Effects of a Biotinylated Squamocin Molecule on Apoptosis in Human Nasopharyngeal Carcinoma Cells

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Abstract

Background: Squamocin is one of the Annonaceous acetogenins isolated from the Annonaceae family. In recent years, it has been found that it can inhibit the growth of various tumor cells. In previous work, to improve the therapeutic potential of squamocin for cancer therapy, we directly coupled biotin to different carbon atoms of squamocin and acquired a set of squamocin derivatives. The effect of biotinylated squamocin on tumor cells has not been reported. In this study, we will describe the effects of a biotinylated squamocin called Sq-6 in nasopharyngeal carcinoma cells. Methods: To understand the biological function of Sq-6 in nasopharyngeal carcinoma cells,we used MTT assay to observe the effect of Sq-6 on cell proliferation of apoptosis of nasopharyngeal carcinoma cells. The Annexin V-FITC / PI double staining assay in flow cytometry was used to detect the effect of Sq-6 on the CNE and CNE-1 cells . Western blot was used to detect the expression levels of proteins related to apoptosis and JNK pathway. Results: Sq-6 inhibited the proliferation of CNE and CNE-1 cells in a dose and time-dependent manner. By flow cytometry and western blot assay, we found Sq-6 induced cell apoptosis in a does-dependent manner and increased the expression of cleaved- PARP , cleaved-caspase9 and cleaved-caspase3. Western blot results showed that the expression of p-JNK and p-c-Jun was up-regulated after the treatment of Sq-2. Conclusion: Sq-6 inhibits the proliferation and induces apoptosis of human nasopharyngeal carcinoma cells by activating JNK signaling pathway.

Keywords

Squamocin,Apoptosis, JNK pathway, Nasopharyngeal carcinoma.

1. Introduction

Nasopharyngeal carcinoma (NPC) originates from the nasopharynx epithelium and is highly prevalent in South-East Asia [1]. NPC is associated with Epstein-Barr virus (EBV) infection with a high incident of treatment failure and overall poor prognosis[2]. Treatment of NPC is an integrated treatment that includes chemotherapy, radiotherapy, and chemoradiotherapy[3]. Although some chemotherapeutic drugs such as cisplatin have a certain clinical effect, the side effects of these chemotherapies have caused some harm to patients[4]. Therefore, it is very urgent to seek new and low-toxic side effects natural medicines for the treatment of nasopharyngeal carcinoma.

Squamocin is a monomer extracted and purified from Annonaceous acetogenins, which can inhibit the proliferation of various cancer cell lines, such as breast cancer, colon cancer, gastric cancer, pancreatic cancer and so on[5].Biotin is an essential nutrient for the human body, which can serve as a targeting ligand in the design of tumor drugs, and then absorb the drug connected to the ligand into tumor cells to improve the targeting of the drug[6,7].Therefore, based on previous research, in order to improve the effectiveness of squamocin in cancer treatment, we connected biotin and Squamocin through chemical synthesis methods, and obtained a series of biotinylated squamocin monomers[8]. One of the biotinylated squamocin monomers was later named Sq-6, and its effect on cancer cells has not been reported. This experimental study will describe the investigation of the molecular mechanism of nasopharyngeal carcinoma cells under Sq-6 treatment.

2. Materials and Methods

2.1 Materials and reagents

The human nasopharyngeal carcinoma cell lines CCNE, CNE-1, CNE-2 and S18 were purchased from American Type Culture Collection (ATCC). Sq-6 was modified by squamocin, an ortho-bis-tetrahydrofuran lactonide which was chemically coupled to biotin at positions 24. The structure is shown in Fig. 1A. The drug powder was prepared into a 25 mM stock solution using DMSO (Sigma, USA), and stored at -80°C. The BCA protein concentration determination kit and skimmed milk powder were purchased from Shanghai Biotech Biotechnology(Shanghai). The antibodies against c-Jun, Phospho-c-Jun,SAPK/JNK, Phospho-SAPK/JNK(Thr183/Tyr185), PARP, cleaved-PARP, Caspase3, cleaved-Caspase3, Caspase9, cleaved-Caspase9 and GAPDH were purchased from Cell Signaling Technology (MA, USA). In addition, Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were also purchased from Cell Signaling Technology (MA, USA).

2.2 Cell culture

The human nasopharyngeal carcinoma cells CNE, CNE-1, CNE-2 and S18 were cultured with RPMI 1640 medium(Gibco, USA) supplemented with 10% FBS (Serana Europe GmbH, Germany) at 37°C under a humidified 5% CO2 and 95% air at an atmosphere. The medium was changed every 2 days.

2.3 Mearsurement of cell viability

Cells were seeded into 96-well plates at a density of 5000 cells per well, and cultured in the culture medium for overnight to adhere to the wall. And then, cells were incubated with different concentrations drug-containing culture medium for 48 hours or 72 hours. After that, 10 μl MTT (5 mg/mL,KayGEN Biotech, Jiangsu) was added into each well and incubated at 37°C for 4 to 6 hours. Removed the cell culture medium and added 100 μl DMSO to each well and shook to dissolve. Finally, a microplate reader was used to detect the absorbance at 570nm and then calculated the cell survival rate.

2.4 Annexin V-FITC/PI double staining assay

Fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (KayGEN Biotech, Jiangsu) was used to detect the apoptotic percentage according to the manufacturer’ s standard protocol. CNE and CNE-1 cells were seeded into 6-well culture plates and incubated overnight. The cells were treated with Sq-6 with diffierent concentrations for 72 hours. Then cells were harvested and washed twice with ice-cold PBS and collected by centrifugation at 1000 r/min for 5 minutes at 4°C. 500μL binding buffer was added to suspend the cells, then 5μL Annexin V-FITC and 5μL PI were added and mixed gently. Then the cells were incubated for 15 minutes at room temperature in dark. The apoptosis rate was analyzed using a flow cytometer.

2.5 Western blot assay

Cells were plated on 6-well plates and treated with different concentrations of Sq-6 for 72 hours. After cell culture medium was removed, cells were washed with ice-cold 1x PBS and lysed in RIPA buffer (Beyotime Biotech, Shanghai) for 30 min. Total protein samples (40 μg/well) were loaded onto 10-12% SDS polyacrylamide gels and transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 hour before incubation with primary antibodies at 4°C overnight. After washing three times with Tris-buffered saline-Tween buffer, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Protein bands were detected using the ECL chemiluminescence kit (Millipore, Billerica, MA, USA) and visualized with a Gel Imaging system (Bio-Rad), and the intensity of the protein bands were analyzed using Image J software.

2.6 Statistical analysis

The data were expressed as the mean ± standard error of the mean and were analyzed using GraphPad Prism Version 8.0 (GraphPad Software Inc., San Diego, CA) with an independent-samplest-test, one-

way aalysis of variance, and student-Newman-Keuls test. $p < 0.05$ was considered statistically significant. All experiments were designed as three independent experiments.

3. Results

3.1 Sq-6 inhibits proliferation of nasopharyngeal carcinoma cells in a dose and time-dependent manner

Cell survivability was evaluated using the MTT assay to determine the cytotoxic effect of Sq-6 on nasopharyngeal carcinoma cells. When the cells were exposed to several concentrations of Sq-6 (0, 1.25, 2.5, 5,10, and 20μM) for 48, and 72 hours, the inhibition rates were increased in a dose and time-dependent manner, as shown in Figure 1B、C and D. The half-maximum inhibitory concentration (IC50) values of CNE cells treated with Sq-6 at 48 and 72 hours were 6.697 ± 0.194 and 2.981 ± 0.237 μM, and the IC50 values of CNE-1 cells were 7.341 ± 0.143 and 5.390 ± 0.360μM for 48, and 72 hours. The results were shown in Table 1. The above results showed that Sq-6 inhibits nasopharyngeal carcinoma cells proliferation in a dose and time-dependent manner.

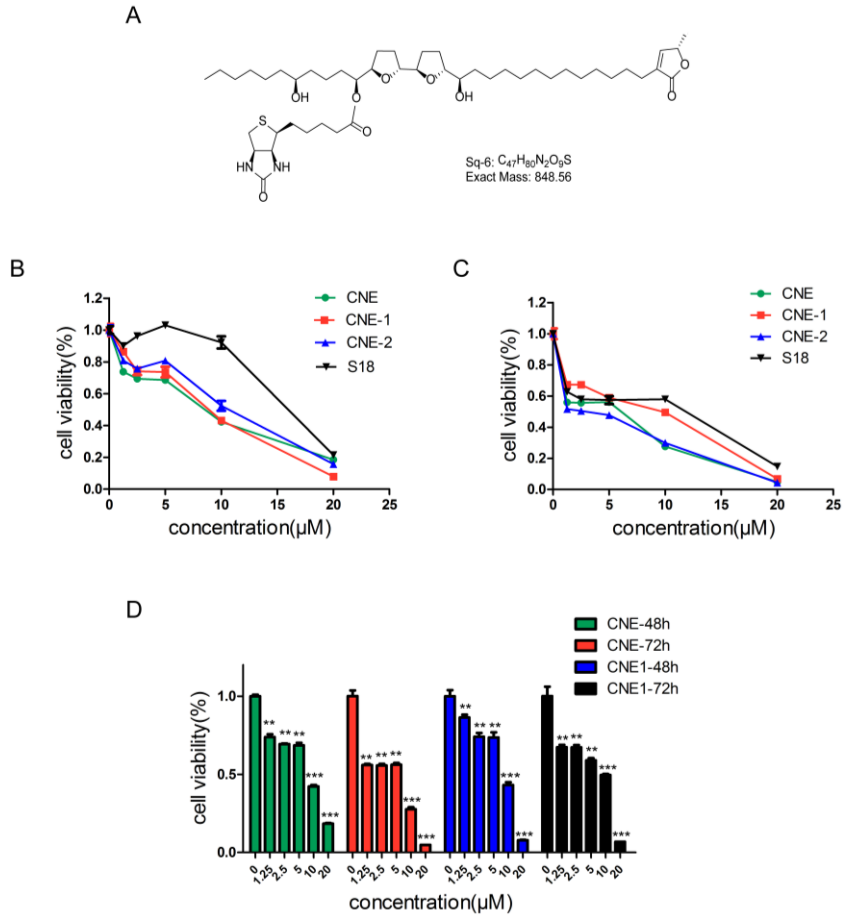

Fig. 1 Sq-6 inhibits cells proliferation of nasopharyngeal carcinoma

A: The chemical structural formula of Sq-6

B: Cells were treated with different concentrations of Sq-6 for 48hr, then MTT assay was used to detect the cell viability.

C: Cells were treated with different concentrations of Sq-6 for 72hr, then MTT assay was used to detect the cell viability.

D: Histogram of cell viability detection of CNE and CNE-1 cells in B and C.

Table 1 IC50 (μM) of Sq-6 on nasopharyngeal carcinoma cells for 48 and 72 hours (Means ± Standard deviation)

Cell lines	IC50(μM) 48hr	IC50(μM) 72hr
CNE	6.697 ± 0.194	2.981 ± 0.237
CNE-1	7.341 ± 0.143	5.390± 0.360

3.2 Sq-6 induced apoptosis of nasopharyngeal carcinoma CNE and CNE-1 cells

To ascertain whether Sq-6 could inhibit the growth of CNE and CNE-1 cells by inducing apoptosis, the cells were incubated with serial concentrations of Sq-6 for 72 hr. Then, we used the Annexin V-FITC/PI double staining assay to detect the apoptotic cells. As shown in Figure 2, the percentage of Annexin V-positive cells increased from3.20% to 31.4% when treated CNE cells with 4 μM Sq-6. Besides,the percentage of Annexin V-positive cells increased from 0.42% to 31.92% when treated CNE-1 cells with 8μM Sq-6. In addition, we used western blotting to detect cell apoptosis induced by Sq-6. In Figure 3, the results revealed that when cells were exposed to different concentrations of Sq-6 for 72 hours, the protein expression levels of cleaved-Caspase9, cleaved-Caspase3, and cleaved-PARP were increased significantly.Together, these results suggested that Sq-6 could induce the apoptosis of CNE and CNE-1 cells.

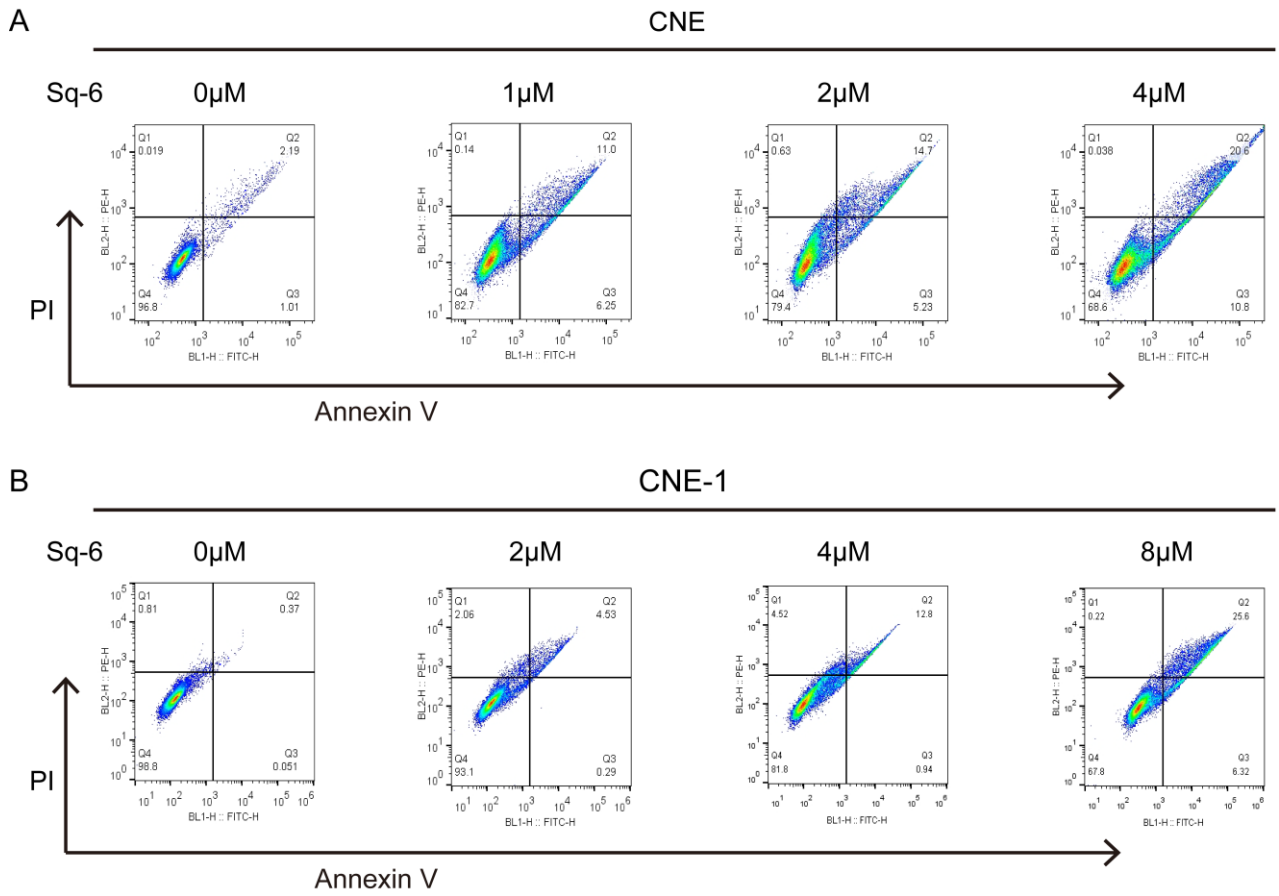

Fig. 2 Sq-6 induced apoptosis in CNE and CNE-1 cells.

A: Annexin V-FITC analysis of CNE cells treated with several concentrations of Sq-6 for 72 hr.

B: Annexin V-FITC analysis of CNE-1 cells treated with several concentrations of Sq-6 for 72 hr.

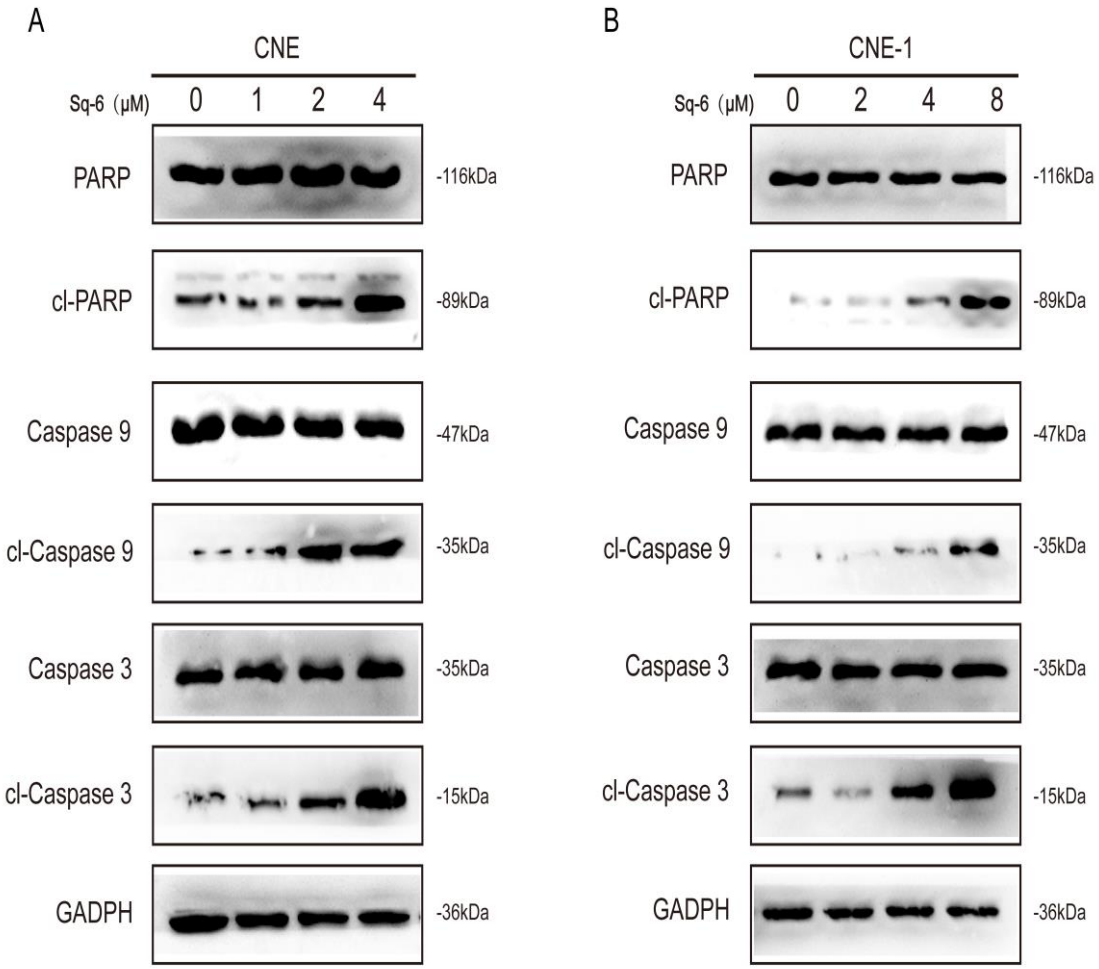

Fig. 3 Expression levels of apoptosis-related proteins

A: Expression levels of apoptosis-related proteins in CNE cells treated with different concentrations Sq-6 for 72hr

B: Expression levels of apoptosis-related proteins in CNE-1 cells treated with different concentrations Sq-6 for 72hr

3.3 Sq-6 activated the JNK signaling pathway in CNE and CNE-1 cells

c-Jun N-terminal kinase (JNK), also known as a stress-activated protein kinase (SAPK) of the MAPK family[9]. The activated JNK can regulate a variety of transcription factors such as c-Jun, c-Fos, ATF-2, activator protein 1 (AP-1), p53 and so on[10-12]. Subsequently, a plethora of cellular processes are triggered, including cell proliferation, apoptosis, autophagy, motility, metabolism and DNA repair[13]. In order to verify whether Sq-6 has an effect on the JNK signaling pathway of nasopharyngeal carcinoma cells, we next used western blot to detect protein levels. As shown in Figure 4, when CNE and CNE-1 cells were exposed to different concentrations of Sq-6, the expression levels of p-JNK and p-c-Jun significantly increased in a dose-dependent manner.The above results revealed that Sq-6 could activate the JNK signaling pathway in nasopharyngeal carcinoma cells.

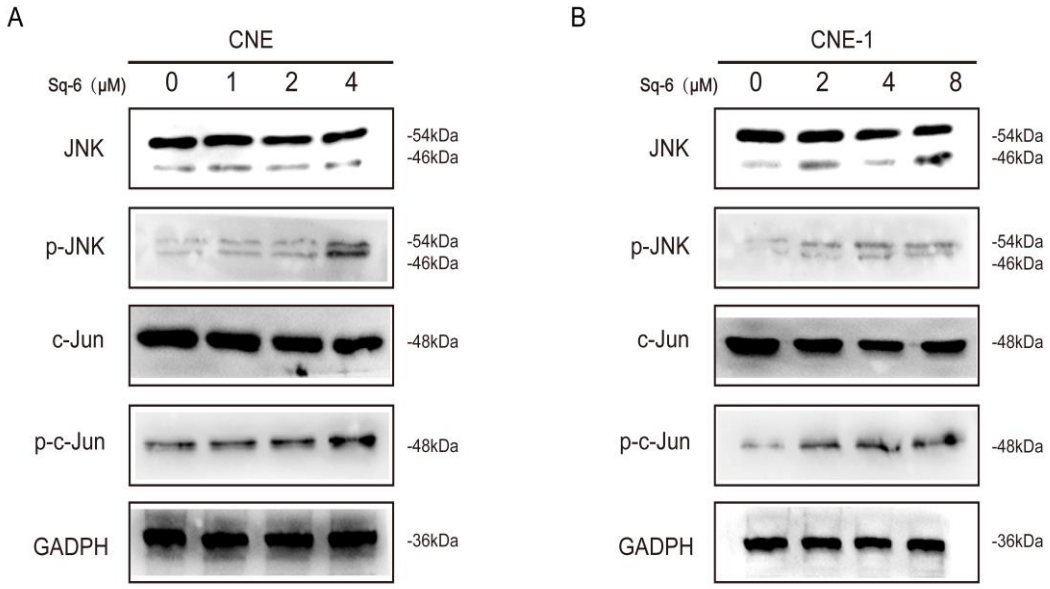

Fig. 4 Sq-6 activated the JNK signaling pathway in CNE and CNE-1 cells

A: Expression levels of JNK pathway proteins in CNE cells treated with different concentrations Sq-6 for 72hr

B: Expression levels of JNK pathway proteins in CNE-1 cells treated with different concentrations Sq-6 for 72hr

4. Dicussion

Nasopharyngeal carcinoma (NPC), formerly known as lymphoid epithelioma, is a malignant tumor caused by nasopharyngeal epithelium. It is characterized by Epstein-Barr virus and is sensitive to radiation and chemistry. Although radiation therapy is still the main treatment method for the high cure rate of early diseases, systemic chemotherap is an indispensable and important part of NPC treatment[14]. As we all know, on the one hand, chemotherapy drugs can control the progression of cancer in patients, on the other hand, its side effects also damage the body of patients. Some Chinese herbal medicines have shown great advantages in inhibiting tumor progression, increasing the sensitivity of chemotherapy, radiation therapy or targeted therapy, improving the immune system function of organisms, and reducing the damage caused by these therapies[15]. Therefore, looking for some natural and effective drugs to treat nasopharyngeal carcinoma has great clinical potential.

Squamocin is an anti-tumor monomer purified and isolated from Annonaceous acetogenins. It has the functions of good proliferation inhibition and apoptosis promotion in cancer cells[16]. However, the low tumor specificity and high toxicity to normal cells make it difficult to apply to clinical tumor treatment. In previous studies, we directly coupled biotin to multiple different carbon atoms of squamocin to obtain a series of monomers with anti-tumor effects, one of which was named Sq-6[8]. So far, the molecular mechanism of biotinylated squamocin on tumor cells has not been reported.

In this study, Sq-6 was used as the research object to explore its mechanism of action on tumor cells. The results of the MTT experiment showed that Sq-6 could significantly inhibit the proliferation of nasopharyngeal cancer cells. Under the same concentration gradient, each nasopharyngeal cancer cell line showed different drug sensitivity. After data comparison and analysis, we found that Sq-6 was more sensitive to CNE and CNE-1 in nasopharyngeal carcinoma cells, and that it had better inhibition of proliferation at 72 hours of drug action. The half-maximum inhibitory concentration (IC50) values

of CNE cells and CNE-1 cells treated with Sq-6 at72 hours were 2.981 ± 0.237 μM and 5.390 ± 0.360 μM (Fig.1).

Many anti-tumor drugs treat cancer by inducing apoptosis (Programmed cell death) in cancer cells. To ascertain whether Sq-6 could inhibit the growth of CNE and CNE-1 cells by inducing apoptosis, we used the Annexin V-FITC/PI double staining assay to detect the apoptotic cells. As we guessed, the results showed that under the effect of Sq-6, the apoptosis rate of cells increased significantly and was dose-dependent. The percentage of Annexin V-positive cells increased from3.20% to 31.4% when treated CNE cells with 4 μM Sq-6. Besides, the percentage of Annexin V-positive cells increased from 0.42% to 31.92% when treated CNE-1 cells with 8μM Sq-6(Fig.2). At the same time, western blot results verified that the expression levels of apoptosis-related proteins such as cleaved-Caspase9, cleaved-Caspase3, and cleaved-PARP increased significantly (Fig.3). All in all, the above results indicated that Sq-6 could indeed induce cell apoptosis.

C-Jun N-terminal kinases (JNKs) regulate cell survival and apoptosis by distinct mechanisms. In cancer cells, JNK and c-Jun play an important role in tumorigenesis and metastasis of cancer cells. RAS-induced transformation requires JNK activation and c-Jun phosphorylation. In nearly 30% of human cancers, Ras, an oncogene, is activated due to the induction of c-Jun phosphorylation or JNK phosphorylation. One of the functions that c-Jun may contribute to tumor development is to suppress the transcription of the gene encoding p53 tumor suppressor[17]. Here, we found that the level of p-JNK and p-c-Jun was upregulated when CNE and CNE-1 cells were exposed to various concentrations of Sq-6(Fig.4). Altogether, these results agreed that Sq-6 activated JNK signaling pathway in CNE and CNE-1 cells.

5. Conclusion

In summary, Sq-6 has a growth inhibitory effect on nasopharyngeal carcinoma cells and induces dose-dependent apoptosis in CNE and CNE-1 cells. In addition, Sq-6 has been found to activate p-Jnk and p-c-Jun, which have been shown to regulate tumorigenesis and development. Therefore, these findings indicates that Sq-6 is a potential clinical drug candidate for nasopharyngeal carcinoma with the support of more research in the future.

Acknowledgements

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