Dean&Francis

ISSN 2959-409X

Grammostola spatulata Spider Venom Peptide GsMTx-4 TFA Shows Anti-cancer Effect on Liver Cancer Cells in Vitro.

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Abstract:

Liver cancer is a common type of cancers that are building great threat to humanity. Many of the peptides derived from spider venoms have been proven to exhibit anti-cancer effect against a variety of cancers. In this study, we investigated the anti-cancer effects of a peptide, named GsMTx-4 TFA, which is derived from spider Grammostola spatulata. We first examined its cytotoxicity in 4 different doses to Escherichia coli, a common type of bacteria. Results manifested that the GsMTx-4 TFA has no cytotoxicity to Escherichia coli under any doses we have utilized in the experiment. Next, we conducted an experiment to test the cytotoxicity of GsMTx-4 TFA to cells of liver cancer, HepG-2. We found out that the peptide exhibits anti-cancer effects in a dose-dependent manner. Under relatively low doses, the cytotoxicity is pretty low, and the survival rate of cancer cell was even bigger than the control group. Moreover, it seemed that the cytotoxicity of the peptide had been lowering between 4h to 24h, showed by the rising survival rates in each group from 4h to 24h, which manifested its relatively low cytotoxicity, poor drug persistence, and the strong adaptability of liver cancer cells. Nonetheless, if the cells were exposed under high doses, their viability was obviously decreased, showing the anti-cancer effect of GsMTx-4 TFA under high concentration. Considering the previous experiments with Escherichia coli, it is possible that the GsMTx-4 TFA has selectivity to cancer cells but not normal cells like Escherichia coli. But to know whether it is also toxic to normal cells in human body, and its anti-cancer mechanisms, further research and experiments are needed to be conducted. Furthermore, the results of mRNA analysis showed that the peptide can also inhibit the growth of cancer cells by regulating the expression of some important proteins, like BAX, BCL2 and mTOR, implying one potential deep mechanism of the anti-cancer effects of the peptide.

Keywords: Spider venom peptide; liver cancer; anti-cancer effect; cytotoxicity;

1. Introduction:

Cancer is one of the most life-threatening deceases, and it is a huge problem in global public health, especially in China and other developed countries[1]. In China, for instance, according to the National Cancer Center's National Cancer Report released in 2024, about 2,574,200 new cancer deaths occurred in China in 2022. Lung cancer, colon-rectum cancer, thyroid cancer, liver cancer and stomach cancer were the top five cancer types, accounting for 57.42% of new cancer cases. Lung cancer, liver cancer, stomach, colon-rectum cancer and esophagus cancer were the five leading causes of cancer deaths, and they are accounted for 67.50% of total cancer deaths. Cancer remains a major public health problem in China, seriously affecting the health of Chinese people, the economy and the social development in China [2].

Liver cancer is one of the most diagnosed cancers around the world. It is the sixth most common cancer worldwide and the third leading cause of cancer-related deaths, and it is estimated that 905,677 people were diagnosed with liver cancer globally in 2020, with an age-standardized incidence of 9.5 per 100,000 people [3]

To date, the current anti-cancer therapies, such as conventional chemotherapy, radiotherapy, and targeted biological therapies are not satisfactory. The main causes for such outcome are low target selectivity (primarily in chemo- and radiotherapy), ineffectiveness to metastatic disease, drug resistance, and severe side effects [4]. Thus, the need for new anti-cancer therapies is urgent.

In recent years, increasing research on the anti-cancer effects of animal toxins has brought a new breakthrough. Many scientists who devoted themselves in cancer treatment research have turned to peptides derived from animal toxins, finding that many of the peptides from animal toxins have strong anti-cancer effects and high medicinal value. Take the scorpion toxins as a case in point, the peptides extracted from the venom of Rhopalurus princeps, a common scorpion, induced apoptosis in the BxPC3 cell line, one of the human prostate cancer cell lines, at very low concentrations, and the peptides significantly inhibited BxPC3 proliferation in a concentration- (≥500 μg/mL) and time-dependent (≥48 hr) manner [4]. In addition, there is also a large number of research programs about the anti-cancer effects of spider venoms. For instance, one of the pore-forming spider venom peptides, named LaFr26, purified from the venom of the Lachesana sp spider, was able to selectively be incorporated into K + channel expressing hyperpolarized cells, and then largely decrease the viability of cancer cells [5]; Scientists also found out that spider venom derived peptide JZTX-14 is able to inhibit the migration and invasion of MDA-MB-231 breast cancer cells via modulation of sodium current through the Nav1.5 channel [6]; LVTX-8 is one type of spider venom-derived peptide with linear amphipathic alpha-helical conformation, which is designed and synthesized from the cDNA library of spider Lycosa vittata, and it can regulate causal genes' expression in p53-related pathways of lung cancer cells, and hence induce apoptosis of cancer cells [7]. In summary, using spider venom-derived peptides as anti-cancer drugs is a plausible new approach in cancer treatment.

GsMTx-4 TFA is a 35 amino acid spider venom-derived peptide isolated from the toxin of the spider Grammostola spatulate. It was first identified by a team led by Thomas M. Suchyna and reported in March 2000 in a paper. According to researchers, GsMTx-4 is able to produce a near complete block of the volume-sensitive cation-selective current, but did not affect the anion current [8], which manifests that GsMTx-4 TFA is a perfect cation channel blocker. Considering that many of those spider venom peptides prevent the growth and migration of cancer cells by block certain ion channels of the cells and regulate the amount of expression of certain DNA, we can reasonably assume that GsMTx-4 TFA is also able to block certain channels or change DNA expression to inhibit the growth of cancer cells. No study had investigated the anti-cancer effects of GsMT-4 TFA before. So, this study presented in this paper will offer another potential new anti-cancer agent for cancer treatment.

2. Materials and Methods:

2.1 Materials:

The powder of spider venom peptide GsMTx-4 TFA was purchased from Shanghai Aladdin Biotechnology Co., LTD, and it was stored under -20℃; Spider venom peptide powder is made into 1,000 micrograms per milliliter of solution, and the solution was stored under -4℃ and away from light; The fetal bovine serum was purchased from the Zhejiang Tianhang Biotechnology CO., LTD; The Dulbecco's Modified Eagle Medium(DMEM) was purchased from Zhejiang Senrui Biotechnology Co., LTD; E. coli was cultured using LB solid and liquid media. The former was prepared by LB nutrient Agar (purchased from Qingdao Hi-Tech Industrial Park Haibo Biotechnology Co., LTD.) with distilled water and autoclaved at 121℃ for 5 minutes; The latter is prepared by LB broth (purchased from Qingdao Hi-Tech Industrial Park Haibo Biotechnology Co., LTD.) with distilled water and autoclave at 121℃ for 15 minutes; The MTT solution used in the MTT assay was stored under -20℃ and away from light; The DMSO used in the MTT assay was stored at room

ISSN 2959-409X

temperature away from light.

2.2 The culture of Escherichia coli:

The E. coli came from the laboratory of the Institute of Food Bioscience and Technology, School of Biosystems Engineering and Food Science, Zhejiang University. E. coli was cultured for 3 generations before the experiment. The first two generations were cultured in LB liquid medium, and the third generation was cultured on LB solid medium and tested. All three generations were cultured at 37℃。

2.3 Disk Test:

Disk test was used to examine the viability of E. coli. We put 2 round small paper discs symmetrically in each E. coli petri dish, adding different doses of venom peptide solution to small paper discs. By observe the size of the bacteriostatic circle generated around the paper, we can roughly test the survival rate of E. coli.

2.4 Cancer Cell Culture:

Human hepatocellular carcinoma cells Hepg2 were obtained from a typical culture preservation center in China. The HepG-2 liver cancer cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. All cells were incubated at 37°C under 5% CO2 in incubator. The cells were cultured for 2 generation. After the cells were cultured, they were moved to the 96-well plate, and the 36 holes in the outer circle of the 96-well plate were injected with PBS buffer to prevent drying inside the plate. The 6*10 holes in the inner circle were evenly divided into 4 groups according to 3*5 modules, and were divided into 4 groups. From the upper left corner of the module clockwise are 4 hours, 24 hours, 48 hours and 72 hours after the venom peptides were added. (The actual experiment only measured the 4 hours and 24 hours groups. Because 24 hours after the data was enough to reflect the results)

2.5 MTT Assay:

The survival rate was measured at 4 hours and 24 hours after the solution of venom peptides were added. After draining the liquid from the hole of the corresponding module, 90 microliters of DMEM high-sugar medium and 10 microliters of MTT mother liquor were added in each hole, and then the cells were incubated under 37℃ and 5% CO2 in incubator. 4 hours later, the 96-well plate is taken out of the incubator, the MTT diluent is removed, and 150uL DMSO solution is added to each hole. After mixing with vibration for 10 minutes, the solution is put into the enzyme marker and measured at the absorption value of 570nm. The absorbance reflected the number of cells that survived.

2.6 The extraction of RNA and the detection of the expression of different genes:

The treated cells were washed once with PBS after discarding the medium. 1mL of TransZol Up was added to every 10cm^2 of cultured cells and placed horizontally for a while to make the lysate evenly distributed on the cell surface and lysate the cells, and then the cells were blown off with a pipette gun. After that, the cell lysate was transferred to a centrifuge tube and repeatedly blown with a pipette until there was no obvious precipitation in the lysate. Let stand at room temperature for 5 minutes. Use 1mL TransZol Up (purchased from website www.transgen.com.cn), add 0.2mL chloroform (TransZol Up: chloroform = 5:1), shake violently for 30 seconds, incubate at room temperature for 3 minutes. Centrifuge 10000g at 2-8℃ for 15 min. After centrifugation, transfer the colorless water phase to a new centrifuge tube, add the same volume of anhydrous ethanol, and gently reverse and mix. The obtained solution and precipitation were added to the centrifugal column, centrifuged at 12000g at room temperature for 30 seconds, and the effluent was discarded. Add 500μL CB9(purchased from website www.transgen.com.cn), centrifuge at room temperature 12000g for 30 seconds, discard the effluent, and repeat. Then add 500μL WB9(purchased from website www.transgen.com.cn), centrifuge at room temperature 12000g for 30 seconds, discard the effluent, and repeat. Then centrifuge at room temperature 12000g for 2 minutes to completely remove the residual ethanol. Put the centrifugal column into the RNase-free Tube, add 50-200μL RNase-free Water in the center of the centrifugal column, and let it stand at room temperature for 1 minute. Finally, the RNA was centrifuged at 12000g at room temperature for 1 minute, eluted, and then stored at -80℃.

After the RNA was extracted from the cells, the RNA was transcribed into cDNA by reverse transcriptase (purchased from website www.transgen.com.cn), and then amplified by fluorescent quantitative PCR detector for content detection. By processing and comparing the expression levels of the control group and the experimental group, we can see the difference in the expression levels of different proteins.

3. Results

3.1 The cytotoxicity of GsMTx-4 TFA to Esche-

richia coli:

To roughly evaluate the toxicity of GsMTx-4 TFA, we first examined its cytotoxicity to E. coli, a common subject in toxin research. We diluted 1000 micrograms per milliliter of TFA solution to 100 micrograms per milliliter, 50 micrograms per milliliter, 25 micrograms per milliliter and 12.5 micrograms per milliliter solutions, and also prepared normal saline (with a toxin concentration of 0) as a control group. The E. coli was maintained under 37℃ for 24 hours.

We can roughly assess the survival rate of E. coli by observing the size of the bacteriostatic circles generated around the paper.

After 24 hours, it is obvious that no bacteriostatic circle was formed, despite the concentration of TFA, which means that the present of TFA toxin peptides did not affect the growth of E. coli. It manifested that TFA has no cytotoxicity to E. coli (Figure 1).

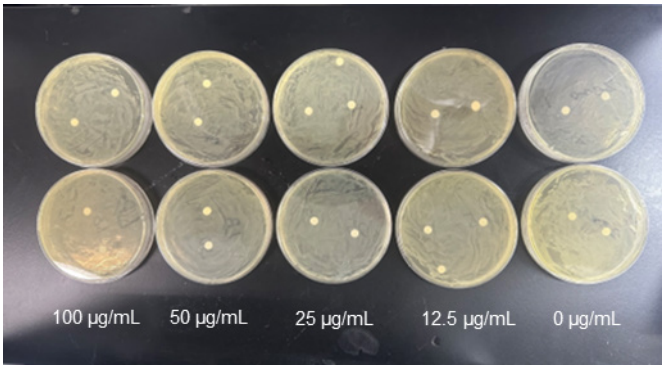

Figure 1 Results of incubating different concentrations of GsMTx-4 TFA with E. coli for 24 hours

3.2 The anti-cancer effects of GsMTx-4 TFA to cancer cells HepG-2:

HepG-2 is a common liver cancer cells used in scientific research and experiments. According to what we observed during the experiment, we assumed that the GsMTx-4 TFA inhibits the growth of cancer cells in a dose-dependent manner. 4 hours later, we first recorded the results. At 12.5μg/ml, the survival rate of cells did not decrease significantly, but increased, that said, the survival rate of cancer cells is bigger than that of the control group (100%). At 25μg/ml, the cell survival rate decreased, but not significantly, and the survival rate was still 96.99%. However, the cell survival rate decreased significantly at 50μg/ml and 100μg/ml, which were 90.14% and 82.8%, respectively (Figure 2A).

After 24 hours, the survival rate of the cells when the concentrations were 12.5μg/ml and 25μg/ml did not decrease but increased, indicating that the adaptability of cancer cells was strong, and low concentration of spider toxin provided an even advantageous environment for cancer cells. When the concentration was 50, the inhibitory effect on cancer cells also became very small, and the survival rate under 100μg/ml is only 83.56%, but there is also a little improvement (Figure 2B).

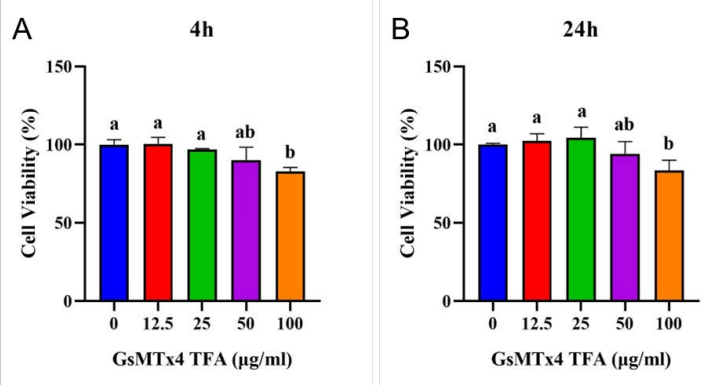

Figure 2 The anti-cancer effects of GsMTx-4 TFA to cancer cells HepG-2. (A) Cell viability of HepG-2 after exposure to GsMTx-4 TFA of different concentrations for 4 hours. (B) Cell viability of HepG-2 after exposure to GsMTx-4 TFA of different concentrations for 24 hours.

ISSN 2959-409X

3.3 The analysis about the change in expression of mRNA in cancer cells HepG-2:

We also investigated the deeper mechanisms of the anti-cancer effect of GsMTx-4 TFA from the perspective of the expression of genes. In this study, we mainly analysis the amounts of expression of 3 types of mRNA. The protein products BAX localize to the cytoskeleton in healthy cells, and follow a death signal. They interact predominantly by heterodimerizing with, and inhibiting, the antiapoptotic proteins, thus initiating apoptosis[9]. By analyzing the change of the expression of BAX, we found out that the expression of BAX in the cells in experimental group (24h after the drugs were added) is more than two times than the control group. This is strong evidence of that the GsMTx-4 TFA can largely increase the expression of BAX, and thus cause the apoptosis of cancer cells (Figure 3A).

BCL2 is an integral mitochondrial membrane protein. It is different from BAX. BCL2 can inhibit apoptosis[9], and it is very important for the survival of cancer cells. The decrease in expression of BCL2 can provide great environment for cell apoptosis. Comparing to the control group, the BCL2 expression in the experimental group (24h after the drugs were added) decreased about 40%, which suggests that the GsMTx-4 TFA can largely inhibit the expression of BCL2, and then facilitate the apoptosis of cancer cells (Figure 3B).

The mechanistic target of rapamycin (mTOR) coordinates eukaryotic cell growth and metabolism. The increase production of proteins, lipid, and nucleotides are essential for cell growth and differentiation. mTOR plays a central role in regulating all of these processes, and it can control the balance between anabolism and catabolism in response to environmental conditions [10] Thus, the expression of mTOR is a crucial factor of the growth of cancer cells. We measured the fold change in the expression of mTOR before and after the drug was added. The data analysis shows that in the experimental group, which was treated with GsMTx-4 TFA peptide, expressed mTOR less than the control group. This decrease in the expression can block the growth of cancer cells (Figure 3C).

In conclusion, GsMTx-4 TFA can regulate the genetic expression in cancer cells to induce the death of cells, and to inhibit the growth of HepG-2. It implies the anti-cancer mechanism of GsMTx-4 TFA.

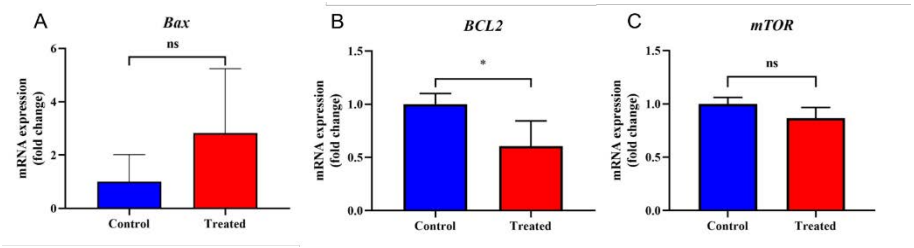

Figure 3 The effect of GsMTx-4 on expression of three types of mRNA in cancer cells HepG-2. (A) Bax. (B) BCL2. (C) mTOR.

4. Discussion

The analysis of results in experiment with cancer cells HepG-2:

Obviously, the GsMTx-4 TFA has inhibitory effects against cancer cells, depending on the concentration of the peptide solution we used. When the doses were relatively low, the results are pretty surprising: the survival rates of cancer cells were even bigger than the control group, the group without a single bit of peptide. Nonetheless, according to the results presented when the concentrations were higher, like when the concentration reached 100μg/ml, the GsMTx-4 TFA had obvious inhibitory effect, showing that the peptide has anti-cancer effect, not a particle of doubt. We think the survival rates of cancer cells were bigger than the control group mainly due to the following factors: First, the toxicity of TFA itself is relatively low. This can also be seen from its lack of any inhibitory effect on E. coli at high doses. Thus, when the doses were low, the inhibitory effects were not significant. Second, the adaptability of liver cancer cell HepG-2 is very strong. We assume that when the cancer cells perceived the presence of a dangerous and risky peptides, their adapting ability was further stimulated and activated, and what's more, the peptides GsMT-4 TFA is not living things like phages or cheetahs. As a result, it is impossible for them to co-evolve with cancer cells. All of these factors together created the surprising results. Of course, this assumption needs further experimental proof. In addition, the results give us some instructions for GsMTx-4 TFA medication, if it is possible for it to be utilized in medication one day. As we can see, the survival rates in all groups had raised between 4h to 24h, which indicates that the anti-cancer effect of GsMTx-4 TFA had decreased during this period. Therefore, to ensure the effect of medication in actual ap-

plication, patients should take medication intermittently for long periods of time, and of course, the dosage should not be too low (depending on the situation of patients).

The low toxicity of GsMTx-4 TFA is not, however, necessarily a disadvantage. Comparing to some other toxins with strong cytotoxicity, LVTX-8, for instance. LVTX-8 is a really strong spider venom-derived peptide. In the experiment held by researchers, by comparison with the control cells, the percentage of apoptotic cells among cancer cells increased from 5.24% to 21.08%, and from 14.56% to 31.87%, in 5M of LVTX-8-treated A549 cells and H460 cells (both are cancer cells), respectively[7]. This suggests that the LVTX-8 is pretty harmful to cancer cells, and the anti-cancer effect of it is much more stronger comparing with GsMTx-4 TFA. Nevertheless, according to their results, LVTX-8 only showed strong cytotoxicity, rather than selectivity. In spite of LVTX-8 showing strong cytotoxicity towards cancer cells, it also showed this activity towards to non-cancer cells, such as Hek293T cells[7]. This finding suggests that if LVTX-8 is used in medication, it will attack both cancer cells and normal cells in human body. What's even worse, since it is so toxic to cells, the side effects of this medication will be much bigger than that of GsMTx-4 TFA. Hence, the low cytotoxicity of GsMTx-4 TFA is not a disadvantage. On the contrary, comparing to those peptides that have strong toxicity, it is more feasible for pharmaceutical use, since the side effects of it are smaller than those peptides.

The analysis of results in experiment with E. coli:

As we presented above, the GsMTx-4 TFA has no inhibitory effect to E. coli. However, afterwards, we did some technical analysis, and we found out that it is unprecise to say that the peptide has no inhibitory effect. We used solid medium in the experiment, rather than liquid. This may lead to the inefficiency of the delivery of drugs.

Another possible reason is that the adaptability of bacteria like E. coli is very strong, and thus the E. coli can adapt to the drug environment and survive.

In conclusion, it is no absolutely precise to say that the GsMT-4 TFA has no cytotoxicity to E. coli. Further improvements need to be done to get a certain result.

Further improvements:

First, we cannot ensure that the GsMTx-4 TFA has no harm to normal cells in human body, since we did not conduct such experiment. Considering the previous experiments with Escherichia coli, it is possible that the GsMTx-4 TFA has selectivity to cancer cells but not normal cells like Escherichia coli, but we cannot ensure until specific experiments are conducted. Therefore, its potential for drug use needs further research. More experiments need to be conducted to investigate whether it will have strong side effects to normal cells.

Second, in this experiment, we mainly investigated one single type of cancer cells, the liver cancer cell HepG-2. Although we have proved that the peptide has obvious anti-cancer effects toward this cancer cells, its anti-cancer effects against other types of cancers can only be proven through further investigation.

Third, in this experiment, we mainly investigated the anti-cancer effects of the peptides in vitro. However, we did not conduct more experiments to check its effects in vivo. The anti-cancer effects of one drug can be different between in vitro and in vivo, since in vivo, the biological environment can be much more complicated, and there will be a lot of other variables existing. Hence, more research should be done to investigated the anti-cancer effects of the peptides in vivo.

Finally, we previously predicted that the GsMTx-4 TFA could probably block certain ion channels in cancer cells to decrease their viability, since it is a great blocker for cation channels. However, in the study, after analyzing the expression of DNA in cancer cells, we found out that the spider venom peptide can also kill cancer cells by regulating the expression of some genes. Though the peptide is a very good ion-channel blocker, we cannot ensure whether it inhibit the growth of cancer cells due to regulation of genetic expression, or blocking the ion channels, or through both. More research needs to be held to investigate its deeper mechanisms.

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