Antioxidant Stress and Anticancer of Peptide Chelate Selenium Element *in vitro*

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Abstract

This contribution reports a facile synthesis of anticancer bioactive peptides (ACBP) - functionalized selenium particles (ACBP-S-Se) with enhanced anticancer activities and a detailed mechanistic evaluation of its regulation of oxidative stress *in vitro*. Structural and chemical characterizations were proved by ultraviolet absorption spectrum (UV), Fourier transform infrared spectroscopy (FT-IR), X-ray photoelectron spectroscopy (XPS), Nuclear magnetic resonance carbon (¹³C NMR) and hydrogen spectra (¹H NMR), scanning electron microscope (SEM), energy dispersive X-ray spectrom (EDX) and inductively coupled plasma mass spectrometry (ICP-MS). The results show that ACBP are effectively sulfhydrylation modification with S-acetylmercaptosuccinic anhydride (SAMSA) *via* chemical absorption. After the polypeptide was modified by sulfhydrylation, there were many sulfhydryl groups on the molecule, and the sulfhydryl group was used as the binding site of Se. A panel of selected human cancer cell lines demonstrated high susceptibility toward ACBP-S-Se and displayed significantly reduced proliferative abilities. Finally, the results presented herein suggest bioactive peptide chelate selenium element effectively inhibited the proliferation of MKN-45 and MKN-74 cell *in vitro*, which in turns allowed the successful application of the ACBP-S-Se in highly complex human cell lines. The related the regulation of oxidative stress gene is *CDKN1A*, *CCNB1*, *TXN* and *MAP3K5*, while *CDKN1A* and *TXN* have ability to protecting cells to reduce oxidative stress and promoting cell growth arrest. Therefore, the great potential exhibited by ACBP-S-Se could make them an ideal candidate as a chemotherapeutic agent for human cancers, especially for gastric cancer.

1. Introduction

Oxidative stress refers to the aberrant production of active substances including reactive oxygen species (ROS) and nitrogen free radicals under various stress stimuli, which leads to unbalanced physiological status or oxidative stress-related damage via oxidation-reduction reactions¹. Rigorous studies have revealed that reactive oxygen species (ROS) generated by cells under oxidative stress (OS) are involved in gastric tumorigenesis².

Previous studies have confirmed that ROS is an actuation factor in gastric carcinogenesis both in clinical studies and in mouse models *in vivo*. The dysregulation of ROS promotes tumorigenesis and the development of abnormalities due to its ability to increase aberrant cell proliferation, survival, and cell migration *in vivo*³. Meanwhile, the aberrant production of ROS also induces DNA damage, leading to genetic disease, tumorigenicity and subsequent tumor progression⁴. If the accumulation of ROS overwhelms the death threshold, cellular apoptosis levels significantly increase, which enhance the antitumor effects of ROS production⁵. Denicola *et al.* confirmed that ROS alleviate certain specific cancers and that the redox state in cells is an important factor for the subsequent tumor formation potential, so the redox states of cells are likely to be potential therapeutic targets of cancers⁶. Finally, reactive oxygen species (ROS) may contribute to the regulation of apoptosis.

Selenium (Se), with a narrow range between deficiency and toxic effects⁷, is an important oligoelement and one of the most important trace elements in organisms⁸. Meanwhile, Se is a key component of the antioxidant system⁹, which makes an indelible contribution to human health. The chemical forms of Se regulate its diverse biological effects, toxicity and bioavailability, and supplementation with Se improves the redox system, promotes proper immune system function and has anticarcinogenic effects¹⁰. Se deficiency leads to heart disease, hypothyroidism and a weakened immune system, but excess exposure to Se results in gastrointestinal disturbances, hair and nail changes and neurologic manifestations^{11,12}. The presence of Se enhances the functions of the immune system, improves the proliferation and biofunctions of immune system cells, and enhances the phagocytosis of immune system cells, thereby improving the body's immune function and fundamentally improving its ability to resist disease^{13,14}. In addition, at higher concentrations, Se has pro-oxidant properties and is essential for the antioxidant activities involved in anticarcinogenic actions¹⁵. Meanwhile, Se inhibits hepatocyte necrosis and DNA damage by inhibiting CTX-induced oxidative stress¹⁶.

Previous studies have confirmed that Se is a promising therapy for cancer^{17,18} via the formation of an internal environment that inhibits cancer proliferation. After cancer operation, Se supplementation obviously controls cancer deterioration, reduces the toxicity and side effects of chemotherapy and radiotherapy, and helps anticancer drugs improve the curative effect^{19,20}.

Furthermore, Se, as an essential component of selenocysteine-containing proteins, is involved in most aspects of cell biochemistry and functions^{21,22}. Younis *et al.* reported that supplementation with insulin transferrin selenium (ITS) to semen extenders benefits the cryopreservation of chimpanzee (*Pan troglodytes*) spermatozoa²³.

Selenoproteins are essential for life, and several selenoproteins have been characterized as antioxidant enzymes, protecting against damage caused by free radicals, as already stated²⁴. Further studies on selenoprotein gene expression and cytokine content in the chicken thymus have shown that a significant decrease in the expression levels of selenoproteins could result in oxidative stress in chicken thymus²⁵. In addition, Se-containing molecules exert antioxidant properties associated with tumor growth, metastasis, angiogenesis, and drug resistance²⁶. One of the more exciting areas of research in drug design is the synthesis of so-called polypeptide selenium chelate, with the added advantage of cancer stem cell line inhibition and antioxidant activity²⁷. Here, the anticancer bioactive peptide (ACBP) is a low-molecular-weight active substance that was extracted from goat liver and was produced by the Clinical Medicine Research Center, the Affiliated Hospital at Inner Mongolia Medical University²⁸. Our former studies found that ACBP has superior antineoplastic activities and inhibits tumor growth in nude mice with Dutch gallbladder carcinoma. It also increases the chemotherapeutic sensitizing effect and reduces the side effects associated with chemotherapy²⁹. A series of nonnatural selenium-containing amino acid derivatives and peptides have been synthesized in a flexible and modular strategy to establish an anti-gastric cancer system for treating diseases³⁰. On the other hand, selenocarbohydrates, selenoamino

acids, and selenopeptides take part in the synthesis of a large number of biological compounds. However, the related oxidative stress genes and potential antitumor efficacy of the as-synthesized ACBP chelate selenium have not been previously demonstrated. Furthermore, the molecular mechanism of the antioxidant regulatory system of the peptide chelate selenium is very important to reveal the regularity of tumorigenesis and drug resistance.

Here, sulfhydrylation modifications of ACBP were performed with S-acetylmercaptosuccinic anhydride (S-AMSA) via chemical absorption. After sulfhydrylation, there were many sulfhydryl groups on the molecule, and the binding site of Se was used to link the sulfhydryl group. The molecular mechanism of the antioxidant regulatory system and antitumor effect of ACBP-S-Se on tumor cell lines were investigated *in vitro*.

2. Experimental Methods

2.1 Materials

ACBP was provided by the Clinical Medicine Research Center, Affiliated Hospital of Inner Mongolia Medical University. S-acetylmercaptosuccinic anhydride (S-AMSA) and hydroxylamine hydrochloride were purchased from Sigma.

2.2 Cell culture

The normal human gastric cell line (GSE-1) and human gastric cancer cell lines (MKN-45 and MKN-74) were purchased from the Institute of Cell Biology of the Chinese Academy of Sciences (Shanghai, China). GSE-1 and MKN-74 cells were cultured in DMEM (10566-016, Invitrogen, Thermo Fisher Scientific, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, 10091148, Gibco, Shanghai, China) and 100 U/mL penicillin and streptomycin (P/S) at 37°C in a humidified chamber containing 5% CO₂. MKN-45 cells were cultured in RPMI1640 growth medium (61870036, Invitrogen, Thermo Fisher Scientific, Shanghai, China) supplemented with 10% FBS and 100 U/mL P/S at 37°C in a humidified chamber containing 5% CO₂.

2.3 Synthesis of ACBP-S-Se

MOPS (2.08 g) was dissolved in ddH₂O (200 mL) to prepare a 3-morpholine-propanesulfonic acid (MOPS)-buffered solution. ACBP (1.0 g) was dissolved in a MOPS solution with a pH of 7.4 to obtain the ACBP solution. S-AMSA was added to the ACBP solution and incubated for 2 h, during which the pH was maintained at 7.4. Next, the sample was stored in nitrogen to avoid air oxidation products. Then, 0.05 mol/L hydroxylamine hydrochloride was added for further reaction for 3 h. After the isolation of the sulfhydrylated ACBP, purified ACBP-S was obtained by gel chromatography. Here, 0.66 g sodium selenite was added to the ACBP-S solutions for 6 h to obtain the raw product of ACBP-S-Se, and then gel chromatographic column purification was performed again to produce a pure ACBP-S-Se solution.

After vacuum freeze drying the ACBP-S-Se solution, the ACBP-S-Se particles were obtained for chemical structure characterization.

2.4 Physical and chemical property analyses

2.4.1 FT-IR spectroscopic analysis

FT-IR spectra were recorded using a Nicolet FT-IR 5700 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) under ambient conditions. The characterization of the four components (ACBP and ACBP-S-Se) was performed, and the samples were triturated with KBr at a ratio of 1:100 and pressed to form pelleted samples for FT-IR spectroscopic analysis at 500-4000 cm^{-1} .

2.4.2 X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS; Thermo Fisher, ESCALAB 250Xi, USA) was used to determine the elemental composition of ACBP and ACBP-S-Se, for which full (pass energy, 100 eV) and high-resolution spectra (pass energy, 20 eV) were recorded.

2.4.3 Particle morphology

The particle morphology of the ACBP-S-Se particles was examined by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDX). ACBP-S-Se was carefully lyophilized to maintain the surface structure. ACBP-S-Se was mounted, sputter-coated with gold, and observed under a Hitachi S-570 SEM microscope (Hitachi, Tokyo, Japan).

2.4.4 Carbon nuclear magnetic resonance (^{13}C NMR) and hydrogen nuclear magnetic resonance (^1H NMR) spectra

^1H -NMR and ^{13}C -NMR spectra were recorded in deuteriooxide (D_2O) at 17°C using a Bruker DRX 500 spectrometer. For each spectrum, approximately 1500 transients were collected using the following acquisition parameters: 3 s delays between pulses, 90° pulse of 5.4 μs , and contact time of 1 ms. Data were acquired and processed using Topspin 2.1 (Bruker, Rheinstetten, Germany).

2.4.5 SDS-Polyacrylamide gel electrophoresis

The molecular weights of ACBP, ACBP-S and ACBP-S-Se were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 16% separating gel and a 8% stacking gel according to previous studies³¹. The ACBP, ACBP-S and ACBP-S-Se powders were mixed in 0.1 M Tris-HCl buffer (pH = 6.8) containing 5% β -mercaptoethanol, 2% SDS, 0.02% bromophenol blue and 10% glycerin, with a sample concentration of 2 mg/mL. The mixed solutions were heated at boiling temperature for 5 min, while 15 μL of the prepared samples was injected into each well and then ran to the bottom of the gel at 100 V. The gel was stained using the Coomassie Blue (R-250) staining method, and the molecular weight was determined by comparison with the MW standard markers (BIO-RAD) (MW 10.0~180.0 kDa).

Images were analyzed by Image Lab software version 5.0 (2000 Alfred Nobel Drive, Bio-Rad Laboratories Inc., WDC, USA).

2.4.6 ICP-MS

The selenium content of ACBP-S-Se was determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies 7900ICP, Beijing Tai Chi Flying Co., Ltd.). After ignition, the tested elements and internal standard (10 bbp TuneA) were set up under standard model. The parameters were set up as follows: H₂/He was used as a collision gas with a flow velocity of 5.5 mL/min, the Co sensitivity was adjusted to 20000 CPS/ppb, the U signal maximum was adjusted, and then the Se in ACBP-S-Se was measured. The amount of selenium bound to ACBP was determined by the following formula:

$$n_{\text{Se}}/n_{\text{ACBP}} = (m_{\text{Se}}/M_{\text{Se}})/(m_{\text{ACBP}}/M_{\text{ACBP}}) = (C_{\text{Se}}/M_{\text{Se}})/(C_{\text{ACBP}}/M_{\text{ACBP}}) = 1228C_{\text{Se}}/C_{\text{ACBP}}$$

where C_{Se} is the concentration of Se determined by ICP-MS; C_{ACBP} is the concentration of ACBP determined by spectrophotometry (Nanodrop 2000C, Thermo Scientific, and Singapore).

2.5 Drug treatments

Before drug treatments, three cell lines were preplated in 6-well culture dishes (Corning, China) at a density of 5×10⁵ cells/well and randomly divided into the following groups based on the ACBP-S-Se supplement to the basic culture medium. When the cell confluence reached 70%, drug treatment was carried out. The optimization of the ACBP-S-Se concentration applied in this study was performed according to our former studies of BAPT³².

2.6 Cell proliferation

The proliferation ability of MKN-45 cells or MKN-74 cells after ACBP-S-Se treatments was determined by the IncuCyte Live Cell Analysis System according to our previous studies. Briefly, MKN-45 and MKN-74 cells were inoculated into a 96-well plate at a density of 5×10³/well and cultured for 24 h. After ACBP-S-Se treatment, the 96-well plates were placed in the designated location of the IncuCyte live cell workstation, and cell proliferation was recorded for 24 h at intervals of 2 h. Here, for MKN-45 and MKN-74 cells, the control group was not treated with any additional ACBP-S-Se (0 mg/mL), while the experimental group was treated with different concentrations of ACBP-S-Se (5 mg/mL, 10 mg/mL, 20 mg/mL, 40 mg/mL and 80 mg/mL). After the assay, the cell fusion degree was counted, and the cell proliferation rate was calculated.

2.7 Cell migration

The cell migration of MKN-45 cells and MKN-74 cells after ACBP-S-Se treatments was determined by the IncuCyte live cell workstation according to our previous studies. Briefly, MKN-45 and MKN-74 cells were inoculated into a 96-well plate at a density of 5×10⁴/well. After scratching, the cells were treated with ACBP-S-Se. Here, for MKN-45 and MKN-74 cell the control group was not treated with any additional

ACBP-S-Se (0 mg/mL), while the experimental group was treated with different concentrations of ACBP-S-Se (1 mg/mL and 5 mg/mL). The 96-well plate with drug treatment after the scratch was placed in the IncuCyte live cell workstation, and cell proliferation was recorded for 36 h at intervals of 2 h. After the assay, the original data were analyzed according to the scratch wound healing protocol.

2.8 Functional enrichment of oxidative stress-related genes

The screening and enrichment analysis of oxidative stress-related genes, network diagram of functional enrichment and association of genes with oxidative stress were evaluated by biological information analysis.

2.9 Reverse transcription PCR and Real-time PCR Analyses

After drug treatments for 24 h, the total RNA was extracted with TRIzol reagent (79306, Gibco, Shanghai, China), and then cDNA was synthesized according to the manufacturer’s instructions (Prime Script™ RT reagent kit with gDNA Eraser, RR047A, Takara, Dalian, China). For PCR amplification, specific primers were designed on the NCBI website and commercially synthesized by Invitrogen (Shanghai, China). Real-time PCR was performed within a Thermo Pikoreal machine with commercial kits (RR820A, Premix Taq™, Takara, Dalian, China).

The PCRs started with an initial melting cycle for 5 min at 95°C, followed by 35 cycles of melting at 95°C for 30 s, annealing at 58°C for 30 s and expansion at 72°C for 30 s. The ubiquitously expressed *β-actin* gene was used as an internal control. The qualities of the PCRs were confirmed by melting curve analyses, and all experiments were performed in triplicate. The relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method³³. The primers for reverse transcription PCR and real-time PCR analyses are shown in Table 1.

Table 1
Primers for reverse transcription PCR and real-time PCR analyses.

Primer	Primer sequences
hCDKN1A-F	GGGTGCGGTGATGGATAAA
hCDKN1A-R	ACTGCTGAGAACAGGAAGAAC
hCCNB1-F	GATGCAGAAGATGGAGCTGAT
hCCNB1-R	TCCCGACCCGTGGTTTTT
hTXN-F	GAAGCTCTGTTTGGTGCTTTG
hTXN-R	CTCGTCTGCTTCCCTCTT
hMAP3K5-F	CCCAGAGAGAGACAGCAGATA
hMAP3K5-R	CTCACTGAAAGAGCCCAGATAC

3. Results

3.1 Determination of the chemical structure of ACBP-S-Se by UV and FT-IR spectroscopy

As shown in Fig. 1A, the UV absorption value of ACBP was 203 nm. The ultraviolet absorption of selenium chelated by ACBP was obvious at 206 nm and 210 nm. It is predicted that there are new forms of -NH and -OH. There was weak absorption at 220 nm, and the formation of -CNO and -C-S bonds may have occurred. The absorption intensity of the carbonyl groups was increased because -NH and -OH are cosolor groups. Therefore, as shown in figure A to B, the $\lambda_{\max}$ of UV absorption increased from 0.12 to 0.62 mm. Compared with the maximum absorption peak of pure ACBP ($\lambda_{\max} = 206$ nm), the maximum absorption peak of ACBP-S-Se ($\lambda_{\max} = 210\text{--}220$ nm) moved to the long wave direction (4 ~ 14 nm). The height of the maximum absorption peak obviously increased. In the range of 210–220 nm, there were two high absorption peaks, which represent the characteristic absorption peaks of -C = O and -NH, -OH in -RCONHR'. There may also be secondary amide functional groups in the product after the sulfhydrylation and deacetylation of ACBP. From the FT-IR analysis of Fig. 1C, the distinction between the two maps (ACBP and ACBP-S-Se) was very obvious, indicating that the ACBP-S-Se curve represents the formation of a new substance, which is different from ACBP. In the ACBP-S-Se curve, 535 cm^{-1} and 640 cm^{-1} are the vibrational peaks of the Se-Se bond, and 736.8 cm^{-1} , 721 cm^{-1} and 775 cm^{-1} belong to the absorption peak of the C-Se bond. Moreover, 2336 cm^{-1} is the unique absorption peak of selenium, while 2600 cm^{-1} is the characteristic absorption peak of -SH, and 3391 cm^{-1} , 1585.5 cm^{-1} and 1406.2 cm^{-1} are the characteristic absorption peaks of secondary amide, these peaks are different from those in the ACBP curve as the absorption strength is enhanced. The above results indicate that ACBP binds with selenium to form ACBP-S-Se, and the ACBP chain is linked with sulfhydryl groups by amide bonds to form the ACBP chelate selenium complex.

3.2 Determination of the chemical structure of ACBP-S-Se by ^{13}C NMR and ^1H NMR

In the ^1H NMR spectra, the absorption peaks of -OH, -NH and -SH can be found in the attachment of $\delta 2.31$, $\delta 6 \sim 8.2$ and $\delta 1.43$, respectively (Fig. 1D). At the same time, there are different absorption peaks and different chemical shifts. In the ^{13}C NMR spectra, in the attachment of $\delta 180$, there are absorption peaks of CHO and -C-S at $\delta 100$ and absorption peaks of different degrees and different chemical shifts at the same time. The results showed that there were different amounts of binding between bioactive polypeptides and selenium in different proportions, so the molecular structure of ACBP-S-Se was inferred.

3.3 Determination of the chemical structure of ACBP-S-Se by XPS

As observed in Figure. 2, both types of polymers composed of pure ACBP and ACBP-S-Se exhibited significant differences in all four peaks in the XPS spectra: C 1 s (284.0-285.0 eV), N 1 s (399.0-400.0 eV), S 2p (166.8-167.57 eV) and Se 3d (55–60 eV) (Table 2). As seen from the XPS spectra of nitrogen, the binding energy decreased from 399.65 eV in ACBP to 399.02 eV in ACBP-S-Se. However, as seen from the XPS spectra of sulfur, the binding energy increased from 166.8 eV in ACBP to 167.57 eV in ACBP-S-Se.

Additionally, the binding energy of Se formed at 56.2 eV in ACBP-S-Se, the new -SH = Se bond in ACBP-S-Se was formed, the electronegativity of Se 3d increased, and the binding energy also increased.

Table 2 Assignments of the main special bands in ACBP-S-Se based on binding energy.			
Element	ACBP/eV	ACBP-S-Se/eV	Assignments
C 1 s	284.7	284.87	C–C
N 1 s	399.65	399.02	NH ₂ , NH ₃ ⁺
S 2p	166.8	167.57	NO ₂ SO ₃ , K ₂ SO ₃ , pNH ₂ C ₆ H ₄ SO ₂ C ₆ H ₄ NH ₂ ⁺
Se 3d	—	56.2	-SH = Se

3.4 SEM-EDX observation and ICP-MS of ACBP-S-Se

As shown in Fig. 3A, the morphology of ACBP is a coarse fibrous structure. From the morphology of the ACBP-S-Se in Fig. 3C, the particle structure has an average uneven grain of 5 μm in diameter. Compared with ACBP, in ACBP-S-Se, the distribution of the elements C, N and O decreased, while the distribution of elements Se and S increased.

As indicated in Fig. 3D, the selenium content was up to 0.28%. The selenium content was 829.030 [He]/PPb when the ratio of ACBP and selenium was 2:1 (Table 3). The content of selenium in the ACBP molecule was 2.1 per mole.

Table 3
Concentration of selenium in ACBP chelate selenium as determined by ICP-MS.

Sample	Concentration of selenium (Se[He]/PPb)	Concentration of ACBP (mg/mL)	RSD	nSe/nACBP
ACBP:Se (2:1)	829.030	0.05	0.3	2.1

3.5 The inhibitory effect of ACBP-S-Se on tumor cell lines

As shown in Fig. 4 (A and B), the growth of MKN-45 and MKN-74 cells was inhibited after treatment with ACBP-S-Se, and the rates of apoptotic cells were increased in a dose-dependent manner. The inhibition of MKN-45 and MKN-74 cell proliferation was not obvious with increasing concentrations of ACBP-S-Se from 5 mg/mL to 80 mg/mL.

The wound healing ability of MKN-45 and MKN-74 cells was detected in the control group and after treatment with different concentrations of ACBP-S-Se and was increased with increasing ACBP-S-Se concentrations, especially after treatment with 1 mg/mL ACBP-S-Se (Figure C and D). The results indicated that from 1 mg/mL to 5 mg/mL ACBP-S-Se has the ability to inhibit cell migration.

3.6 Functional Gene Selection Principles

The results of functional gene analyses showed 911 upregulated genes and 1122 downregulated genes after ACBP-S-Se treatment in MKN-45 cells. The screening results and enrichment analysis of oxidative stress-related genes are shown in Table 4.

Table 4
Screening and enrichment analysis of oxidative stress-related genes.

Classification	Characterization
GO:0006979	response to oxidative stress
GO:0008631	intrinsic apoptotic signaling pathway in response to oxidative stress
GO:0034599	cellular response to oxidative stress
GO:0036475	neuron death in response to oxidative stress
GO:0043619	regulation of transcription from RNA polymerase II promoter in response to oxidative stress
GO:0097193	intrinsic apoptotic signaling pathway
GO:1900407	regulation of cellular response to oxidative stress
GO:1900408	negative regulation of cellular response to oxidative stress
GO:1902175	regulation of oxidative stress-induced intrinsic apoptotic signaling pathway
GO:1902176	negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway
GO:1902882	regulation of response to oxidative stress
GO:1902883	negative regulation of response to oxidative stress
GO:1903202	negative regulation of oxidative stress-induced cell death
GO:1903204	negative regulation of oxidative stress-induced neuron death
GO:1903376	regulation of oxidative stress-induced neuron intrinsic apoptotic signaling pathway
GO:1903377	negative regulation of oxidative stress-induced neuron intrinsic apoptotic signaling pathway
hsa04068	FoxO signaling pathway
hsa04115	p53 signaling pathway

From the functional enrichment results of oxidative stress-related genes, a total of 75 genes with significant differences were selected (Fig. 5A). From the gene interaction mapping, *CDKN1A*, *CCNB1*, *TXN* and *MAP3K5* were extracted due to the role of most genes associated with them and the significantly enriched pathways, which can be used as downstream molecules (Fig. 5B).

In Fig. 6A, according to the interaction of genes related to oxidative stress and lncRNA, networks of oxidative stress genes and lncRNA interactions (lncRNA FPKM > 5) were constructed via lncRNA trans and cis analysis (TRANS_ENERGY<-50). The results showed that the network contained 55 genes related to oxidative stress and 37 lncRNAs.

The selected lncRNAs included MSTRG.13268.2, ENST00000508832, ENST00000580180, ENST00000454068, ENST00000607476, ENST00000504539, ENST00000326677, ENST00000448718, ENST00000531126 and MSTRG.5031.1. As shown in Fig. 6B-E and Table 5, the expression of oxidative stress-related genes (the 4 selected genes) in TCGA and the expression of *CDKN1A* increased after treatment with ACBP-S-Se, but the expression of this gene was lower in tumor samples than in paracancerous samples. The expression of the other three genes decreased after treatment with ACBP-S-Se but was lower in tumor samples than in paracancerous samples.

Table 5
Expression of CDKN1A, CCNB1, MAP3K5 and TXN under oxidative stress in TCGA.

Gene name	MKN-45-ACBP	MKN-45	fc	log2(fc)	Regulation
CDKN1A	264.77	9.55	25.92	4.7	up
CCNB1	8.99	25.89	0.33	-1.62	down
MAP3K5	0.99	2.94	0.32	-1.66	down
TXN	38.74	74.24	0.49	-1.03	down

3.7 Relative gene expression levels

As shown in Fig. 6, the *CDKN1A*, *CCNB1*, *TXN* and *MAP3K5* gene expression levels in GSE-1, MKN-45 and MKN-74 cells were analyzed by real-time PCR. In the GSE-1, MKN-45 and MKN-74 cells, the gene expression levels of *CDKN1A* and *TXN* were significantly increased after ACBP-S-Se treatment compared with the control condition. In addition, the gene expression level of *CCNB1* and *MAP3K5* in GSE-1 cells was significantly different between the control group and ACBP-S-Se treatment group; however, the gene expression level of *MAP3K5* in MKN-45 cells was significantly increased, and the gene expression level of *MAP3K5* in MKN-74 cells was significantly decreased after ACBP-S-Se treatment. The results indicated that *CDKN1A* and *TXN* have the ability to protect cells to reduce oxidative stress and promote cell growth arrest.

4. Discussion

Gastric cancer (GC) is one of the major threats to human health worldwide. Previous studies have demonstrated that ACBP combined with oxaliplatin (OXA) significantly inhibited the growth of MKN-45 cells. Recently, the replacement of peptides with chemical modifications to achieve the same therapeutic effects as natural peptide counterpart in combined chemotherapeutics has been rapidly developed. Furthermore, the incorporation of the Se atom in amino acids and peptides is mostly restricted to selenocysteine derivatives, and there is still a need for further developments toward the synthesis of amino acid-derived chiral selenium compounds, including potential candidates for biological evaluations. Here, ACBP was modified by sulfhydrylation to combined Se to form the ACBP selenium chelate, and its properties and inhibitory effect on GC cells *in vitro* was investigated.

From the FT-IR, XPS, ¹³C NMR and ¹H NMR, SEM, EDX and ICP-MS analyses, the results show that new functional groups were formed, such as -C-Se, Se-Se, -SH = Se and -CONH. ICP-MS techniques are used to determine the single elements and multielement analysis in synthetic drugs. The higher content of Se in ACBP-S-Se than in ACBP indicates that Se may have been successfully incorporated into the molecular chain of ACBP. Meanwhile, Se is a trace element that is beneficial to humans depending on its concentration and chemical speciation³⁴. Studies have shown that different Se compounds could reduce cancer growth as potential anticancer drugs³⁵. Selenium deficiency led to increased cell apoptosis and decreased cell viability. Selenium supplementation may mitigate these alterations³⁶. Interestingly, in this study, we showed that ACBP-S-Se at 5 mg/mL effectively inhibited the proliferation of MKN-45 and MKN-74 cells. In addition, the wound healing of MKN-45 and MKN-74 cells after ACBP-S-Se treatment reduced cell migration.

The polypeptide has high glutathione peroxidase (GPX) activity and can clear the free radicals in human body³⁷. Although several functions of selenoproteins are unknown, many disorders are related to alterations in selenoprotein expression or activity. Selenium insufficiency and polymorphisms or mutations in the genes encoding selenoproteins and synthesis cofactors are involved in the pathophysiology of many diseases, including immune dysfunctions and cancer²⁴. Considering the perspective of oxidative stress to explore the ceRNA mechanism of ACBP-S-Se in the treatment of GC, it is necessary to determine the changes in the expression of oxidative stress-related genes after ACBP-S-Se treatment. By carefully studying oxidative stress-related pathways and genes, functional genes were detected.

From all these genes, the ones that were consistently regulated were the selenoprotein genes. Furthermore, the Se-specific effects noted were caused primarily by Se deficiency and not high Se³⁸. A total of 75 genes with significant differences were selected. From the gene interaction mapping, *CDKN1A*, *CCNB1*, *TXN* and *MAP3K5* were selected. The cyclin-dependent kinase inhibitor 1 A (*CDKN1A*, also known as p21), a cell-cycle-dependent kinase suppressor molecule, has been shown to protect cells from oxidative stress damages³⁹. Cyclin B1 (*CCNB1*), also inhibits certain specific factors in ubiquitin-proteasome⁴⁰. For example, hydrogen peroxide prevents ubiquitin (Ub) from binding to late-promoting

complex (APC/C) substrates, which also prevents the degradation of *CCNB1* and thus promotes cell growth arrest caused by oxidative stress. Increased *CCNB1* expression has been observed in several cancers; for example, the expression of *CCNB1* in gastric cancer tissues was higher than that in normal gastric tissues⁴¹. However, ACBP-S-Se treatment decreased the expression of *CCNB1* in MKN-45 and MKN-74 cells. The thioredoxin protein gene (*TXN*) plays an important role in cellular antioxidant defense^{42, 43}. Mitogen-activated protein kinase kinase kinase 5 (*MAP3K5*), also known as apoptotic signal-regulated kinase 1, regulates the biological and physiological processes of apoptosis, immunity and gastric emptying by regulating the flow of apoptotic kinases⁴⁴. Furthermore, *MAP3K5*, activated in response to stress signals, plays an important role in oxidative stress regulation, cell proliferation, differentiation and death, and immune response⁴⁵. It has been shown that a molecular target of reactive oxygen species (ROS), thioredoxin (TXN/Trx), is an inhibitor of *MAP3K5*⁴⁶. Moreover, oxidation via ROS disrupts the binding of *Trx* to *MAP3K5*, resulting in apoptosis⁴⁷. The results show that the formation of ACBP-S-Se negatively regulates *MAP3K5* activity, thus attenuating the proapoptotic signal in MKN-74 cells. Inhibition of apoptosis via this mechanism would result in increased proliferation and survival, increasing the probability of tumorigenesis and/or metastases. Therefore, oxidative stress is closely related to the occurrence and development of cancer. Oxidative stress results in cell apoptosis/death, stressors alter the miRNA expression profile, and miRNAs play a role in the cell response to stress.

Conclusion

In summary, the application of sulfhydrylation and deacetylation is an effective method to enhance the chelating efficiency of ACBP and Se. According to the UV, FT-IR and ¹³C NMR, ¹H NMR, XPS, EDX, and ICP-MS analyses, Se was chelated into ACBP via sulfhydrylation; the sulfhydryl group was used as the binding site of Se and increased the chelating rate. Finally, the results presented herein suggest ACBP-S-Se effectively inhibited the MKN-45 and MKN-74 cell growth and migration *in vitro*, which in turns suggests that ACBP-S-Se could be applied in the clinic.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

XL, XJW, GL, YNX and XLS analyzed and interpreted the patient data. RY, FJ, CLS and XLW do the experiment. XL was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 2

The XPS results of ACBP-S-Se, including the C, N, O, S, and Se elements.

Figure 3

The SEM and EDX results (distribution of the elements C, N, O, Se, S) of ACBP (A) and ACBP-S-Se (B) after vacuum freeze drying; the elemental content of ACBP (C) and ACBP-S-Se (D).

Figure 4

The proliferation of MKN-45 (A) and MKN-74 cells (B) after treatment with different concentrations of ACBP-S-Se was detected by the IncuCyte live cell workstation. Wound healing of MKN-45 (C) and MKN-74 cells (D) treated with different concentrations of ACBP-S-Se as detected by the IncuCyte live cell workstation after the cell wound scratch assay. ACBP-S-Se (0 mg/mL) was used as the control group.

Figure 7

The relative hCDKN1A, hCCNB1, hTXN and hMAP3K5 gene expression levels after treatment with 5 mg/mL ACBP-S-Se were detected by RT-PCR. ACBP-S-Se (0 mg/mL) was used as the control group. *, represents $P < 0.05$ compared with the control group.

Figure 8

Mechanism of the synthesis of ACBP, S-SAMS and sodium selenite. First, ACBP was submitted to sulfhydrylation modification by S-AMSA and then participated in chelation reactions with sodium selenite.