The Inhibition of H1N1 influenza virus-induced apoptosis by functionalized Selenium nanoparticles with β -Thujaplicin through ROS-mediated P53 and AKT signaling pathways

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Abstract

β -Thujaplicin possess a variety of biological activities. The use of modified biological nanoparticles (NPs) to develop novel anti-influenza drugs has increased in recent years. Selenium nanoparticles (SeNPs) with antiviral has attracted increasing attention for biomedical intervention. Functionalized SeNPs by β -Thujaplicin (Se@TP) surface modified with superior antiviral were synthesized in this study. β -Thujaplicin decoration of SeNPs obviously inhibited H1N1 infection and were less toxicity. Se@TP could inhibit H1N1 from infecting Madin Darby Canine Kidney (MDCK) cells and block chromatin condensation and DNA fragmentation. Se@TP obviously prevented MDCK cells from generating reactive oxygen species (ROS). Furthermore, Se@TP prevent lung injury in H1N1 infected mice through eosin staining and hematoxylin *in vivo*. Additionally, when treated with Se@TP, the DNA damage of lung tissues reduced substantially by TUNEL-DAPI test. Mechanistic investigation revealed that Se@TP inhibited H1N1 influenza virus from infecting MDCK cells through induction of apoptosis via suppression AKT and p53 signaling pathways through Immunohistochemical assay. Our results suggest that β -Thujaplicin modified SeNPs as carriers is an efficient way to achieve antiviral pharmaceutical candidate for H1N1 influenza.

Introduction

As a segmented RNA virus, Influenza virus affects millions of people and is still a serious contagious pathogen in seasonal epidemics.^{1,2} H1N1 influenza virus is a highly infections respiratory disease which belongs to influenza A type viruses.³ H1N1 Influenza virus was discovered and identified in US and Mexico in 2009 which infected more than 8768 deaths in 207 countries.⁴ Due to the arising mutation of antigenic shifts and the genome in different species, influenza virus may emerge a novel influenza among humans in the future.⁵ The influenza infection cycle included three steps: First, the influenza viruses attach to the host cell surface receptor and fuse with the endosomal membrane. Second, uncoating of nucleocapsid and multiplication of the genetic material occurs. Finally, the influenza protein and new viron is expressed and released.^{6,7} On the surface of influenza virus, there are two important glycoprotein: Hemagglutinin (HA) and Neuraminidase (NA).⁸ In the early stage of viral infection, HA combined sialic acid-containing receptors on host cells and mediated the entry and fusion of virus.^{9,10} when mature viruses separate from host cell surface, NA plays an important role in assisting virus cleave the linkage between sialic acid and Hemagglutinin.¹¹ Although, the conventional way to restrain the spread of influenza infections is vaccination. But, the restraining the spread of influenza infections is the long period between the rapid virus evolution and vaccine development.¹²

β -Thujaplicin (2-hydroxy-4-isopropyl-2,4,6-cycloheptatrieneone) is an antimicrobial tropolone derived from geranyl pyrophosphate (GPP) and monoterpene intermedicate.^{13,14} β -Thujaplicin have been reported to possess antibacterial and antifungal activities. However, anti-influenza virus effects of β -Thujaplicin remain unclear.^{15,16} The nanotechnology provided a new prospect to solve these problems, and the new antiviral nanodrug should effectively inhibit viral infection with fewer cytotoxicity.¹⁷⁻¹⁹ Gold

nanoparticles conjugated to consensus M2e peptide against influenza A viruses was reported by Tao et al.²⁰ Different sizes of polyvalent nanoparticles inhibited virus was reported by Vonnemann et al.²¹ Wang et al reported that SiO₂@LDH nanoparticles enhance the response of hepatitis B virus DNA vaccine.²² Shen et al reported efficient Encapsulation of Fe₃O₄ Nanoparticles into genetically engineered Hepatitis B core virus-like particles.²³ Zhang et al reported silver nanoparticle treatment ameliorates biliary atresia syndrome in rhesus rotavirus inoculated mice.²⁴ among of them, selenium nanoparticles (SeNPs) attract much attention due to the unique antimicrobial activities.²⁵ Selenium is integral component of several selenoproteins which control several crucial biological processes.^{26,27} The deficiency of selenium could enhance the susceptibility to infections including respiratory virus infections.²⁸ Therefore, the aim of the present study was to exposure novel functionalized selenium nanoparticles which can inhibit the infection of H1N1 virus. We hypothesized that β -Thujaplicin modified SeNPs (Se@TP) have excellent antiviral activity against H1N1 virus. Though several research groups have described the antimicrobial effects of SeNPs, the antiviral mechanisms is still unclear. This study was to verify how β -Thujaplicin modified SeNPs to inhibit H1N1 influenza virus in vitro and in vivo.

Materials And Methods

Materials

Na₂SeO₃, Vitamin C, β -Thujaplicin, propidium iodide, 6-coumarin were provided from Sigma. Fetal bovine serum (FBS) and Dulbecco's modified eagle medium (DMEM) and were purchased from Gibco. lyso tracker, Thiazolyl blue tetrazolium bromide (MTT) were from Sigma. phosphorylated p53 (p-p53), Phosphorylated AKT (p-AKT) and β -actin antibodies were purchased from Cell Signaling Technology (CST). Madin darby canine kidney cells (MDCK) were obtained from American Type Culture Collection (ATCC® CCL-34TM). H1N1 influenza virus was provided by Guangzhou Women and Children's Medical Center, Guangzhou Medical University. Mice were obtained from the Guangdong Medical Laboratory Animal Center (Guangdong, China). All animal procedures were performed according to the guidelines of Guangdong Medical Laboratory Animal Center.

Preparation and Characterization of Se@TP

β -Thujaplicin modified Selenium nanoparticles were prepared as follow: 0.25 ml stock solution of Na₂SeO₃ (0.1 M) was gradually added into 2 ml stock solution (50 mM) of Vitamin C. Then, 10 μ l 40 mg/ml of β -Thujaplicin was added into the selenium nanoparticles solution. The excess β -Thujaplicin, Vitamin and Na₂SeO₃ were removed by dialysis for overnight. Se@TP nanoparticles were sonicated and then filtered through 0.2 μ m pore size. The concentration of SeNPs and TP was measured by ICP-AES. The morphology and elemental composition of Se@TP nanoparticles were characterized by Transmission Electron Microscopy (TEM, H-7650) and EDX (EX-250 system, Horiba). Malvern Zetasizer Software was used to monitor the zeta potential and size distribution of Se@TP.

Determination of cell viability

The cytotoxic of Se@TP nanoparticles was performed as previously reported.^{29,30} First, H1N1 influenza virus was added to MDCK cells for 2 h, and then the indicated concentrations of β -Thujaplicin with or without SeNPs were added to MDCK cells for 24 h. Then, MTT (20 μ l/well) was added for 5 h. The formazan crystals were recorded at the absorbance of 570 nm.

Intracellular localization of Se@TP

MDCK cells were treated with lyso tracker for 60 min as previous reported.³¹ DAPI and coumarin-6 labelled Se@TP were added for various periods of time incubation. Fluorescence microscope was used to obtain and analysis the image.

Caspase-3 activity and TUNEL-DAPI co-staining assay

The caspase-3 activity with the wavelengths at 380 nm (excitation) and 460 nm (emission) was detected as previously described.³² DNA fragmentation was examined with fluorescence staining by the TUNEL apoptosis detection kit.³³ MDCK cells were confirmed with TUNEL for 1 h and incubated of DAPI for 15 min at 37 °C for nuclear staining.

Thin sections of MDCK cells and ROS generation

TEM analysis of MDCK cells were detected as previous described.³⁴ After incubation with H1N1 influenza virus for 2 h, MDCK cells were washed with PBS and incubated with Se@TP. Se@TP-treated MDCK cells induced ROS accumulation was detected as previously described.³⁵ The ROS generation was monitored through the fluorescence intensity (excitation 500 nm and emission 529 nm).

Animals infection and treatment

Fifteen female BALB/c mice (aged 4–6 weeks) were randomly divided into five groups as follow: control group, H1N1 virus, virus + TP, virus + SeNPs, virus + Se@TP. All mice were anesthetized with 10% chloral hydrate at a dose of 3 μ l/g. Then the control group was treated with 20 μ l physiological saline by nasal dripping while the other four groups with 20 μ l H1N1 virus by nasal dripping as well. 24 h later, TP, SeNPs and Se@TP were administered to anesthetized mice respectively via intranasal absorption every 24 h thereafter for a total of three times. The lungs were extracted, affused with physiological saline and fixed in paraformaldehyde before hematoxylin and eosin (HE) staining, tunel test and immunohistochemistry staining. All mice experiments were approved and guided by the Ethics Committee of Guangzhou Medical University.

Statistical analysis

All the data are presented as mean $\pm$ SD. One-way analysis of variance (ANOVA) was used in multiple group comparisons. Difference with $P < 0.05$ (*) or $P < 0.01$ (**) was considered statistically significant.

Results And Discussion

Preparation and Characterization of Se@TP

The light images and Tyndall effect of Se@TP were as shown in Fig. 1A, 1B and 1C, the results indicated that Se@TP nanoparticles were synthesized. The morphology of Se@TP presented spherical particles in Fig. 2A shape. TP modified with SeNPs formed more compact and stable globular nanocomposites. As shown in Fig. 2B, EDX indicated the signal of C (10%) and O (8%) that from TP, the percentage of Se atoms was 82%. As shown in (Fig. 2C and 2D), Se@TP was decreased from 200 nm to 80 nm which indicated much smaller size. The zeta potential of SeNPs (-25 mv) was lower than Se@TP (8 mv), demonstrating the higher stability of Se@TP than SeNPs.

Antiviral of Se@TP

MDCK cells infected by H1N1 influenza was showed reduction in cells numbers and loss of cell-to-cell contract as shown in Fig. 3A. When co-treatment with Se@TP, the cells morphological were slightly changed. In Fig. 3B, the cell viability were 26% (virus), 43% (virus + TP), 67% (virus + SeNPs) and 88% (virus + Se@TP). The results indicated that the antiviral of SeNPs was effectively amplified by TP. Minimum inhibitory concentration (MIC) of drug A combination present in Se@TP of Se (125 μ M); MIC of drug B combination present in Se@TP of TP (2.5 μ M); MIC of drug A alone corresponded to free SeNPs (1 mM); MIC of drug B alone corresponded to free TP (10 μ M). Fractional Inhibitory Concentration (FIC) was calculated as (MIC drug A combination/MIC drug A alone) + (MIC drug B combination/ MIC drug B alone) = 125 μ M / 1 mM + 2.5 μ M / 10 μ M = 0.375. FIC was 0.375, below 0.5, indicating synergy. In this study, the FIC index was basically interpreted as follows: FIC < 0.5, synergy; FIC between 0.5 and 2, indifference; FIC > 2, antagonism. The results suggest that Se@TP effectively inhibited the proliferation of H1N1 influenza virus.

Intracellular localization of Se@TP

The lysosomes was found and increased in a time-dependent manner in MDCK cells as shown in Fig. 4. After 30 min, Se@TP escapes from lysosomes and transported in the cytosol. Then distributed into the cells after 60 min. This result showed the target organelle of Se@TP was lysosome.

Detection of Caspase-3 activity

The caspase-3 activities were 451% (virus), 332% (virus + TP), 290% (virus + SeNPs) and 190% (virus + Se@TP) as shown in Fig. 5. The treatment of H1N1 influenza virus infected MDCK cells remarkably increased the activity. TP and SeNPs slightly inhibited the caspase-3 activity, Se@TP significantly decreased the caspase-3 activity. The result showed that the Se@TP inhibits the H1N1 influenza virus through capase-3 activity.

Inhibition of H1N1 influenza virus infection

Typical apoptotic features with H1N1 influenza virus with nuclear condensation (blue fluorescence) and DNA fragmentation (green fluorescence) was exhibited by MDCK cells in Fig. 6. After treatment with H1N1 virus for 24 h, the number of TUNEL-positive cells was significant increased. Co-treatment with Se@TP obviously inhibited the H1N1 influenza virus-induced changes. The results indicated that Se@TP inhibited the apoptosis of MDCK cells by H1N1 influenza virus.

Inhibition of ROS generation by Se@TP

Microvilli and mitochondria were observed in MCK cells. As shown in Fig. 7A When MDCK cells was incubated with H1N1 influenza virus, TEM image indicates distorted organelles, shrinking cytoplasm and condensed chromatin. The mitochondria of MDCK cells recovered the shape after treatment with Se@TP in Fig. 7B. The ROS generation was conducted by DCF determination to indicate the action mechanisms of Se@TP. The intracellular ROS generation were 430% (virus), 332% (virus + TP), 260% (virus + SeNPs) and 130% (virus + Se@TP) as shown in Fig. 8A. TP and SeNPs slightly inhibited the ROS generation. However, Se@TP remarkably decreased the ratio of ROS generation. The fluorescent intensity of DCF treated with H1N1 influenza virus was much stronger than TP, SeNPs and Se@TP in Fig. 8B. The results indicate the ROS participated in the antiviral action.

In vivo antiviral

Mice were infected with H1N1 virus and treated with TP, SeNPs and Se@TP, followed by HE staining, tunel analyses and immunohistochemical test of lungs tissues after being executed (Fig. 9A). As the HE staining present (Fig. 9B), H1N1 infected group manifested as alveolar collapse, perivascular and peri-bronchiolar edema. When treated with TP or SeNPs, the symptoms lessened. Se@TP attenuated the histopathological manifestations substantially. The result indicated that Se@TP protected the lungs from being injured. Se@TP nanoparticles prevent the DNA damage during H1N1 infection. Meanwhile, the HE stain and tunel analysis illustrated that Se@TP inhibited MDCK cells infection by H1N1 influenza virus. Caspase-3, P-AKT, T-AKT, P-P53 and P53 proteins were detected and positive after H1N1 virus infection as showed in Fig. 10. The results demonstrated that Se@TP inhibited H1N1 influenza virus-induced MDCK cell apoptosis by ROS mediated AKT and p53 signalling pathway (Fig. 11).

Conclusions

In conclusion, Se@TP with lower toxicity exhibits superior antiviral abilities to prevent H1N1 influenza virus infection in this study. The mechanisms of antiviral showed that Se@TP inhibited caspase-3 mediated apoptosis through ROS generation. In vivo antiviral result showed that Se@TP inhibited MDCK cells apoptosis through regulating the AKT and p53 signalling pathways. In summary, the nanosystem of Se@TP might provide a promising selenium species with antiviral activity to against H1N1 influenza virus.

Abbreviations

FBS: Fetal bovine serum; DMEM: Dulbecco's modified eagle medium; MTT: Thiazolyl blue tetrazolium bromide; p-p53: phosphorylated p53; p-AKT: Phosphorylated AKT.

Declarations

Availability of Data and Materials

All data are fully available without restriction.

Authors' contributions

Changbing Wang, Mingqi Zhao and Zhengfang Lin designed the study, analyzed the experimental data and drafted the manuscript. Min Guo, Tiantian Xu and Ying Tang carried out the experiments. Yi Chen, Liang Hua, Jiayu Zhong, Tao Lin, Guangwan Lian and Huanhui Chen participated in the design. Bing Zhu and Yinghua Li refined the manuscript and coordination. All authors read and approved the final manuscript.

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Disclosure

The authors report no conflicts of interest in this work.

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Figures

Figure 1

Light images of Se@TP, SeNPs and TP.(A) Color changes in Se@TP, SeNPs and TP. (B) and (C) Tyndall effect in Se@TP.

Figure 2

Characterization of SeNPs and Se@TP. (A) TEM images of SeNPs and Se@TP. (B) EDX analysis of Se@TP. (C) Size distribution of SeNPs and Se@TP. (D) Zeta potentials of SeNPs and Se@TP.

Figure 3

Effects of Se@TP on the growth of H1N1 infection of MDCK cells by MTT assay. (A) Morphological changes in H1N1-infected MDCK cells observed by phase-contrast microscopy. (B) Antiviral activity of Se@TP. Concentration of SeNPs was 1 mM and TP 10 μ M. Bars with different characters are statistically different at * $p < 0.05$ or ** $p < 0.01$ level.

Figure 4

Localization of coumarin 6 loaded Se@TP in MDCK cells. The cells were treated with coumarin 6 loaded Se@TP for different periods of time and were observed under a fluorescent microscope s tained with lysotracker (red fluorescence, lysosome) and DAPI (blue fluorescence, nucleus)

Figure 5

Inhibition of caspase 3 activity by Se@TP. Cells were treated with TP, SeNPs and Se@TP, and caspase 3 activity was detected by synthetic fluorogenic substrate. Concentration of SeNPs was 1 mM and TP 10 μ M. Bars with different characters are statistically different at * $p < 0.05$ or ** $p < 0.01$ level .

Figure 6

Se@TP induced apoptosis in H1N 1 infection of MDCK cells. DNA fragmentation and nuclear condensation as detected by TUNEL DAPI co staining assay. Concentration of SeNPs was 1 mM and TP 10 μ M.

Virus

Virus+Se@TP

Figure 7

TEM images of thin sections of MDCK cells treated with Se@TP. (N: nucleus M: mitochondria Mv: microvillus)

Figure 8

ROS overproduction induced by Se@TP in H1N1 infection of MDCK cells. (A) ROS levels were detected by DCF fluorescence intensity. (B) H 1N1 infection of MDCK cells preincubated with 10 μ M DCF for 30 min and then treated with Se@TP. Concentration of SeNPs was 1 mM and TP 10 μ M. Bars with different characters are statistically different at * $p < 0.05$ or ** $p < 0.01$ level .

Figure 9

Istopathological analysis of Se@TP effects on the lung tissue sections of mice.(A) Photos of extra lung. (B) In vivo antiviral efficiency of Se@TP. Pathologic lesions of lung tissues of HE and TUNEL DAPI staining.

Figure 10

In vivo antiviral efficiency of Se@ TP by immunohistochemistry of. Mice without infection performed as control.

Figure 11

Apoptotic signaling pathways by TP in H1N1 infection of MDCK cells. The main signaling pathway of ROS mediated AKT and P53 signaling pathways.