxide, cytarabine, vincristine, bortezomib, anthracyclines, histone deacetylase inhibitors, etc.[52-57]

Many redox-active small molecules are being investigated in preclinical and clinical stages like phenethyl isothiocyanate, β-lapachone and SeCs.[58-60] Of notice, the well-known antioxidant vitamin C could turn into a pro-oxidant if applied according to the proper regimen. In a pioneering clinical trial, high-dose of vitamin C demonstrated significant therapeutic efficacy in patients with non-small-cell lung cancer and glioblastoma.[61] The use of high-dose vitamin C in leukemia patients is yet to be explored. Various nanomedicine and photodynamic agents that could generate ROS in a controlled manner are also under preclinical development.[41,62,63]

Heme oxygenase-1 is an indirect antioxidant enzyme and works by synthesis of bilirubin for ROS neutralization. Its specific inhibitor protoporphyrine was shown to increase ROS level and kill leukemia cells.[64] Thioredoxin pathway is vastly implicated in redox regulation, and many direct or indirect inhibitors of this pathway are preclinically proved to be effective against leukemia cells, like auranofin, 13-hydroxy-15-oxo-zoaptlin and 3-deazaneplanocin A.[65-67]

GSH is ubiquitously present in cancer cells at millimolar range. Compounds that could quickly deplete GSH have also exhibited strong cytotoxicity to leukemia cells.[68] GSH is a tripeptide and synthesized from glutamine, cysteine, and alanine with the help of several enzymes. Molecules that reduce the availability of building amino acids or inhibit synthase activity could well exacerbate oxidative stress. For instance, Cramer et al. has engineered a cysteine-depleting enzyme, cyst(e)inase, and validated its efficacy in a leukemia mouse model without apparent side effects.[69] γ-Glutamylcysteine ligase is the rate-limiting enzyme in GSH synthesis. Its specific inhibitor, buthionine sulfoximine, could decrease GSH level and significantly suppress leukemia progression stand-alone and in combination with other treatments.[70,71] Noteworthy, the strategies listed above are indeed not exclusive and many molecules feature multiple functions to exacerbate oxidative stress.

1.4 Redox-Active Selenocompound

1.4.1 Introduction to selenium

In 1817, selenium (Se) was discovered by the Swedish chemist, Jöns Jacob Berzelius, following the analysis of an impurity in the sulfuric acid that was produced at a particular factory in Sweden. The impurity was originally believed to be tellurium; however, Berzelius noticed that it was actually a newly discovered element. Since the new element resembles tellurium (Tellus is Earth in Latin), Berzelius named it selenium after ‘Selene,’ the Greek goddess of the moon.

The toxicity of Se in farm animals was firstly reported in 1930's as "Alkali disease" and "Blind Staggers" caused by the excess intake of Se.[72,73] Alkali disease was noted as severe damage to the hoofs and hair loss; while Blind Staggers was manifested by wandering, circling, loss of ability to swallow, and blindness.[74] In human being, inhalation of Se can cause respiratory membrane irritation, pulmonary edema, bronchial inflammation and pneumonia. On the other hand, acute toxicity can be manifested as acute respiratory distress syndrome, myocardial infarction, renal failure, tachycardia, and neurological features (including tremors, irritability, and myalgia).[73]

During 1950's, researchers observed that Se is an essential element for bacteria and animals. Lack of nutritional Se was later associated with "Keshan Disease" (an endemic cardiomyopathy; firstly reported in 1979) and "Kashin-Beck Disease" (a chronic, endemic type of osteochondropathy; firstly reported in Siberia during 1849 and later in China during 1908). Both diseases were reported to occur in low-Se areas of China and Russia.[75,76] A total of 25 selenoproteins (protein that incorporates Se in the form of selenocysteine [SeCys]) are discovered in human,[77] while biological functions of most selenoproteins are still unclear. Given the redox activity of Se, selenoproteins are supposed to have redox-modulating abilities. For instance, glutathione peroxidase, thioredoxin reductase, and iodothyronine deiodinase are all implicated in redox homeostasis. The benefits of long-term supplementation of Se have been associated with redox homeostasis mediated by selenoproteins.[78]

1.4.2 Selenocompound as chemotherapeutic candidate

SeC refers to the selenium-containing molecule. The rationales behind the pursuit of SeCs as chemotherapeutics have two aspects. Firstly, SeCs could produce superoxide anion through a chemical process without involvement of particular enzymes.[79] Secondly, higher vulnerability of cancer cells to further oxidative stress compared to healthy cells could confer selective cytotoxicity.[49, 80, 81]

Diverse SeCs are under preclinical and clinical investigations for cancer chemotherapy[38], and the list continues to expand. Noteworthy, many synthetic SeCs derive from prototypic structures, like selenocyanate, diselenide, isoselenazolone as shown in the examples below (Figure 4).

Inorganics

Several inorganic forms have shown anti-leukemic potential including selenite and selenium dioxide.[82,83] Selenite features the most prominent cytotoxicity and is the best studied inorganic SeCs in terms of mechanisms of action and cancer types. Epigenetic study reveals that selenite could affect histone function and molecules related to oxygen and hypoxia response in the leukemia cell line K562.[82] In difference, another study found that selenite triggers the apoptosis of leukemia stem cells through modulation of arachidonic acid metabolism.[84]

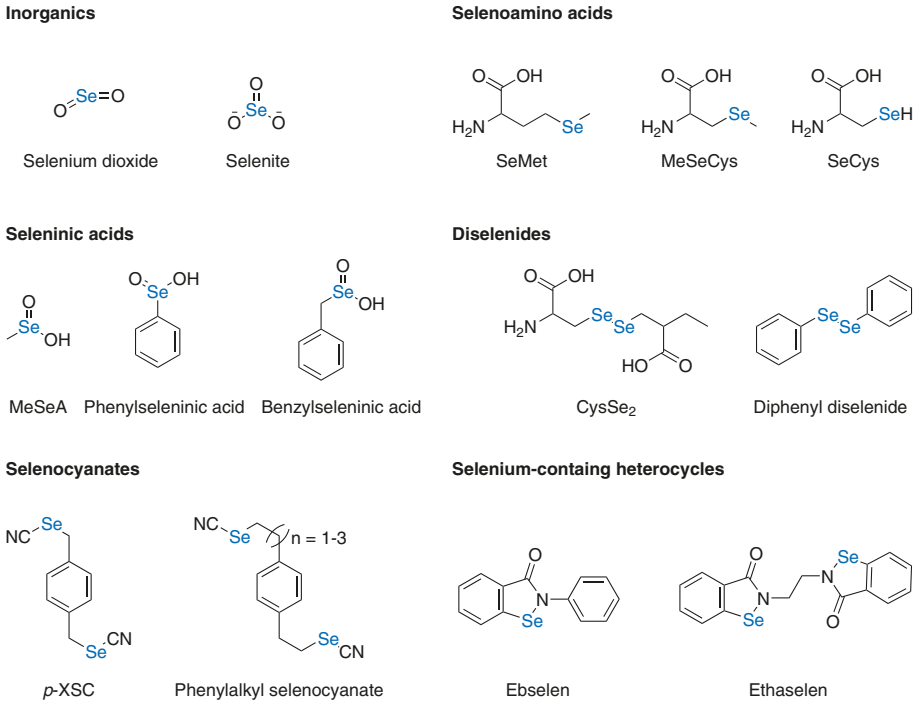

Figure 4. Selenocompounds with chemotherapeutic potential

Selenoamino acids

Selenomethionine (SeMet) and methyl selenocysteine (MeSeCys) are naturally existing amino acids in form of monoselenide. Although both are reported to inhibit the growth of cancer cell, much higher doses are required in comparison to other redox-active SeCs.[85,86] This is linked to their poor potency in generation of methylselenol (the key active metabolite) due to the deficiency of γ-lyase and β-lyase in mammals.[87] In accordance, replenishment of specific lyase was shown to potentiate the cytotoxicity.[88,89]

Seleninic acids

Methylseleninic acid (MeSeA) is a precursor of methylselenol and its cytotoxicity has been well documented against lung, prostate and breast cancer.[90] It is also reported to enhance the efficacy of conventional chemotherapeutics like paclitaxel.[91] In leukemia cells, available data suggest that MeSeA may modulate adhesion and migration pathways and interfere with crosstalk with stroma cells.[82] Its analogs like benzylseleninic acid, phenylseleninic acid have been synthesized while their the biological functions remain to be assessed.[92]

Diselenides

Selenocystine ($CysSe_2$) is the oxidized form of selenocysteine. Its anticancer efficacy as well as mechanism of action have been explored *in vitro* and *in vivo*.[93] $CysSe_2$ could also potentiate the effects of 5-fluouracil and doxorubicin for cancer treatment.[94,95] One drawback of $CysSe_2$ is the low solubility in water and also organic solvent. To overcome this problem, researchers have encapsulated $CysSe_2$ into mesoporous silica nanoparticles.[96] A plethora of symmetric or asymmetric diselenides with strong anticancer effect have been synthesized.[97,98] Among them, diphenyl diselenide is another promising candidate due to the efficacy on several cancer cell lines.[99]

Selenocyanates

Organic selenocyanates emerge as important anticancer candidates. The first selenocyanate described for cancer treatment was *p*-xyleneselenocyanate (*p*-XSC).[100] It was shown to significantly prevent development of prostate cancer and oral carcinoma. Later on, several phenylalkyl selenocyanate analogs were synthesized and tested *in vitro* and *in vivo*.[101-103] Researchers also established structure-activity relationship and concluded that longer alkyl chain length corresponds to higher tumor-inhibiting effect.[101] However, the application of selenocyanates for leukemia treatment is limited and the underlying mechanism of action is poorly understood.

Selenium-containing heterocycles

Selenium-containing heterocycles represent a large class of SeCs, and ebselen is the first and most studied one in this group. It was originally developed as an antioxidant but later recognized as a pro-oxidant while used at higher concentration.[104] Several studies have proven its ability to treatment solid tumor,[105,106] and recently Laverdière et al have demonstrated that ebselen also targets leukemia stem cells.[107] However, ebselen is poorly soluble in water and alternative formulation is required for therapeutic usage. Immerse efforts have been made to develop new heterocyclic SeCs using ebselen as a prototype.[108-110] For instance, ethaselen demonstrated remarkable efficacy against leukemia as well as many other types of cancer and has entered phase II clinical trial.[111,112]

1.4.3 Cellular uptake

SeCs exert selective toxicity on cancer cells in relative to normal cells. In addition to the differential basal ROS level between cancer and normal cells, another underlying mechanism was thought to be selective uptake. Early studies have shown that radiolabeled SeCs (^{75}Se-selenite and ^{75}Se-SeMet) have preferable distribution into the tumor in comparison to healthy tissues and could be used for

tumor diagnostics.[113,114] After intravenous administration of radiolabeled selenite, a large percentage of selenium was detected in red blood cell.[115] In addition, it was reported that presence of reducing agents, either cysteine[116] or glutathione[117], could facilitate uptake of selenite into the cancer cell, implying that reduced forms of selenite are preferable. However, assessment of SeCs uptake is based on quantification of total elemental Se intracellularly post exposure; therefore the exact uptaken form of Se is unknown. So far, only a few reports have suggested that anion transporters play a role in mediating the uptake of selenite and SeMet.[114,115] For the majority of SeCs, the uptake mechanism is not fully understood.

1.4.4 Mechanism of action

In the presence of GSH, SeC could transform into selenol or selenolate (the deprotonated form of selenol) which then reacts with molecular oxygen and yields superoxide anion. Along with other downstream ROS derived from superoxide, cellular ROS level is eventually elevated. Multiple intracellular molecules including proteins and nucleic acids are potential targets of ROS, resulting into oxidative damages (Figure 5).[38] Low degree of damage could be partially or completely offset by the endogenous antioxidative system and exogenous antioxidants. In addition, emerging evidences suggest that the activity of SeCs might be carried out through direct protein modification by redox-active metabolites, for example NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells).[118]

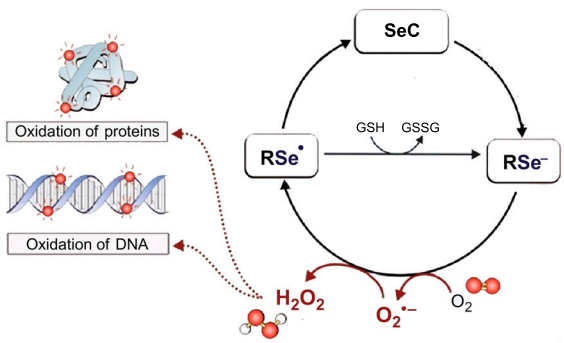

Figure 5. Mechanism behind the cytotoxicity of selenocompounds

Redox-active SeC could transform into the active selenolate intermediate (RSe−) which then transfers one electron to oxygen molecule, yielding superoxide. In proximity with other enzymes or molecules, superoxide can generate downstream forms of ROS for inducing oxidative damages to proteins and DNA. This redox cycle also produces selenyl radical (RSe•) which can in turn refuel selenolate.

Reprinted with permission from Elsevier and adapted.[119]

Mechanisms underlying cell death caused by oxidative damage are diverse.[61] Different modes of cell death like apoptosis, necrosis, autophagy, and necroptosis might take place alone or in parallel, which are structure-, concentration and time-dependent.[120] Chemoresistance due to the loss-of-function of certain components in cell death pathways is one of the major hurdles for current treatments. However, this hurdle might be less concerned with for SeCs which could prime diverse cell death machinery.

1.4.5 Speciation and quantification

The biological activities vary between different SeCs and their metabolites. To improve the use of SeCs in chemotherapy, it is a prerequisite to quantify the different forms of Se, rather than total Se as an element.[121] Substantial efforts are being dedicated to speciation and quantification of SeCs as summarized below (Table 2).[122,123]

Table 2. Features of different selenium speciation and quantification strategies

Strategy	Chromatography	X-ray absorption spectroscopy	Chemical probe
Advantage	• High specificity and sensitivity • Wide applicability • High availability in instrument	• Minimal sample preparation • Wide applicability	• Minimal sample preparation • Utility in cells and animals non-invasively • High availability in instrument
Limitation	• Complex and bias-prone sample preparation • Need prior knowledge for specie identification	• Semi-quantitative • Low availability in instrument • Complex data analysis	• Qualitative • Only detect total selenol

Chromatography

Chromatography is a traditional separation technique based on varying partition coefficient of compounds between mobile and stationary phases. The most adopted separation techniques are gas chromatography (GC) and liquid chromatography (LC).[124] GC is useful for separation of volatile species, like methylselenol, dimethyl selenide and dimethyl diselenide; while LC has much more general utility.

Before separation, sample pretreatment is required to enrich the species of interest and remove irrelevant components (like lipids and proteins).[123,125] The choice of pretreatment method is based on matrix type, species of interest and instrumental requirement. Many Se species are known to be unstable (i.e. SeMet and SeCys),

thus species interconversion/loss during pretreatment is expected. In additional, extraction efficiency might vary for different species, which would introduce great bias to data interpretation. To minimize variation during pretreatment, a usual way is to apply internal standard that has similar physiochemical property as the species of interest. However, this is hampered by shortage of internal standard and poor knowledge of Se species in complex matrix.

Identification of species follows samples pretreatment and separation. In the simplest situation, this could be achieved by comparing the retention time of the sample to that of reference. Alternatively, mass spectrometry (MS) is a powerful tool in identification of new Se species. In fact, tandem GC/LC-MS is the major methodology in the field of Se speciation and quantification. Furthermore, there are other atomic detectors which could enable extremely high sensitivity, e.g. inductively coupled plasma atomic emission spectroscopy and microwave induced plasma atomic emission spectrometry.[126]

X-ray absorption spectroscopy

Although chromatography remains the mainstream method in Se speciation, it holds several intrinsic limitations. Synchrotron X-ray is a type of extremely bright light of high energy. The electrons of Se could be readily excited by the high energy from X-ray and generate spectrometric properties that are distinguishable from other elements. The spectrometric feature is also dependent on the type of bond that Se forms.[127] During analysis with X-ray absorption spectroscopy (XAS), sample preparation procedure is rather facile. In this regard, the possibility of species interconversion is minimized, making synchrotron X-ray a promising tool for Se speciation. Prince RC et al. carried out one of the pioneering endeavors by acquiring distinct absorption spectrograms of several selenoamino acids.[128] After obtaining the spectra of unknown samples and pre-selected reference materials, linear regression analysis can be carried out to perform semi-quantification of Se species. Using this strategy, Harris HH et al. have recently succeeded to illustrate the dynamic metabolic profile of selenite in human lung cancer cells.[129]

Chemical probe

Selenols are the active metabolites of SeCs and also critical sites for many seleno-proteins. Visualization of selenols has received tremendous interests in the last decades with a plethora of fluorescent selenol probes been reported so far. In principle, all probes take advantage of the exceptional reactivity of selenol.[130-133] Those probes have been tested in cell cultures or living animals like zebrafish and mouse, which contributed to a better understanding of the fate of selenols *in vivo*. One major limitation of those chemical probes is that none of them are able to distinguish different selenol forms.

1.4.6 Metabolism and pharmacokinetics

Among the diverse SeCs investigated for cancer chemotherapy, only a few were particularly studied in terms of metabolism and pharmacokinetics. The metabolism of selenite in the lung cancer cells has been studied using XAS semi-quantitatively.[129] With a dedicated chromatography method, Terlinden et al found that after oral administration ebselen quickly became undetectable in human plasma and two major metabolites were identified.[134] El-Bayoumy's group also used chromatography method to study the metabolism of orally administered *p*-XSC and identified tetraselenocyclophane as a major metabolite in mouse feces.[135]

In general, the metabolic pathway and pharmacokinetics of SeCs are poorly investigated, which most probably is due to the fact that most preclinical studies and clinical trials utilize the quantification methods that only detect the total amount of Se as an element.[136,137] More efforts are warranted to depict how SeCs are metabolized *in vivo* as an important part of translational endeavors.

2 AIMS

Even though current treatment strategies have greatly improved the survival of patients suffering from hematological malignancies, complete and long-term remission of leukemia is still far from reality, demanding further endeavors to improve the clinical outcome. Selenocompounds have exhibited therapeutic potential in the treatment of solid tumors through induction of oxidative stress; however, their roles in leukemia treatment are poorly explored.

The general aim of the present thesis was to explore the anti-leukemic activity of selenocompounds. Our specific aims were:

- To study the anti-leukemic efficacy of selenocompounds *in vitro*
- To selectively quantify selenocompounds in plasma
- To investigate the mechanism of cellular uptake of selenocompounds
- To explore the pharmacokinetics and evaluate the anti-leukemic effect of selenocompounds *in vivo*

3 MATERIALS AND METHODS

3.1 Reagent

MeSeA (Catalog No. 541281), $CysSe_2$ (Catalog No. 545996), N-ethylmaleimide (NEM; Catalog No. 389412), tris(2-carboxyethyl)phosphine (TCEP; Catalog No. 75259), bovine serum albumin (BSA; Catalog No. A7030), and human serum albumin (HSA; Cat. No. A1653) were purchased from Sigma-Aldrich. Ebselen (Catalog No. ALX-270-097) and *p*-XSC (Catalog No. ab142600) were bought from Enzo Life Sciences and Abcam, respectively.

Different solvents were applied to prepare the stock solutions for the SeCs depending on particular use. For cell and animal experiments, $CysSe_2$ and MeSeA were dissolved in DPBS (0.7-1 mg/mL) and the pH thereof was always adjusted till around 7.4; while the solvent was H_2O in speciation & quantification section. For Ebselen and *p*-XSC, the solvents used were DMSO (for cell experiment; 1.5 mg/mL), acetonitrile (for speciation & quantification section), and DMSO/Cremophor EL (w/v of 1:1; for animal experiment; 5 mg/mL).

3.2 Cell Experiment

3.2.1 Cell culture condition

C1498 (ATCC; Catalog No. TIB-49) and C1498-*luc* (PerkinElmer, prepared upon request) were cultured in DMEM (ThermoFisher; Catalog No. 41966) supplemented with 10% fetal bovine serum (FBS; ThermoFisher; Catalog No. 41966) and penicillin (100 U/mL)-streptomycin (0.1 mg/ml) (Sigma-Aldrich; Catalog No. P4333). HL60 (DSMZ; Catalog No. ACC3), KG-1a (DSMZ; Catalog No. ACC421) and K562 (ATCC; Catalog No. CCL243) cells were cultured in RPMI1640 (ThermoFisher; Catalog No. 41966) supplemented with 10% FBS and 1X penicillin-streptomycin. HUVEC (kindly provided by Eva Wärdell) cells were grown in MV2 medium (PromoCell; Catalog No. C22022). The medium with appropriate full supplements was hereafter referred as complete medium.

All cells were maintained at 37 °C with 5% CO_2 and the viability was assayed by trypan blue routinely. Only cells under exponential growth phase and with trypan blue positivity ≤ 5% were used.

3.2.2 Determination of cell viability

Leukemia cells (1×10^5 cells/mL; 0.1 mL/well) were treated with SeC in 96-well plates for different periods. For HUVEC cells, a total of 5×10^3 cells were seeded 24 hr before treatment. Cell viability was determined using either CellTiter-Glo

kit (Promega; Catalog No. G7571; 100 µL/well) or WST-1 kit (Sigma-Aldrich; Catalog No. 11644807001; 10 µL/well plus 2 hr incubation). Signals were quantified using the microplate reader (SpectraMax i3x). In combination treatment *p*-XSC was added 2 hr or 1 hr later after NAC (Sigma-Aldrich; Catalog No. A7250) or Z-VAD-FMK (Enzo; Catalog No. ALX-260-020-M005), respectively. In some cases, treatment was performed in medium without FBS and without/with BSA.

3.2.3 Determination of oxidative stress

Two different assays were used to determine oxidative stress in cells. In the first assay, C1498-*luc* cells (1×10^6 cells/mL) were treated with 20 µM of the fluorescent ROS probe, DCFDA (Abcam; Catalog No. ab113851) for 30 min. The DCFDA-loaded cells (1×10^5 cells/mL) were then treated with *p*-XSC for 4 hr. In combination treatment with NAC, cells were successively treated with NAC (5 mM; 2 hr), DCFDA and *p*-XSC with rinsing step between each treatment. Cellular fluorescence images were captured using microscope (Olympus IX81; cellSens software 1.16) and the signals were also quantified using microplate reader. Phenol red-free DMEM (ThermoFisher; Catalog No. 31053) was used to avoid interference.

The second assay refers to measurement of glutathione level. Briefly, C1498-*luc* cells (1×10^6 cells/mL; 5 mL) were treated with *p*-XSC and harvested at different time points. The cells were lysed using 80 µL of hydrochloric acid (10 mM) and glutathione level in the lysate was determined using a colorimetric kit following the manufacturer's instructions (Sigma-Aldrich; Catalog No. 38185).

3.2.4 Determination of intracellular selenium element

C1498 cells (2×10^6 cells/mL; 4 mL) were treated with SeC and pelleted 30 min later. The pellet was rinsed and lysed with 0.4 mL lysing buffer (Promega, Catalog No. E153A). The supernatant afforded through centrifugation (1×10^4 g, 10 min) was diluted with H_2O till 4 mL and subjected to Se measurement on inductively coupled plasma atomic emission spectrometer (iCAP 6500, ThermoFisher). The standard curve was acquired using selenium reference solution (Sigma-Aldrich; Catalog No. 50002).

3.2.5 Transmission electron microscopy

C1498 cells (1×10^6 cells/mL; 1 mL) were treated with *p*-XSC and harvested at different time points. The cells were immediately fixed in 2.5% glutaraldehyde overnight and immersed in 2% osmium tetroxide for 2 hr. After dehydration and embedding, ultrathin sections (~ 50 nm) were cut and examined using microscope (Tecnai 12 Spirit BioTwin; FEI Company) at 100 kV.

3.2.6 Western blot

C1498-*luc* cells (1×10^6 cells/mL; 20 mL) were treated with *p*-XSC and harvested at different time points. The cell pellet was lysed using RIPA buffer (CST; Catalog No. 9806) supplemented with proteinase/phosphatase inhibitor (CST; Catalog No. 5872) and concentration of total protein thereof was quantified using BCA kit (ThermoFisher; Catalog No. 23227). Around 15-20 μg of protein were loaded on 10% precast TGX polyacrylamide gels (Bio-Rad; Catalog No. 4561036) and transferred onto a PVDF membrane (GE healthcare; Catalog No. 10600023). The membranes were blocked using 5% non-fat milk (CST; Catalog No. 9999) and incubated with primary antibodies overnight at 4 °C. The primary antibodies were against Actin (Sigma-Aldrich; Catalog No. 2066, 1: 400) and AIF (CST; Catalog No. 4642, 1: 500). Protein band was stained with fluorescent second antibody at room temperature for 45 min (anti-mouse or rabbit IRDye 680RD/800CW, 1: 10,000; Li-cor) and captured (Odyssey 9120, Li-cor).

3.2.7 Flow cytometry

C1498-*luc* cells (1×10^5 cells/mL; 2 mL) were treated with *p*-XSC and harvested at different time points. The cells were stained with 10 μL of PE-Annexin V and 10 μL of 7-AAD solution (BD; Catalog No. 556547) for 15 min at room temperature. Data were acquired on 1×10^4 cells and analyzed using flow cytometry (BD FACSArray).

3.3 Speciation and Quantification

3.3.1 Synthesis of selenol derivative

To synthesize the selenol derivative (SeC-NEM), SeC solution was mixed with NEM and then TCEP for 30 min at room temperature. Detailed loading amount of the materials was available in our publications.[138,139] The derivative was purified using preparative liquid chromatography and validated using ^{1}H-NMR (nuclear magnetic resonance, Bruker DRX-400, 400 MHz) and ESI-MS (electrospray ionization-mass spectrometry, Thermo TSQ Quantum Ultra).

3.3.2 Quantification of selenocompound

Two different strategies were utilized to extract SeC for subsequent quantification: (1) direct deproteinization; (2) RECID (REductive Cleavage and Instance Derivatization). During direct deproteinization, 50 μL of acetonitrile was mixed with 20 μL of matrix. In RECID, NEM (10 μL, 0.4 M in dimethylacetamide) and TCEP (5 μL, 0.2 M in H_2O) were successively added into 15 μL of matrix, followed

by vortex for 30 sec and reaction for 10 min at room temperature. The mixture was centrifuged (3×10^4 g, 10 min) and 2 μL of the supernatant afforded was injected into the LC-MS analyzer (Thermo TSQ Quantum Ultra). Detailed LC conditions and MS parameters were available in our publications.[138,139]

3.3.3 Determination of albumin binding degree

SeC solution (40 μg/mL; 200 μL) was mixed with equal volume of H_2O (control) or 5% BSA. The mixture was added to ultrafiltration tube (cut-off 10 kDa; Sigma-Aldrich; Catalog No. 13239E) and centrifuged (2×10^3 g and 5 min). SeC in the ultra-filtrate was measured directly using LC-MS. Albumin binding degree was calculated as the percentage of SeC peak area in BSA in relative to that in control.

3.3.4 X-ray absorption spectroscopy

The conjugate between selenocompound and albumin (SeC-albumin) was firstly prepared. Briefly, SeC solution was mixed with albumin and kept at room temperature for 30 min. The loading amount of the materials was detailed in our publications.[138,139] The conjugate was obtained after purification using gel permeation chromatography (Superdex 200 Increase 10/300 GL column) and lyophilization. SeC-HP or SeC-FBS samples were prepared through mixing SeC and human plasma or FBS, respectively, and subsequent lyophilization.

The samples including SeC, SeC-albumin, SeC-FBS and SeC-HP were formulated into small pellets (diameter 5 mm). Se K-edge XAS measurements were performed in QEXAFS mode using a Si(311) double-crystal monochromator in CLAESS beamline at ALBA synchrotron light facility (Cerdanyola del Valles, Spain). All measurements were performed at liquid N2 temperature to avoid radiation damage. Data were analyzed using Demeter software package (version 0.9.26).

3.4 Animal Experiment

3.4.1 Pharmacokinetic study

Animal experiments were approved by the Stockholm Southern Ethical Committee (ethical permit no. ID 1031). SeCs were intravenously or intraperitoneally injected into C57BL/6 albino mouse ($CysSe_2$ and MeSeA: 5 mg/kg; ebselen and *p*-XSC: 2 mg/kg). Blood samples were collected at different time points post administration and SeCs concentration in plasma was analyzed using RECID as described above. The concentration-time curves were modeled using WinNonLin software (version 2.0) to calculate pharmacokinetic parameters.

3.4.2 Leukemia mouse model

The leukemia mouse model was established through intravenous inoculation of C1498-*luc* cells (1×10^6 cells in 100 µL DPBS) into C57BL/6 albino mouse. Disease progression was longitudinally monitored using *in vivo* imaging system (IVIS® Spectrum; PerkinElmer) after i.p. injection of D-luciferin (15 mg/mL in DPBS; PerkinElmer). *p*-XSC treatment (daily i.v.; 2 mg/kg) was initialized 11 days post cell inoculation and continued for 5 days. All mice were sacrificed 22 days after cell inoculation; bone marrow from both femurs was immediately flushed. Bioluminescence signal in bone marrow samples was measured by mixing 100 µL D-luciferin (3 mg/mL) with 100 µL bone marrow flush and captured using IVIS. Data were analyzed using Living Image software (version 4.5.5).

3.5 Statistics

Results were shown as mean ± standard deviation if appropriate. Two-sided Mann-Whitney test was used to compare the means between two groups. $p \leq 0.05$ was considered statistically significant.

4 RESULTS

4.1 Selenocompounds Have Prominent Anti-Leukemic Effect *in Vitro*

Most SeCs currently under investigation derive from structural modification of prototypic compounds and have common structures such as monoselenide, selenocyanate, seleninic acid, diselenide and isoselenazolone. Therefore, to gain a better understanding of their anti-leukemic activity, we selected six candidates representing different classes of SeCs (Figure 6). All the SeCs selected have shown anti-cancer activity according to previous reports.[38]

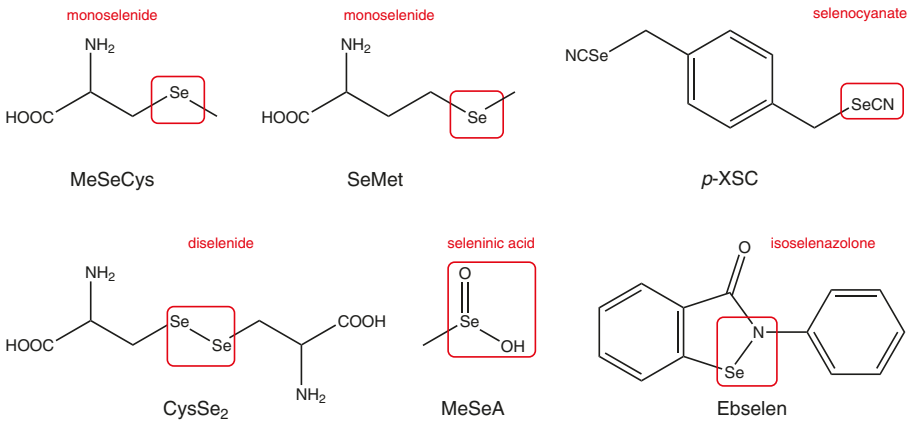

Figure 6. Chemical structures of selenocompounds used in this thesis
Prototypic structures were highlighted in red.

4.1.1 p-Xyleneselenocyanate has superior toxicity on leukemia cells

C1498-*luc* is an aggressive murine AML cell line and was utilized as the primary model for assessing the anti-leukemic efficacy of SeCs. Among the six SeCs studied, *p*-XSC displayed the highest cytotoxicity against C1498-*luc* cells with half-inhibitory concentration (IC50) of 4.8 μM after 24 hr treatment (Figure 7A) and thus was used in further investigations. To confirm a general anti-leukemic efficacy of *p*-XSC and increase clinical relevance, we characterized the efficacy of *p*-XSC on several leukemia cell lines of human origin: K562 (chronic myelogenous leukemia), HL-60 (acute promyelocytic leukemia) and KG-1a (acute myelogenous leukemia). *p*-XSC was similarly effective in killing human leukemia cells with sharp dose-effect profiles (IC50 ranged 4.9-11.3 μM; Figure 7B). In comparison, higher concentrations of *p*-XSC were required to kill the non-cancerous fibroblasts and endothelial cells (IC50 over 15 μM; data not shown). These findings suggest the selective cytotoxicity of *p*-XSC against leukemia over non-cancerous cells.

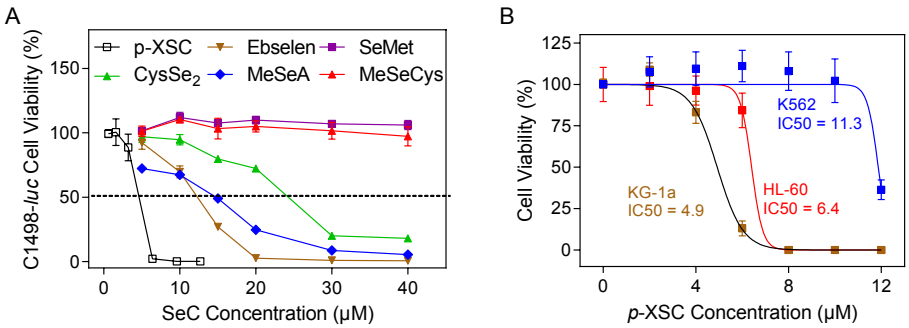

Figure 7. Cytotoxicity of selenocompounds on leukemia cell lines

(A) C1498-luc cells were treated with different SeCs for 24 hr and cell viability was determined using CellTiter-Glo kit. (B) KG-1a, HL60 and K562 cells were treated with p-XSC for 24 hr. Cell viability was determined using WST-1 kit. Cell concentrations were all 1×10^5 cells/mL. N = 3.

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4.1.2 p-Xyleneselenocyanate induces oxidative stress of leukemia cells

To understand the underlying mechanism of action, we exploited a cell-permeable probe, DCF-DA, which fluoresces upon exposure to reactive oxygen species (ROS) in C1498-*luc* cells. Compared to the vehicle control, much stronger fluorescence signals were detected in *p*-XSC-treated cells (Figure 8A). N-acetylcysteine (NAC), a commonly used antioxidant, significantly reduced cellular ROS (Figure 8B). In accordance, the cytotoxicity of *p*-XSC was completely abrogated by NAC, while NAC itself has no effect on cell viability at the dose applied (Figure 8C). These findings imply that the cytotoxicity of *p*-XSC on leukemia cells is mediated by induction of ROS generation.

Alteration of intracellular glutathione is one of the best markers during oxidative stress and was also examined in C1498-*luc* cells (Figure 8D). Since higher cell mass is required for glutathione measurement, *p*-XSC concentration was escalated to the range of 5-25 μM to achieve the same phenotype. In line with the absence of cytotoxicity for 5 μM of *p*-XSC (data not shown), total glutathione level returned to basal level in spite of an initial decrease by 1 hr. Glutathione variation in case of 15 μM exhibited a clear time-dependence as demonstrated by the steep decline during the period of 2-3 hr. Increasing *p*-XSC concentration till 25 μM led to a fast exhaustion of glutathione. Overall, the pattern in glutathione depletion corroborates the findings from cytotoxicity as well as mechanistic studies above.

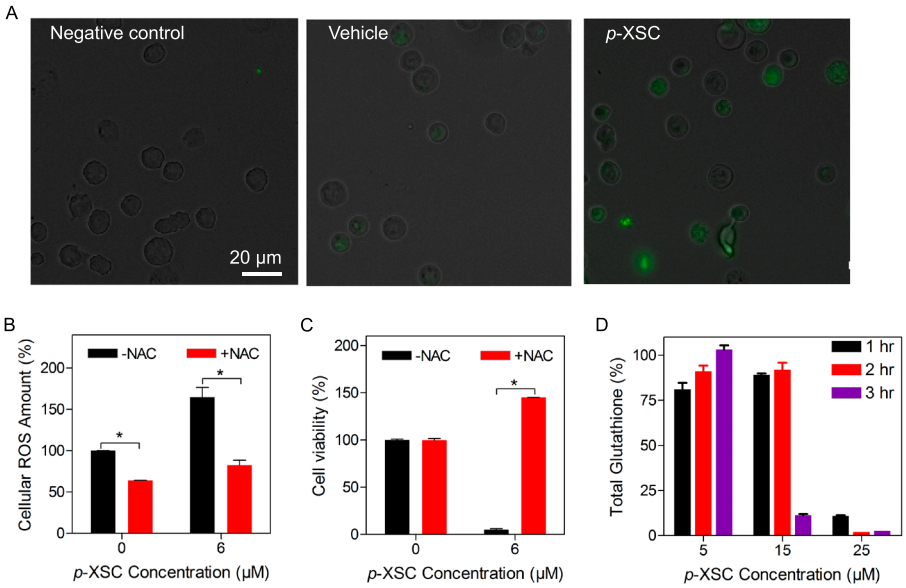

Figure 8. Implication of oxidative stress in the cytotoxicity of p-xyleneselenocyanate

(A) C1498-luc cells (1×10^5 cells/mL) were treated with 8 μM of p-XSC or vehicle for 4 hr. Intracellular ROS was stained using a green fluorescent dye DCF-DA. Representative microscope pictures were shown. The scale bar applies to all pictures. (B) C1498-luc cells (1×10^5 cells/mL) were treated with 5 mM of NAC for 2 hr and then with p-XSC for 4 hr. Intracellular ROS were quantified. N = 3. (C) C1498-luc cells (1×10^5 cells/mL) were treated with 5 mM of NAC for 2 hr and then with p-XSC for 24 hr. Cell viability were determined using WST-1 kit. N = 6. (D) C1498-luc cells (1×10^6 cells/mL) were treated with p-XSC and intracellular glutathione level was determined. N=3.

4.1.3 Mitochondrion is the primary target of p-xyelenselenocyanate

To reveal specific organelle affected by *p*-XSC, we investigated ultrastructural alteration using transmission electron microscopy (TEM; Figure 9A) in C1498-*luc* cells. Mitochondria were filled up with big vacuoles after treatment with 25 μM of *p*-XSC for 30 min. In fact, the phenotype was already present with less severity at an early time point (5 min). No major ultrastructural change was observed at 5 μM which was not toxic (Figure 8D). Next we used Annexin V/7-AAD staining to reveal whether the cell death is in mode of apoptosis. Again, we found that apoptosis did not initiate until 2 hr post treatment (8 μM of *p*-XSC) and all cells became apoptotic in the next hour, confirming critical time-dependence of the cytotoxicity (Figure 9B). Furthermore, noticeable upregulation of apoptosis-inducing factor (AIF) was detected upon *p*-XSC treatment (25 μM; Figure 9C). In accordance with the knowledge that AIF acts through caspase-independent pathway, the pan-caspase inhibitor, Z-VAD-FMK, did not compromise the cytotoxicity of *p*-XSC at all (Figure 9D). In total, the cytotoxicity of *p*-XSC was pertinent on massive mitochondria damage and apoptotic cell death.

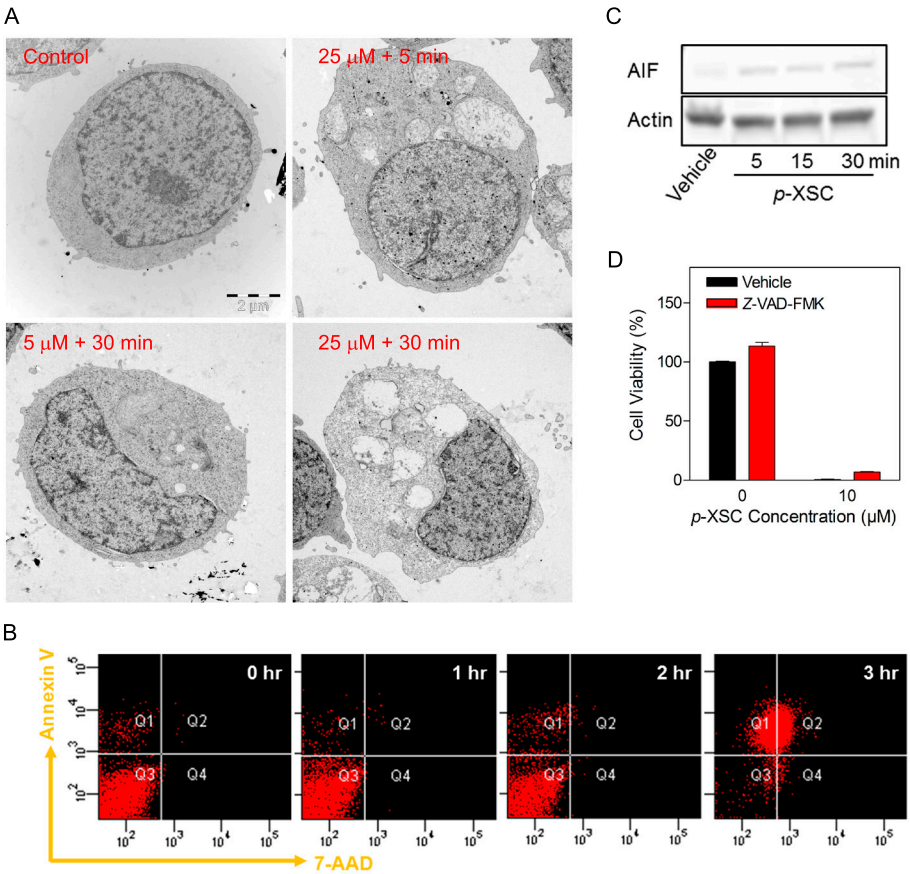

Figure 9. Cellular mechanism underlying the cytotoxicity of p-xyleneselenocyanate

(A) C1498-luc cells (1×10^6 cells/mL) were treated with p-XSC and the ultrastructure was imaged using transmission electron microscope. The scale bar applies to all pictures. (B) C1498-luc cells (1×10^5 cells/mL) were treated with 8 μM of p-XSC and then stained for apoptosis analysis. Representative scatter plots from flow cytometry were shown. (C) C1498-luc cells (1×10^6 cells/mL) were treated with 25 μM of p-XSC. Protein level of apoptosis-inducing factor (AIF) in the whole cell lysate was determined using western blot. (D) C1498-luc cells (1×10^5 cells/mL) were treated with Z-VAD-FMK (50 μM) for 1 hr and then together with p-XSC for 24 hr. Cell viability was determined using WST-1 kit. N = 6.

4.2 Quantification of Selenocompounds in Plasma

Results above highlighted that the activity of SeCs is determined by compound type, concentration as well as treatment duration. To ensure adequate drug exposure, one practical measure is to optimize treatment regimen on basis of the pharmacokinetic properties. However, there is no citable report on SeCs to the best of our knowledge, motivating us to establish a quantification method that is amenable to plasma setting.

4.2.1 Direct deproteinization fails to extract selenocompounds

Deproteinization is the most common method to extract drugs in free and non-covalently bound forms from plasma prior to analysis. SeCs were firstly extracted from plasma through direct deproteinization and their levels were measured using LC-MS. Quantification of $CysSe_2$ and MeSeA was however prohibited due to strong matrix effect on compound ionization (data not shown) that is indeed commonly present in the interface of LC and ESI-MS. Since albumin is the major plasma protein, we used 5% BSA as the matrix instead of plasma to examine the extraction efficiency. Interestingly, MeSeCys and SeMet were easily extracted with efficiency over 95%; whereas the extraction efficiencies for the other four SeCs were as low as 0-28% (Figure 10A). In consistence, low degrees of albumin binding (< 3%) were observed for MeSeCys and SeMet; while the rest demonstrated high albumin binding ability (Figure 10B).

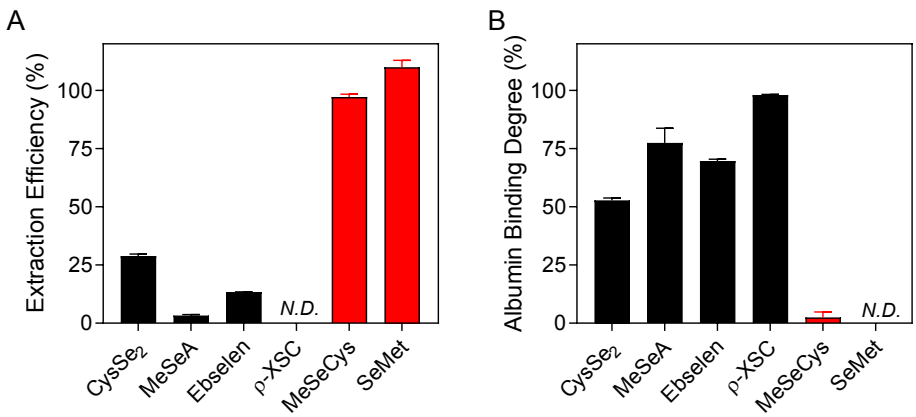

Figure 10. Extraction of selenocompounds from albumin solution

SeC was mixed with 5% BSA solution and extracted using direct deproteinization (A) or ultrafiltration (B) strategy. Final concentration of SeC in matrix was 20 μg/mL. SeC was quantified using LC-MS. N.D. refers to not detected. N = 3.

4.2.2 Selenocompounds bind to albumin through selenium-sulfur bond

In order to confirm the binding type, SeCs were firstly made to react with serum albumin from bovine (BSA) or human (HSA). The selenocompound-albumin conjugate (SeC-albumin) was purified and analyzed using XAS. Both the X-ray absorption near edge spectrum (XANES; Figure 11A) and extended X-ray absorption fine structure (EXAFS; Figure 11B) of SeC-albumin markedly differed from that of the corresponding SeC, indicating changes in the coordination of Se. Noteworthy, the EXAFS signals were similar for the eight SeC-albumin samples (Figure 11B).

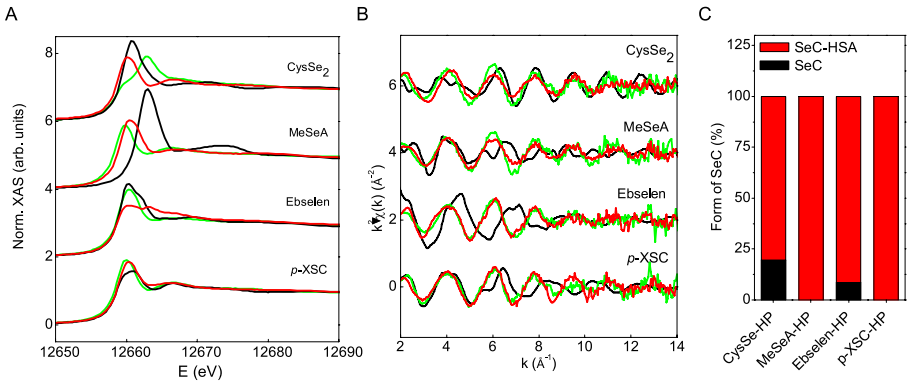

Figure 11. X-ray absorption spectroscopy measurement

(A-B) X-ray absorption near edge spectrum and extended X-ray absorption fine structure of SeC (black), SeC-HSA (red), and SeC-BSA (green). (C) Amount of different forms of SeC in SeC-HP estimated from linear combination fitting. Total concentration of SeC in SeC-HP was 20 μg/mL.

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Further modeling of the pseudo-radial distribution function of EXAFS supported that Se coordinated with sulfur (S; Table 3). Moreover, linear combination fitting using the reference spectra of SeC-HSA (the conjugate between selenocompound and human serum albumin) and SeC was carried out to assess the amount of each form in the mixture of SeC and human plasma (SeC-HP). Results showed that SeC-HSA was always a major constituent in SeC-HP (Figure 11C).

Table 3. Coordination of selenium in the conjugate between selenocompound and human serum albumin

The analysis was based on modelling of the EXAFS signal. The k-range was 2.6-13.3 $Å^{-1}$ (exception: 2.6-10.1 $Å^{-1}$ for MeSeA-HSA). The amplitude reduction factor (S_0^2) was fixed to 0.85.

SeC-HSA	Atom	Number	E_0 (eV)	R (Å)	σ^2 ($Å^2$)	R-factor
CysSe-HSA	C S	1 1	6.7	2.016 2.191	0.003 0.001	0.047
MeSeA-HSA	O S	1 1	5.8	1.745 2.178	0.030 0.002	0.004
Ebselen-HSA	C S	1 1	7.0	1.957 2.205	0.002 0.002	0.024
p-XSC-HSA	C S	1 1	6.9	1.972 2.190	0.009 0.003	0.036

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To further understand how Se-S bond forms, we used a thiol-reactive agent, NEM, to eliminate the effect of albumin thiol. Compared to pristine albumin, higher extraction efficiencies of SeCs were obtained after blocking albumin thiols (Figure 12A). Selenol is the major active metabolite and its abundance has been positively correlated with cytotoxicity. In order to know whether generation of selenol is implicated, we correlated cytotoxicity (Figure 7A) and albumin binding ability (Figure 10B; Figure 11C) of SeCs. Interestingly, only the four compounds with prominent albumin binding (i.e. $CysSe_2$, MeSeA, ebselen and *p*-XSC) were cytotoxic. Moreover, a negative correlation was found between IC50 and albumin binding degree acquired from both XAS and LC-MS measurements (Figure 12B), suggesting a causative role of selenols. Given the vulnerability of Se-S bond to reduction and high reactivity of selenol, we used TCEP/NEM to take a snapshot of the selenol structure. The speculated selenol derivative of SeC (SeC-NEM; Figure 12C) was synthesized and validated using ESI-MS and 1H-NMR (data not shown). On the basis of the results above, we reasoned that cytotoxic SeCs could transform into selenols which react with albumin thiol through Se-S bond.

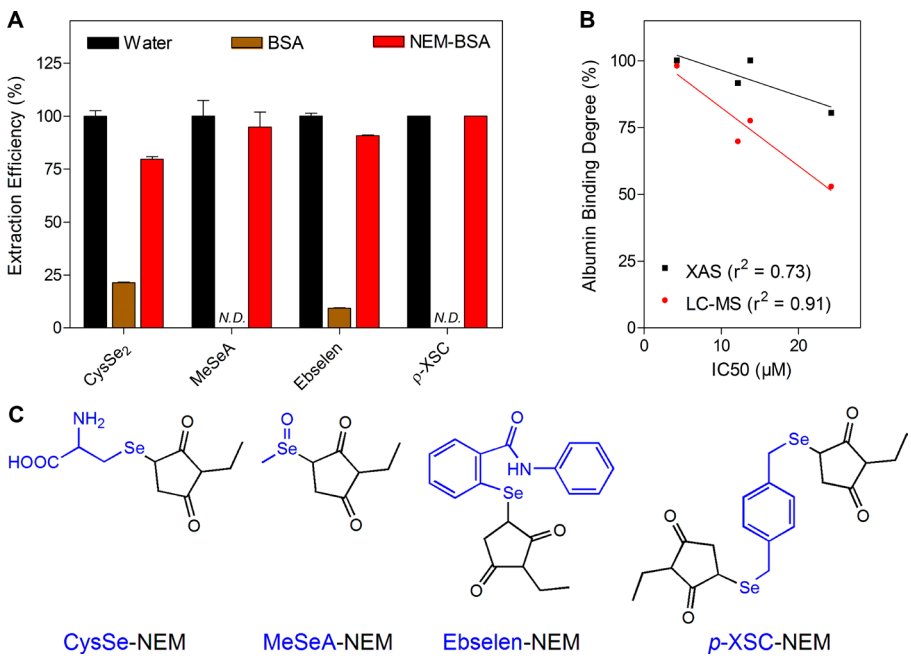

Figure 12. Role of selenol and thiol in albumin binding

(A) Extraction efficiency of SeC from 5% BSA and NEM-BSA (BSA treated with NEM; 5%) through deproteinization in relative to H_2O. SeC concentration was 20 μg/mL. N.D. refers to not detected. N = 3. (B) Linear regression between IC50 and albumin binding degree of SeCs. IC50 was calculated from Figure 7A; albumin binding degree was retrieved from LC-MS (Figure 10B) and XAS (Figure 11C) measurements. The goodness of fitting is shown as r2. (C) Structure of the selenol derivative (SeC-NEM). The selenol intermediate is labeled as blue.

4.2.3 Extraction of total selenocompounds using RECID

In order to quantify the free and albumin-bound SeCs in plasma, we established the REductive Cleavage and Instant Derivatization method (RECID) which contains three consecutive steps: (1) generation of selenol intermediate by TCEP; (2) derivatization of the selenol intermediate by NEM; (3) extraction of the derivative by deproteinization (Figure 13A).

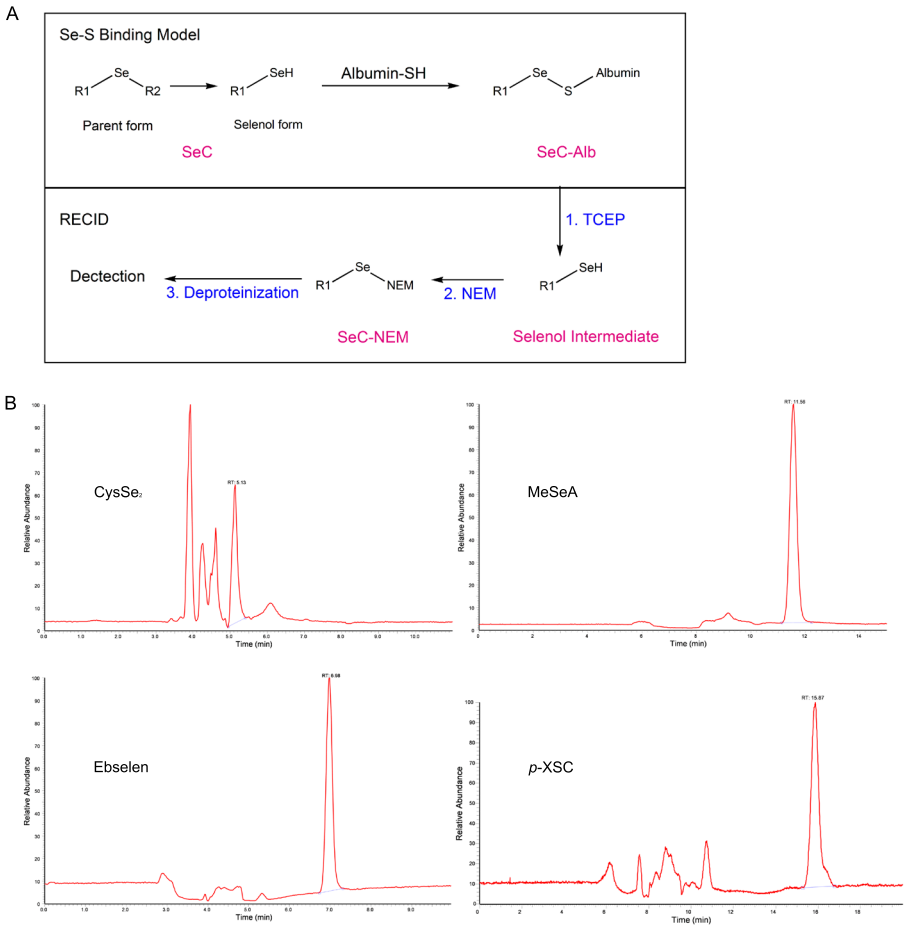

Figure 13. Quantification of selenocompounds in plasma using RECID

(A) Scheme of Se-S binding model and the reductive cleavage and instant derivatization method (RECID). (B) Representative chromatograms of SeCs in human plasma using RECID.

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The reaction time, amount of TCEP/NEM, as well as the order in addition of TCEP/NEM was optimized. RECID was also methodologically validated in terms of accuracy, precision, recovery and stability (data not shown). In general, sample preparation could be finished within 30 min and running time was less than 20 min. Using the optimized RECID and LC-MS conditions, we managed to detect total SeCs of high specificity in plasma (Figure 13B).

4.3 Albumin Sequesters Selenocompounds and Limits Their Efficacy

Given the covalent binding between SeCs and BSA, albumin binding was assumed to prevail in cell culture medium that is universally supplemented with fetal bovine serum (FBS; 10% in most cases). We are thus interested in elucidating the extent as well as the way in which albumin binding affect the activity of SeCs.

4.3.1 Selenocompounds form conjugates with serum albumin

Firstly of all, we investigated the transformation of SeCs at a concentration of notable cytotoxicity. SeCs were mixed with FBS (SeC-FBS) rather than complete medium to ease sample preparation and then subjected to XAS measurement. Similarly, we found that Se coordinated with S in SeC-FBS samples (Table 4). Moreover, EXAFS fitting indicated that 40% of $CysSe_2$ and ebselen added in FBS formed Se-S bond; while all *p*-XSC and MeSeA were transformed.

Table 4. Coordination of selenium in the mixture of selenocompound and fetal bovine serum

The analysis was based on modelling of the EXAFS signal. The k-range was 2.5-13.0 $Å^{-1}$. The amplitude reduction factor (S_0^2) was fixed to 0.85.

SeC-FBS	Atom	Number	E_0 (eV)	R (Å)	σ^2 ($Å^2$)	R-factor
CysSe-FBS (300 μM)	C	1	4.8	2.003	0.006	0.022
	S	0.4		2.139	0.004	
	Se	0.6		2.322	0.001	
MeSeA-FBS (200 μM)	O	1	4.0	1.682	0.003	0.008
	C	1		2.359	0.004	
	S	1		2.203	0.008	
Ebselen-FBS (300 μM)	C	1	5.0	1.920	0.004	0.016
	S	0.4		2.191	0.002	
	Se	0.6		2.355	0.004	
p-XSC-FBS (100 μM)	C	1	6.5	2.009	0.003	0.017
	S	1		2.185	0.002	

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Besides albumin thiols, small molecule thiols like GSH and Cys are present in fresh and conditioned cell culture medium. In order to clarify whether these small molecule thiols could participate in transforming SeCs, we characterized its conjugate with SeCs (SeC-SM) if present. *p*-XSC had high reactivity to GSH and Cys (the conjugates were collectively abbreviated as *p*-XSC-SM)[133] and was chosen for this purpose.

Free *p*-XSC in complete medium was extracted using direct deproteinization and quantified. It was undetectable when *p*-XSC concentration was below 12 μM; and addition of extra 1% BSA further depleted free *p*-XSC (Figure 14A). To facilitate the characterization of *p*-XSC-SM, *p*-XSC concentration was fixed to 10 μM where no free *p*-XSC was present. The macromolecular conjugate *p*-XSC-BSA (the conjugate between *p*-XSC and BSA) was then filtered using a centrifugation tube (molecular weight cut-off 10 kDa; Figure 14B). The filtrate was analyzed in terms of *p*-XSC-SM amount, cellular uptake as well as toxicity profiles. Actually, there was no sign of *p*-XSC-SM (data not shown), *p*-XSC uptake (Figure 14C) or cytotoxicity (Figure 14D) for the filtrate, suggesting the absence of SeC-SM.

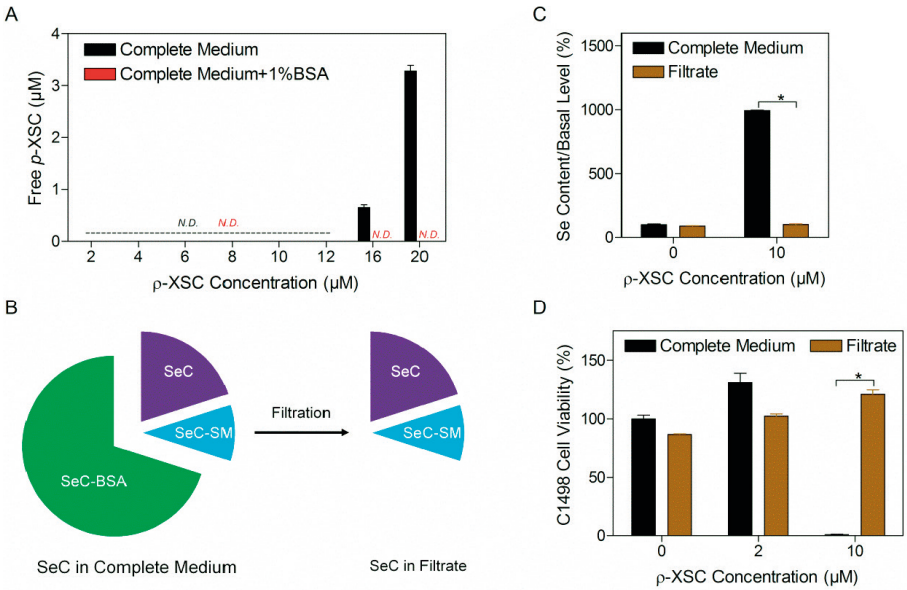

Figure 14. Transformation of selenocompounds in cell culture medium

(A) Quantification of free p-XSC in complete DMEM medium without (black bar) or with (red bar) 1% extra BSA. N.D. refers to not detected. N = 3. (B) Forms of SeC in complete medium and the filtrate. (C) C1498 cells (2×10^6 cells/mL) were treated with p-XSC (provided in complete medium or the filtrate) for 30 min. Intracellular Se level was then determined. N = 3. (D) C1498 cells (1×10^5 cells/mL) were treated with p-XSC (provided in complete medium or the filtrate) for 24 hr. Cell viability was then assayed using WST-1 kit. N = 3. * indicates $p \leq 0.05$ (Two-sided Mann-Whitney test).

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4.3.2 Albumin reduces cellular uptake of selenocompounds

Since macromolecular SeC-BSA is canonically thought to enter cells less efficiently compared to free SeCs, we asked whether the presence of albumin could reduce cellular uptake of SeCs. C1498 cells were treated with SeC at the conditions when no cell death occurred (data not shown) to preclude free diffusion of SeC across cell membrane. Results demonstrate substantial increases of intracellular Se element upon treatment with *p*-XSC or ebselen in relative to basal level (Figure 15A). The increases were however abrogated by 1% extra BSA. We also characterized SeC uptake in FBS-free medium to exclude the effect of serum-derived albumin. In comparison to that in complete medium, SeC uptake in FBS-free medium increased by folds. Once again, adding extra BSA into FBS-free medium remarkably down-regulated SeC uptake. In general, we observed that extracellular albumin content was inversely associated with intracellular Se level (Figure 15B).

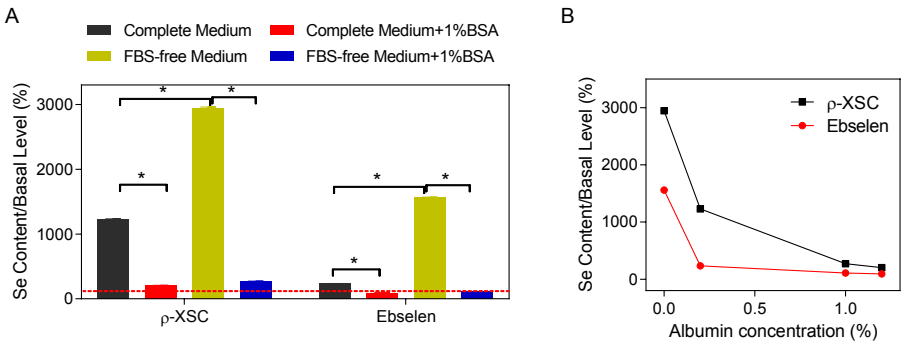

Figure 15. Effect of albumin on the cellular uptake of selenocompounds

(A) C1498 cells (2×10^6 cells/mL) were treated with p-XSC (10 μM) or ebselen (20 μM) for 30 min. The treatment was performed in complete medium (black), complete medium with 1% extra BSA (red), FBS-free medium (yellow), FBS-free medium with 1% extra BSA (blue). Intracellular Se level was then determined. N = 3. The red dash line marks the basal Se level from untreated cells. * denotes $p \leq 0.05$ (Two-sided Mann-Whitney test). (B) Relationship between extracellular albumin content and intracellular Se content. Data were retrieved from panel A.

4.3.3 Albumin antagonizes the efficacy of selenocompounds

Decreased exposure to SeCs in the presence of albumin would reasonably lead to lower cytotoxicity. In our experiments, we also found that co-treatment with 1% BSA completely abolished the cytotoxicity of *p*-XSC in spite of the strong activity of *p*-XSC alone on murine C1498 cells (Figure 16A). Similar patterns were observed on two other human cell lines including HL60 (Figure 16B) and HUVEC (human umbilical vein endothelial cell; Figure 16C). In parallel, the cytotoxicity of ebselen, MeSeA or $CysSe_2$ on C1498 cells was substantially

lower in the presence of 1% extra BSA (Figure 16D-16F). The antagonistic effect of albumin was also observed in FBS-free medium (Figure 16G-16H). In general, SeCs primarily form conjugates with albumin rather than react with small molecule thiols or remain unchanged. Due to the inferior uptake of the macromolecular SeC-albumin in relative to SeC itself, probably partially due to competitive inhibition by pristine albumin, intracellular Se pool and subsequent cell death was decreased.

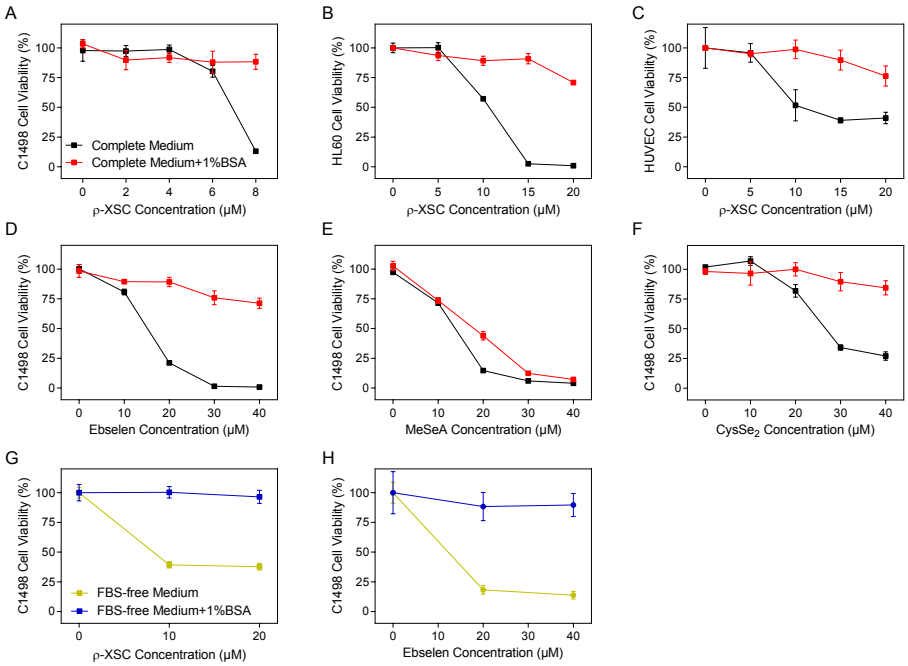

Figure 16. Effect of albumin on the cytotoxicity of selenocompounds

(A-C) Viability of C1498, HL60 or HUVEC cells after treatment with p-XSC. (D-F) Viability of C1498 cells after treatment with ebselen, MeSeA or $CysSe_2$. The treatment was performed in appropriate complete medium for each cell line without (black) or with (red) 1% extra BSA. (G-H) Viability of C1498 cells after treatment with p-XSC or ebselen. The FBS-free medium was either without (yellow) or with (blue) 1% extra BSA. In all experiments, the treatment duration was 24 hr and cell viability was determined using CellTiter-Glo kit. Cell concentrations were 1×10^5/mL except for HUVEC. N = 4.

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4.4 Pharmacokinetics and Anti-leukemic effect of Selenocompounds in Mouse

On basis of the encouraging anti-leukemic efficacy *in vitro* and robust quantification method established above, efforts was dedicated to exploring the pharmacokinetic properties of SeCs and further examining their anti-leukemic efficacy in animal models.

4.4.1 Pharmacokinetics of selenocompounds in mouse

The four cytotoxic SeCs were administered intravenously, and similar concentration-time curves were observed except for MeSeA which remained at very low level during the period examined (Figure 17A). The pharmacokinetic profile of *p*-XSC following intraperitoneal administration was also characterized. The preliminary therapeutic threshold was calculated based on the IC50 *in vitro* (≈ 1.5 μg/mL; Figure 7A) as well as the percentage of plasma volume in mouse blood (≈ 50%). The maximal concentration achieved was well below the therapeutic concentration threshold (~3 μg/mL; Figure 17B).

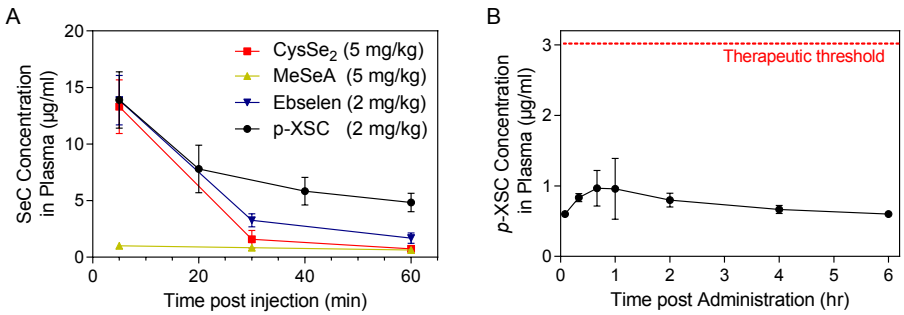

Figure 17. Concentration-time curve of selenocompounds in mouse

SeC was administered intravenously (A) or intraperitoneally (B) and blood samples were taken at different time points. SeC concentration in plasma was then analyzed using RECID. The red line in panel B refers to the preliminary threshold of therapeutic potential. N = 3-5.

Pharmacokinetic modeling of the concentration-time curves shows that *p*-XSC had lower clearance rate (5.8 mL/h) and conferred higher exposure (10.8 μg/mL·h) than the other three compounds following intravenous administration. For *p*-XSC, intraperitoneal route bestowed comparable drug exposure as intravenous route did (Table 5).

Table 5. Pharmacokinetic parameters of selenocompounds in mouse

One-compartment open model was used to calculate the parameters. N = 3-5.

Parameter	$CysSe_2$ (5 mg/kg, *iv*)	MeSeA (5 mg/kg, *iv*)	Ebselen (2 mg/kg, *iv*)	*p*-XSC (2 mg/kg, *iv*)	*p*-XSC (2 mg/kg, *ip*)
AUC (μg/mL·h)	4.1 ± 1.1	N.A.	5.7 ± 0.7	10.8 ± 2.4	10.9 ± 2.2
C_{max} (μg/mL)	20.4 ± 2.7	N.A.	18.1 ± 3.7	14.8 ± 2.7	1.0 ± 0.1
Cl (mL/h)	38.5 ± 11.7	N.A.	10.6 ± 1.3	5.8 ± 1.3	5.5 ± 1.1
V_{ss} (mL)	7.5 ± 1.0	N.A.	3.4 ± 0.6	4.1 ± 0.8	N.A.

Note: *N.A.*: not available; AUC: area under the curve; Cmax: peak concentration; Cl: rate of clearance; Vss: apparent volume of distribution at steady state; iv: intravenous; ip: intraperitoneal.

4.4.2 p-Xyleneselenocyanate suppresses leukemia progression in mouse

p-XSC has poor aqueous solubility which hampered our attempts to increase the dose. In combination with its pharmacokinetic properties, we assume that intraperitoneal route might be unsuitable to confer adequate exposure. In the anti-leukemic study in mouse, we rationally chose tail vein as the route of administration.

The anti-leukemic activity of *p*-XSC *in vivo* was evaluated in a murine AML model which express luciferase reporter. Disease progression was non-invasively tracked using bioluminescence imaging (BLI). The treatment was started when the C1498-*luc* cells inoculated homed to bone marrow and continued for 5 successive days (Figure 18A). In the control group, disease progressed quickly and spread to common leukemia-affected organs including liver and bone marrow. In comparison, *p*-XSC-treated mice had significantly slower disease progression (Figure 18B-18C). After dissection of bone marrow from mice sacrificed, less BLI signals were detected in *p*-XSC group (Figure 18D).

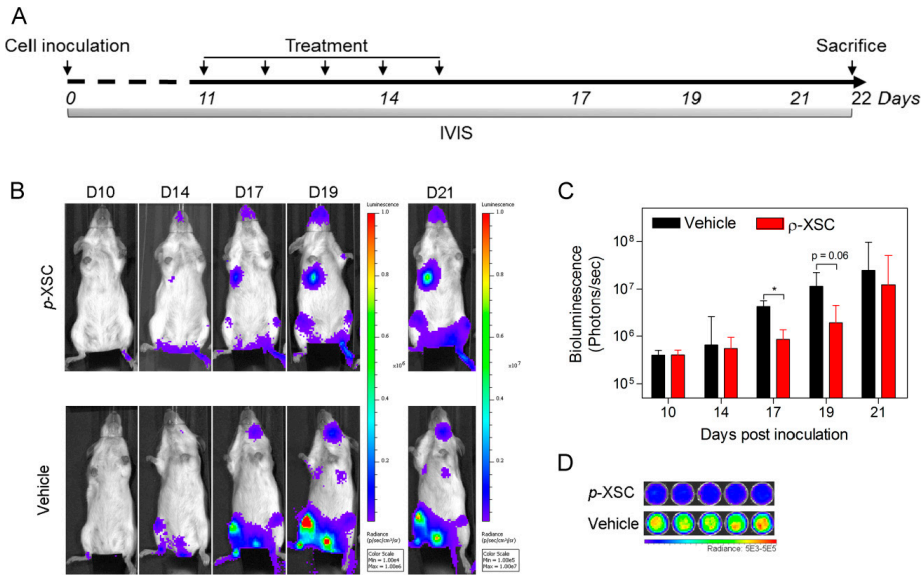

Figure 18. Anti-leukemic effect of p-xyleneselenocyanate in mouse model

(A) Leukemia mice were established through inoculating 1×10^6 C1498-luc intravenously (day 0) and disease progression was monitored using IVIS system. p-XSC treatment (iv, 2 mg/kg) was performed during day 11-15 once daily. All mice were sacrificed at day 22 and bone marrow from femur was collected. (B-C) Representative BLI images and quantification of whole body bioluminescence signal. Mann-Whitney test were used to compare the means between two groups. * denotes $p \leq 0.05$. N = 6. (D) Representative heat maps of bioluminescence signal in bone marrow ex vivo.

5 DISCUSSIONS

5.1 Stress Out Leukemia Cells

Using a panel of human and murine leukemia cell lines, we found that *p*-XSC was the most potent compound in eradicating leukemia cells among the six SeCs studied herein and also those reported elsewhere.[140] Interestingly, *p*-XSC was found to quickly eradicate all the tested leukemia cells (Figure 7A), which might indicate high therapeutic efficacy and less likelihood for disease relapse. Previous studies reported that *p*-XSC generates superoxide anion through the one-electron transfer chemical process (Figure 5).[141] Therefore, induction of oxidative stress is independent of particular enzyme/protein and may confer wide applicability, which could be an advantage over other therapies that target specific antigens, kinases or mutations. In line with this notion, we observed that the four leukemia cell lines, in spite of their distinct oncogene nature (BCR-BCL in K562 cells, C-MYC in HL-60 cells, and FOP2-FGFR1 in KG-1a cells), shared similar susceptibility to *p*-XSC (Figure 7B).

The toxicity of *p*-XSC on non-leukemia cells was observed at higher concentration, providing a therapeutic window for killing leukemia cells selectively. The superior vulnerability of leukemia cells to further oxidative stress is endowed by higher basal stress level in relative to that in normal cell. Huang et al. had set up an elegant model by transfecting oncogene in hematopoietic cells and found selective cytotoxicity of β-phenylethyl isothiocyanate (a ROS inducer) to oncologically transformed cells.[81] In the present study, we used fibroblasts and endothelial cells instead of normal leukocytes as the controls. More conclusive evidences could be obtained if normal leukocytes were utilized.

As many other chemotherapeutic agents, the cytotoxicity of *p*-XSC was shown to be dependent on concentration and time (Figure 7A; Figure 9B), which was also demonstrated by the pattern how intracellular glutathione level changes (Figure 8D). One possible explanation might be the threshold of ROS that cellular antioxidative system could cope with. Take glutathione alteration as an example, in spite of an initial decrease following treatment with low-dose *p*-XSC (5 μM), glutathione became replenished later on. As a result, cellular viability remained unaffected. Once *p*-XSC-derived ROS exceeds the threshold, cell functions are irreversibly jeopardized and cell death commences. On the other hand, reinforcement of the antioxidative systems by supplementing the antioxidant NAC reasonably reduced the cytotoxicity of *p*-XSC (Figure 8C).

Mitochondria are canonical targets for oxidative damage probably because they are the major sources of endogenous ROS. In this study, we also found that *p*-XSC induced massive damages of mitochondria as early as 5 min after treat-

ment (Figure 9A), suggesting the strong efficacy of *p*-XSC. Oxidative damages can initiate diverse cell death pathways dependent or independent on caspase. Our further analysis of pro-apoptotic factor and caspase corroborated that cell death might be independent of caspases (Figure 9C; Figure 9D). However, more studied are warranted to depict the complete pathway.

5.2 Towards More Selective Quantification

Our present results together with others' have highlighted significant impacts of the chemical form and concentration on the efficacy of SeCs, making speciation and quantification of SeCs in biological matrices of high priority.[121] However, the available methods, particularly for pharmacokinetic studies, are predominately measuring Se for a total elemental concentration. In this study, we combined hyphenated mass spectrometry and X-ray absorption spectroscopy for Se speciation and established a general covalent binding model between cytotoxic SeCs and albumin, irrespective of physiochemical attributes in terms of structure and hydrophilicity (Figure 13A). Furthermore, we have developed a powerful quantification method based on reductive cleavage and instant derivatization (RECID). The newly developed method enabled quantification of both free and albumin-bound SeCs selectively and was applicable with good precision and accuracy to biological samples including plasma.

Our data clearly show a correlation between cytotoxicity and albumin binding degree of SeCs (Figure 12B) as both are dependent on the intrinsic ability of SeCs to transform into selenol.[133] In spite of the poor cytotoxicity of SeMet and MeSeCys, adding an appropriate lyase was reported to enhance their cytotoxic activities.[87,141] Therefore, it is of high importance to keep in mind that albumin-bound selenol should be considered while investigating the mechanism of action and/or tracing the active metabolites of SeCs. More broadly, emerging cytotoxic SeCs are presumably predisposed to bind to albumin and RECID could be used for the quantification as well.

In dynamic contexts like cells and blood circulation, downstream selenol metabolites like methylselenol and hydrogen selenide might be present.[142,143] The high reactivity of selenol *per se* leads to low abundance at steady state and challenges direct measurement. As RECID relies on (re)generation and instant stabilization of selenol intermediates, it might be used for quantification of other selenol metabolites beyond the selenol form of parent compounds. Accordingly, depiction of a clearer metabolic pathway could be envisioned. On the other hand, it should be mentioned that several methods should be combined in order to establish the original formation pathway of selenol. Since albumin is present in various biological matrices (e.g. cell culture medium, tissue homogenate, cell lysate and plasma), we believe

that albumin binding prevails and ubiquitously interferes with quantification of SeCs. Accordingly, the application of RECID could be broadened from plasma to many other biological contexts.

5.3 Albumin, an Overlooked Key Cofounder

Albumin is ubiquitously present in biological contexts, while its critical role in the determination of SeCs cytotoxicity is firstly reported in the present investigations. In fact, albumin content varies among the cell culture media in common use (ranged 0.1-0.4%) and is as high as 3-5.5% in the plasma of laboratory animals and human. Such variability might account for the widely reported heterogeneity in cytotoxicity and alert potential discrepancies in translational studies. Furthermore, albumin level in patients might fluctuate dramatically depending on the pathophysiological conditions, which can in turn determine the treatment efficacy as well as adverse effects of SeCs.

Albumin is widely reported to have anti-oxidative function executed through "free radical-trapping" and "ligand-binding", [144] in contrast to the pro-oxidant SeCs. Considering the ubiquitous presence of the antioxidative system as well as other cellular components, we assumed that the strong antagonistic effect of albumin is unlikely due to trapping of free radical intracellularly. In other words, albumin was supposed to bind SeCs extracellularly and downregulate subsequent cellular uptake and cytotoxicity. In consistence, measurements of intracellular Se demonstrated an inverse relationship between extracellular albumin content and cellular uptake of SeCs (Figure 15B).

Since addition of albumin could decrease SeCs uptake, we assume that other biological and/or pharmacological functions of SeCs besides the cytotoxicity, like antimicrobial activity, might be similarly antagonized. From the therapeutic perspective, preclusion of albumin binding through controlled release of SeCs from nanomaterial or prodrug in combination with targeting moiety could probably improve the efficacy. This notion necessitates further exploration *in vitro* and *in vivo*.

SeCs formed macromolecular conjugates with albumin, while the uptake mechanism of the conjugate is not fully understood but might involve albumin scavenger receptors on basis of the competitive inhibition phenomenon.[145,146] Early studies had found tumor-specific distribution of Se and utilized selenite as radiotracer for tumor detection.[113] Our study might provide a mechanistic perspective that formation of SeC-albumin conjugate *in vivo* directs SeC to tumors that have high demand for albumin.[147] In this sense, exploitation of albumin scavenger receptors might impact uptake and subsequent functions of SeCs.

Noteworthy, 10 μM of *p*-XSC in complete medium was all in form of *p*-XSC-BSA and eradicated leukemia cells (Figure 7A; Figure 14A), indicating that the SeC-albumin conjugate is still internalized by the cells and biologically functional. This is in agreement with the systemic toxicity of SeCs in animals and human despite that the albumin content in circulation is even higher than the maximal concentration examined in the present studies (2.2%-5% *vs* 1.2%). Even though both free SeCs and SeC-BSA could be internalized, it is unclear regarding the relative contribution of each form in the overall cellular uptake and cytotoxicity. The functions of SeC-albumin also stress the importance of quantification of both the free form as well as the albumin-bound fractions.

In addition to albumin, other thiols like GSH and Cys that might participate in SeCs transformation.[135] However, our results indicated the absence of small-molecule conjugates in either fresh or conditioned cell culture medium, highlighting a predominant role of albumin in SeCs transformation. The superior reactivity of albumin might be attributed to Cys34 residue as well as high concentration in common biological matrices. A previous study by Olm et al. demonstrated that extracellular Cys could increase the uptake and cytotoxicity of selenite,[116] which seemingly contrasts with our finding. The disagreement might be due to the stepwise transformation of selenite: 1) reduction into more reduced forms (e.g. selenodiglutathione) in the presence of Cys; 2) binding to albumin. Therefore, the facilitator role of Cys in selenite uptake probably originates from its action on the former transformation step. Moreover, the study of Olm et al. did not specifically consider the effect of albumin.

5.4 Promises and Challenges in Treating Leukemia Mouse

The strong albumin binding property and importance of albumin-bound fraction underline the essentiality to quantify total SeCs. Using the newly established method, we depicted the pharmacokinetic properties of four diverse SeCs, for the first time (Figure 17). Ebselen and *p*-XSC are both highly lipophilic and should theoretically have large distribution volume *in vivo*. To our surprise, their distribution volume was close to blood volume in adult mouse (3.4-4.1 mL *vs* 2 mL; Table 5), supporting significant specie transformation. Secondly, due to the fact that the four SeCs all have strong affinity to albumin, their pharmacokinetic properties were assumed to resemble mouse albumin. On the contrary, rather unique pharmacokinetic properties were observed, particularly in case of MeSeA (Figure 17A). We also found that the half-life of the studied SeCs was much shorter compared to I^{131}-labelled mouse albumin (< 30 min *vs* 21 hr). Likely, SeC-albumin conjugate was more quickly eliminated by albumin scavenger receptors. However, this suspicion

requires further investigation. Apparently, more studies are warranted to delineate how albumin binding impacts ADME (absorption, distribution, metabolism and excretion) of different SeCs.

Since *p*-XSC was the most potent against leukemia cell *in vitro* and also had the highest exposure following intravenous administration, it was selected for treating leukemia mouse. The pharmacokinetic profile after intraperitoneal administration was also examined with the aim to optimize treatment regimen. However, the maximum concentration reached was well below the therapeutic concentration obtained from preliminary experiments (Figure 17B; Table 5). Thus, in later stage *p*-XSC was administered intravenously. The experiences further underline the importance of a proper analytical method as well as thorough pharmacokinetic investigation.

The treatment was started when C1498-*luc* cells homed into the bone marrow. Significant delay in disease progression following the *p*-XSC treatment was observed in comparison to the control group. The treatment efficacy was observed as well in the bone marrow. However, we believe that extension of treatment duration and increase the dose of *p*-XSC will significantly improve the therapeutic efficacy.

6 CONCLUSIONS

In the present thesis, we explored one group of the most redox-active agents, selenocompounds, for leukemia treatment. Among the six selenocompounds tested, *p*-xyleneselenocyanate (*p*-XSC) displayed remarkable cytotoxicity against leukemia cells through induction of oxidative stress. The cytotoxic activity was critically concentration- and time-dependent. Following *p*-XSC treatment, massive mitochondria damage and release of apoptosis-inducing factor were observed.

In pursuit of the pharmacokinetic properties for *in vivo* application, we observed that cytotoxic selenocompounds could transform into selenol intermediates and react with albumin thiols through selenium-sulfur bond. Accordingly, a selective, reproducible and robust method that measures both free and albumin-bound selenocompounds was established and enabled pharmacokinetic investigations.

Moreover, we found that albumin binding not only occurred in plasma but also in cell culture media. The macromolecular conjugate between selenocompounds and albumin was less internalized compared to free compounds, leading to reduced drug exposure intracellularly. As a result, the cytotoxicity of selenocompounds was antagonized by extracellular albumin.

Using the newly developed analytical method, we found that intraperitoneal administration of *p*-XSC would not reach the preliminary therapeutic threshold as established from cytotoxicity experiments *in vitro*. *p*-XSC was therefore intravenously administrated and shown to significantly postpone leukemia progression in the mouse model.

In general, this thesis proved that redox-active selenocompounds could be used for leukemia treatment and in the meanwhile provided that albumin content was a critical factor in determining the therapeutic efficacy.

7 FUTURE PERSPECTIVES

Firstly, the vulnerability of leukemia cells to *p*-XSC treatment in relative to non-leukemia cells was still low as shown by the small difference in the IC50s. Future endeavors should be devoted to expanding the therapeutic window and improving the selectivity of selenocompounds. One potential strategy would be searching for particular cancer subtypes that have defects in antioxidative system.[148-150] Alternatively, it would be worthy combining another strategy of selectivity. For instance, when the ROS inducer β-lapachone was combined with poly (ADP-ribose) polymerase inhibitors, cytotoxicity that is more selective was achieved.[151]

Secondly, our studies suggested that the overall cytotoxicity of selenocompounds partially originates from the SeC-albumin conjugate, but it remains unclear regarding the exact contribution of the conjugate in relative to that observed for the free compound. For free selenocompounds, the internalization efficiency could be correlated to hydrophobicity thereof; however, for SeC-albumin, that was not investigated in the present study. To address this question, it would be helpful to investigate different SeC-albumin conjugates that have the same Se level per molecule as well as absolute absence of albumin.

Thirdly, the antagonistic effect of albumin on the efficacy of selenocompounds is believed to prevail in different conditions. Preclusion of albumin binding through controlled release of SeCs from nanomaterial or prodrug could probably improve the efficacy. This might in parallel resolve the limitation regards poor solubility of selenocompounds, particularly *p*-XSC.

The last but not the least, even though *p*-XSC could eradicate leukemia cells *in vitro*, disease progression and persistence of leukemia lesion in bone marrow were still noticed in the mouse model. To achieve better therapeutic strategies in hematological malignancies, increasing the distribution of *p*-XSC into highly resistant microenvironment, like the bone marrow niche, might be one of the future directions. In addition, although the superoxide anion generated by *p*-XSC could be transformed into more reactive hydroxyl radicals by endogenous chaperones, it would be interesting to explore whether loading *p*-XSC into a well-designed targeted nanocarriers where Fenton reaction occurs could potentiate the treatment efficacy or not.

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