Study on in vivo Anti-hepatoma Activity of Actinidia arguta polysaccharide AAP - 3b

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Abstract

Because of the development and popularization of tissue culture method, the study on anticancer activity by the culture of cancer cells in vitro has become an important method for screening anti-cancer active substances. However, in vitro method has some limitations, mostly the high false positive rate. The growth of cells in vitro is affected by many factors; due to the complexity of in vivo environment, the active substances in vivo are affected by various factors, so there is obvious difference in the pharmacological effect compared with that in vitro. Furthermore, information on drug tissue selectivity, toxicity, metabolism, etc. becomes unobtainable in in vitro experiment. Animal model provides tumor cells with the internal environment for growth which is unavailable in vitro, including nutrition supply, blood circulation, and the interaction with the surrounding substances, similar to the growth pattern of tumors in the human body.

Of all animal models, the model of human transplanted hepatocellular carcinoma in nude mouse is the closest to the biological behavior of human hepatoma, which provides an ideal research tool for basic and experimental research of hepatoma in vivo. The method of subcutaneous inoculation is applicable to tumor formation and anti-tumor drug research, with the advantages of simple operation and high tumor formation[1]. In this study, the in vivo anti-cancer effect of AAP-3b was studied by establishing a model of human transplanted hepatocellular carcinoma HepG2 in nude mice, and the pharmacological activity and possible mechanism of AAP-3b in vivo was evaluated, providing experimental basis and theoretical basis for clinical application and development of health care products.

The results showed that AAP-3b could significantly inhibit the growth of tumor in HepG2 tumor-bearing nude mouse, with tumor inhibition rate being 16.76% and 43.06% respectively at a dose of 50 mg/kg·bw and 200 mg/kg·bw. AAP-3b can significantly suppress the proliferation activity of human hepatoma cells HepG2. The inhibitory rate on HepG2 proliferation were 17.23% and 41.51% at a dose of 50 mg/kg·bw and 200 mg/kg·bw respectively.

Keywords: Actinidia arguta polysaccharide, AAP-3b, in vivo, antitumor.

Material and Methods

Main materials and reagents

Actinidia arguta polysaccharide AAP-3b: The extraction method: The crushed fruit of actinidia arguta was treated with ethanol for impurity removal, and the polysaccharide was extracted with the assistance of microwave. The polysaccharide was then centrifuged and the supernatant was concentrated under pressure. Afterwards, the concentrate was precipitated with absolute ethanol (1:4, by volume), and then stored overnight in the refrigerator (at 4℃). The next day the precipitation was removed from the refrigerator for centrifugation.

After that, the precipitation was soaked and washed with petroleum ether, acetone and absolute ethanol respectively. Finally, polysaccharide AAP-3 was obtained after filtration and freeze-drying. AAP-3 was then passed through a DEAE-5 2 cellulose chromatographic column and a Sephadex G-100 gel chromatographic column. Next, the ingredients were collected, dialyzed, desalted, concentrated and freeze-dried to obtain four components: AAP - 3 a, AAP - 3 b, AAP - 3 c, AAP - 3 d. Among them AAP-3b is a major elution ingredient and a major functional component[2]. They should be freeze-dried after extraction and purification, and kept under -20 ℃ for standby.

Cell line: human hepatoma cell line HepG2 was kindly provided by Shenyang Pharmaceutical University and cultured, passaged, frozen and tested by genetic toxicology laboratory of safety evaluation center of Shenyang Research Institute of Chemical Industry.

Experimental animal: 45 athymic nude mice (BALB / C), male, aged 5 weeks (provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. China).

Main reagents

Cyclophosphamide	Sigma-Aldrich, USA
Tetrazolium thiazolyl tetrazolium (MTT)	Sigma-Aldrich, USA
Dimethyl sulfoxide	Sigma-Aldrich, USA
Fetal bovine serum	Corning, USA

DMEM medium	Corning, USA
Penicillin / streptomycin stock solution	Tianjin Haoang Biological Manufacture Co. LTD., China
Trypsin	Tianjin Haoang Biological Manufacture Co. LTD., China

Main instruments and equipment

Analytical balance (AL204-IC)	**Mettler-Toledo, LLC (Shanghai), China**
Biological safety cabinets	Thermo Fisher Scientific, USA
High-speed refrigerated centrifuge	BECKMAN COULTER
UV-2550 UV-visible spectrophotometer	SHMADZU Corporation, Japan
CO2 Incubator(MCO_18AIC)	SANYO Electric Co. Ltd., Japan
IX71 inverted microscope	OLYMPUS, Japan
CKX31 inverted microscope	OLYMPUS, Japan
Synergy HT Multi-mode microplate Reader	Bio-Tek, USA
Hitachi 7180 biochemistry automatic analyzer	Hitachi Group, Japan
Automated laser-based hematology analyzer (XT-2000iV)	Sysmex Corporation, Japan

Test methods

Cell resuscitation, culture and passage: Cells in logarithmic growth phase were selected for experiments.

Animal rearing: The barrier system was provided for nude mice with temperature at 25 ± 2 ℃ and humidity 45 ~ 50%. Cage, padding, food and drinking water were all autoclaved. Furthermore, the laboratory and the raising environment were regularly disinfected with UV light, and all experimental operations were carried out in clean benches.

Construction of tumor bearing model in nude mice: HepG2 cells in logarithmic growth phase were selected with original culture medium in the petri dish discarded. The HepG2 cells washed twice in PBS were digested with trypsin. The digestion was terminated by adding 10% fetal calf serum into DMEM medium. Afterwards, the cells were harvested, then centrifuged before centrifuged twice in basal medium (DMEM). Finally, the cells were resuspended in basal medium (DMEM) and the cell density was adjusted to 1.0×10^7 cells / mL.

The HepG2 cell suspension (each for 0.2 mL, including 2×10^6 viable cells) was inoculated subcutaneously into the back of the right forelimb of the sterilized nude mice with microinjector. HepG2 cell suspension was inoculated subcutaneously in the dorsal subcutaneous tissue of the right forelimb of the nude mice. Afterwards, the mice continued to be raised in SPF conditions. On day 8 after inoculation, tumor nodules appeared in the inoculation site. With the diameter up to 4-5 mm. Thus, tumor bearing model in nude mice was successfully constructed.

Experimental grouping, drug delivery and sampling: Nude mice with no infection and good mental status were chosen for grouping: 40 nude mice bearing tumors were randomly divided into four groups: model control group, cyclophosphamide control group, AAP-3b low dosage group and AAP-3b high dosage group, 10 in each group. Number, weighing and processing conditions in each group are:

1) Model control group: normal saline, 25 mL / kg.bw daily, a single intraperitoneal injection;
2) Cyclophosphamide control group: cyclophosphamide prepared with sterile water, 20 mg / kg.bw daily, a single intraperitoneal injection;
3) AAP-3b low dosage group: AAP-3b prepared with sterile water, 50 mg / kg.bw daily, a single intraperitoneal injection;
4) AAP-3b high dosage group: AAP-3b prepared with sterile water, 200 mg / kg.bw daily, a single intraperitoneal injection.

Drugs were administered at the same time for 10 days.

After the last administration, fasting was performed with free access to water. After 24 hours, the mice were weighed before blood was collected. Next, the rats were sacrificed painlessly. After sterilization with 75% ethanol, the specimens were cut with sterile operation. The scalpel and forceps were used respectively to remove the tumor and the spleen.

General observation items during the experiment: Nude mice were observed during the administration of the changes in mental state, diet, activity, defecation and weight.

Blood testing: The next day after the last administration, eyeballs of nude mice were taken painlessly for blood which was collected and placed at room temperature for 5-10 minutes before stored in crushed ice. The serum was separated by centrifugation within 1 hour after the collection. Next, the blood was tested with biochemistry automatic analyzer (Hitachi-7180) for biochemical parameters: alanine aminotransferase (AST), urea (UREA) and creatinine (CREA), and with automated laser-based hematology analyzer (XT-2000iV) for blood routine indicators: total red blood cell count (RBC) and total white blood cell count (WBC).

Determination of immune index: The removed spleen was dissected, washed away the blood with normal saline, followed by drying them with filter paper for weighing, then calculate the spleen index, the formula is as (1):

$$I = \frac{m}{M} \quad (1)$$

In the formula I: spleen index; M: spleen weight (mg) and M: nude mice weight (g).

Determination of solid tumor inhibition rate: The surrounding connective tissue of the tumor was removed, then the tumor was weighted after dried with filter paper. Next, calculate the tumor inhibition rate with the formula (2):

$$S\ (\%) = \frac{(A_0\text{-}A_1)}{A_0}\ 100\% \quad (2)$$

In the formula, S: tumor inhibition rate (%); A0: average weight of the tumor in the model group and A1: average weight of the tumor in administration groups.

Determination of tumor cell proliferation activity: The tumor tissues cut into pieces were placed in a petri dish filled with saline. They were stirred, filtered, centrifuged and resuspended in DMEM medium containing 10% fetal bovine serum followed by the adjustment of the tumor cell density to 1×10^6 / mL. The cells were seeded in 96-well plates (200 uL per well) and placed in a CO^2 incubator (at 37 ℃) for 24 hours. Then, they were taken out to add 20μL of MTT solution (5 mg / mL) and placed back the incubator (at 37 ℃) for 4 hours before the culture termination. The 96-well culture plate was removed, and the solution in the wells was discarded with DMSO 150 uL added in each well. After that, multi-mode microplate reader (detection wavelength of 560 nm) was put in to exam the optical absorption (OD) of each well. Optical absorption (OD) indicates the cell proliferation activity in each group. The inhibitory rate on cell proliferation was calculated as shown in formula (3):

$$IR\ (\%) = \frac{(A_0\text{-}A_1)}{A_0}\ 100\% \quad (3)$$

In the formula, IR: tumor inhibition rate; A0: absorbance value of tumor cell control group and A1: absorbance value of tumor cell test group.

Results and Analysis

General observation index of nude mice: Tumor formed in all the 45 Nude mice inoculated with HepG2 cells (tumor formation rate 100%). The average weight of model control group, positive control group (also named cyclophosphamide control group), low dosage group and high dosage group were 18.4 ± 0.6 g, 18.5 ± 0.8 g, 18.3 ± 0.7 g and 18.3 ± 1.0 g respectively before the administration, while the average weight were 24.2 ± 1.8 g, 23.1 ± 1.7 g, 24.5 ± 1.8 g, 25.3 ± 1.8 g respectively at the end of administration. This result indicated that there were no significant differences in weight among the two dosage groups, the positive control group and the model control group (P> 0.05). After the administration ended, there was no significant difference in the weight of the two dosage groups and the positive control group compared with that of the control group (P> 0.05). But there was significant difference in weight between the positive control group and the high dosage group (P <0.01), indicating that phosphamide may be toxic to the body. There were no significant changes in diet, drinking and activity of nude mice in each group during the first three days of administration.

On the fourth day after administration, nude mice in the model group gradually became sluggish in spirit and slow in action, while the performance of the dosage group of actinidia arguta polysaccharide and cyclophosphamide control group was better than that of the model control group. The tumor gradually increased with time. However, the growth of tumors in AAP-3b group and positive control group was slower than that in model group, meanwhile diet, activity and weight of nude mice in administration groups were gradually recovered. In the later period of the experiment, in contrast with the positive control group and the high dosage group, the nude mice in control group and low dosage group showed decreased activity, sluggish spirit and reduced diet on account of their larger transplanted tumor. Nude mice in each group had normal survival and no death.

Blood testing: Red blood cells and white blood cells are the main types of blood cell. Therefore, their changes in number are viewed as one of the auxiliary means for diagnosis. Alanine transaminase (ALT) which exists in the stem cell cytoplasm and aspartate aminotransferase (AST)which exists in mitochondria, both as important enzymes involved in the synthesis of key amino acids in the body, normally maintain the stable level. If the liver is sick, especially with the damage of the membrane system in the liver, it releases these enzymes (AST and ALT) into the bloodstream in larger-than-normal quantities. Hence, Serum ALT level, serum AST (aspartate transaminase) level, and their ratio (AST/ALT ratio) are commonly measured clinically as biomarkers for liver health. Urea and creatinine are indicators of renal function.

After the end of administration, the number of red blood cells and white blood cells was measured by automated laser-based hematology analyzer (XT-2000iv) and the Alanine transaminase (ALT), aspartate aminotransferase (AST), urea and creatinine by biochemistry automatic analyzer (Hitachi-7180). As shown in table 1, blood cells count and function of liver and kidney in nude mice of each group were not changed, in which there were no statistically significant differences among groups by T-test (P> 0.05).

Immune index determination: Immune system combines a range of functions like defense, surveillance and self-stabilization and others. Organs of the immune system make cells, which either contribute in the immune response, or act as sites for the immune function. The spleen, the largest peripheral immune organ, contains a large number of lymphocytes and macrophages, which are closely associated with humoral immunity. Immune function is connected with

the weight of the immune organs, can be measured with relevant organ index. As seen from table 2, spleen index in AAP-3b low dosage group and AAP-3bhigh dosage group increased compared with the model control group, which is statistically different. It indicated that actinidia arguta polysaccharide AAP - 3b could effectively improve the immunological function of HepG2 tumor bearing mice by protecting the spleen index. Spleen index in cyclophosphamide positive control group was significantly lower than that of the model control group, which further confirmed that cyclophosphamide had an inhibitory effect on the immune system.

Determination of solid tumor inhibition rate: Compared with model control group, the inhibitory effect on HepG2 solid tumors of all the administration groups is visible to the naked eye since the volume of solid tumors was reduced in the administration group. The inhibitory rates of AAP-3b on tumors (Table 3) calculated on the basis of the weight of both the tumor and nude mice indicates that AAP- 3b inhibited the growth of HepG2 solid tumors to a certain extent (low dose:16.76%; high dose: 43.06%). The cyclophosphamide positive control group had a significant effect on tumor inhibition in HepG2 nude mice, up to 47.33%. Weight change in nude mice before and after the experiment goes to the point that cyclophosphamide had a great side effect on the body, while AAP-3b basicly resulted in non-toxic and side effect.

Determination of tumor cell proliferation activity: As shown in table 4, the proliferative activity of HepG2 solid tumor cells in each dosage group and the control group was as follows: model control group, 1.25 ± 0.08; low dosage group AAP-3b, 0.65 ± 0.06; high dosage group AAP-3b, 1.03 ± 0.04; cyclophosphamide control group, 0.73 ± 0.05. Compared with the model control group, the proliferation activity of other groups was significantly lower (P <0.001).

Table 1
The results of hematology and clinical chemistry in mice after the test ($\bar{x}$±s)

Group	Number	RBC ($\times10^{12}$/L)	WBC ($\times10^{9}$/L)	ALT (U/L)	AST (U/L)	UREA (μmol/L)	CREA (μmol/L)
Model control	10	8.23±0.47	5.98±0.51	34.45±3.01	180.20±20.58	5.66±1.03	35.33±2.87
50 mg/kg Dose group	10	8.11±0.53	5.89±0.48	35.24±3.12	178.58±15.84	5.78±1.06	36.40±2.40
200 mg/kg Dose group	10	8.17±0.58	5.88±0.66	34.80±3.32	181.62±19.52	5.70±1.09	35.30±2.47
20 mg/kg CP control	10	8.24±0.61	5.92±0.61	35.78±3.34	180.35±19.93	5.62±1.12	35.92±2.80

Note: RBC- Red Blood Cell, WBC- White Blood Cell, ALT- alanine aminotransferase, AST- Aspartate aminotransferase, UREA - blood urea nitrogen, CREA - serum creatinine.

Table 2
Spleen index and thymus index in each group of mice ($\bar{x}$±s)

Group	Number	Spleen index (mg/g·bw)
Model control	10	$3.51\pm0.66^{\#\#}$
50 mg/kg Dose group	10	$4.27\pm0.67^{*\#\#\#}$
200 mg/kg Dose group	10	$5.05\pm0.90^{**\#\#\#}$
Model control	10	$2.20\pm0.81^{**}$

Note: compared with model control group, * P<0.05, ** P<0.01, *** P<0.001 ; compared with CP control group # P<0.05, ## P<0.01, ### P<0.001

Table 3
Inhibition ration of AAP-3b on HepG2 solid tumor in mice ($\bar{x}$±s)

Group	Number	Weight gain（g）	Tumor weight（g）	Inhibitory rate (%)
Model control	10	5.8±2.1	$1.38\pm0.28^{\#\#\#}$	-
50 mg/kg Dose group	10	$6.2\pm1.5^{\#}$	$1.15\pm0.21^{\#}$	16.76±6.54
200 mg/kg Dose group	10	$7.0\pm2.0^{\#\#}$	$0.79\pm0.24^{***}$	43.06±8.57
20 mg/kg CP control	10	4.6±1.6	$0.73\pm0.19^{***}$	47.33±9.91

Note: Value is expressed as MEAN±SD; compared with model control group, * P<0.05, ** P<0.01, *** P<0.001 ; compared with CP control group # P<0.05, ## P<0.01, ### P<0.001.

Table 4
Prolife rative activity results of mice hepatoma cells ($\bar{x}$±s)

Group	Number	Proliferative activity	Inhibition rate（%）
Model control	10	1.25±0.08$^{###}$	-
50 mg/kg Dose group	10	1.03±0.04$^{***\#\#}$	17.23±5.71
200 mg/kg Dose group	10	0.73±0.05$^{***\#\#}$	41.51±6.25
20 mg/kg CP control	10	0.65±0.06***	47.84±6.57

Note: compared with model control group, * P<0.05, ** P<0.01, *** P<0.001 ; compared with CP control group $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001.

Discussion

In recent years, natural bioactive substances have been paid extensive attention due to their safety, non-toxic and side effects, such as some peptides[2,3] flavonoids[4], saponin and polysaccharides[5]. In particular, lentinan has been used generally as an adjuvant in the treatment of tumors. The anti-tumor mechanism of natural active substances has also been widely studied worldwide[6]. The effect and mechanism in vivo of the natural active substance inducing the apoptosis of tumor cells on tumor cell apoptosis need further study, though achievement has been reached in vitro in that direction.

Mainly by establishing the animal tumor model, this research suggests that AAP-3b can significantly inhibit the proliferation of HepG2 cells and induce their apoptosis. Whereas, considering the limitations of in vitro experiments, the establishment of subcutaneously transplanted tumor model in nude mice can better simulate the in vivo biological behavior of human hepatocellular carcinoma, which is beneficial to the study on the antitumor activity of AAP-3b in vivo and provides experimental and theoretical basis for the development and utilization of AAP-3b.

(1) Methods of establishing animal tumor model[7]

Animal spontaneous tumor model, limited only to the spontaneous tumor of cancer clustering animal, is difficult to get enough for the experiment in a short time, let alone greatly different individuals. Induced tumor model, namely, the use of carcinogens acting on animal tissues or organs for a period of time to induce tumors, is time-consuming with obviously different individuals. The use of transplantable tumors to establish models, including transplantation of animal tumors in syngeneic animals in vivo and human malignancies in immunodeficient animals in vivo, is short in time, stable in development process, good in reproducibility, controlled in the number of transplanted cells, even in the growth rate of tumor, small in individual differences and manageable in experimental conditions. Consequently, this model is the most ideal and most practical animal tumor model at present.

(2) Commonly selected animals in human cancer allograft model

Human cancer allograft models are commonly used for in vivo experiments to evaluate pharmacodynamic efficacy and reliability of antineoplastic agents, in which athymic or combined immunodeficient mice are commonly selected. Nude mice were confirmed that they become mutated on the alleles of its 11th chromosome. "Nu" is the symbol of the naked gene and nude mice are homozygous (nu/nu). They are congenitally immunodeficient animals with congenital dysplasia in thymus which has only some residues or abnormal epithelium, unable to normally differentiate T cells and thus lack of inhibition and killing function from mature T cell, resulting in low cellular immunity and decreased number of lymphocytes in the thymus-dependent zone of lymph nodes and peripheral blood[8]. Nude mice in mixed lymphocyte reactiont produce no toxic effector cells, and shows no sensitivity and transplant rejection, which are good receptors in human cancer in vivo experiment.

Rygaard firstly transplanted human tumor into nude mice in 1969, creating a precedent for human tumor xenograft and marking the application of immunodeficiency animal in tumor transplantation[9]. With a variety of human tumor tissue and cells have been successfully transplanted[10,11,12], nude mice have become the ideal animal or the establishment o ideal animal for human tumor model, playing an important role in the study on biological behavior of human tumor and antitumor experiment[13,14,15].

Transplantation of human malignant tumors into nude mice resulted in a high tumor formation and continuous passage in vivo, maintaining primary tumor characteristics, such as the pathological morphology of cells or tissues, the ability to produce characteristic enzymes, chromosomal features, and the function for hormone secretion[16]. Inbreeding nude mice can maintain the same biological and genetic characteristics with the experimental results consistent and small in difference. Moreover, they are closer to the human body's natural condition, thus relatively small number of them in the experiment can meet statistical need. European and American countries advocate that the clinical anti-cancer drugs have to be screened by human tumor transplantation model in nude mice[17]. Commonly selected species of nude mice are BALB / C, C578L / 6, NIH, C3H, etc.

(3) Establishment of human hepatocellular carcinoma transplantation model in nude mice

The success of nude mice with transplanted tumor depends on quite a few factors, such as the conditions related to nude mice themselves, the number of transplanted tissues or cells, proliferation ability, tumor types, transplantation sites, vaccination ways, the status of animal nutrition and so forth. Research showed that young SPF grade nude mice raised under constant temperature and humidity, was low in the cell activity, which was suitable for tumor growth and metastasis. Nude mice with BALB / C genetic background are relatively the lowest in cell activity. Nude mice less than 4 weeks old have not yet weaned, though high in tumor formation, susceptible to death.

The number of normal cells in nude mice increased with age, but the rate of tumor formation began to decrease 8 weeks later, so nude mice aged 4-8 week were usually chosen for the model. Therefore, BALB / C nude mice, 5 weeks old were selected in this study, which were acclimated for one week in the SPF environment barrier system, and the experimental operation and feeding were carried out under sterile conditions. Tumor cells in ascites, tumor cell lines cultured in vitro, etc. can be used for transplantation, the highest success rate being tumor cell lines. The human hepatoma cell line HepG2 is derived from human hepatoblastoma which is high in differentiation degree and stable in biological activity, constituting a important cell line for the study of hepatocyte physiology and the detection of anticancer drugs. It is widely used in experimental studies of hepatocellular carcinom, in vitro and in vivo.

In the paper, HepG2 cells in logarithmic growth phase were selected and the number of subcutaneous inoculation is 2 $\times 10^6$. Subcutaneous inoculation is easy to operate, in favor of the observation and monitor of growth in transplanted tumor and drug inhibition on the tumor. Orthotopic transplantation tumor model is the choice for such observation and study. In this study, we selected the subcutaneous tissue of the back of the right forelimb in the nude mice as the transplanted site. This site is suitable for tumor growth and convenient for the observation and can avoid the influence of lymph node metastasis on the volume of transplanted tumor. The results showed that all of the 45 nude mice were inoculated successfully, up to100%and for about one week, which showed the successful establishment of human hepatocellular carcinoma transplantation model in nude mice.

(4) The inhibitory effect of AAP-3b on the growth of human hepatocellular carcinoma xenograft in nude mice

After the successful establishment in the model, the nude mice were divided into groups and given different doses of AAP-3b. The general and pathological examination in tumor-bearing nude mice and their important tissues and organs could understand whether AAP-3b had adverse effects on the body. The results showed that the growth of transplanted tumor in nude mice was inhibited after AAP-3b administration. After administration, the weight of transplanted tumor in AAP-3b dosage groups was lower than that of the control group, the rate of AAP-3bhigh dosage group even at 43.06%, indicating that AAP-3b was active to a certain degree in anticancer in vivo, which was consistent with that of in vitro cell experiments. The number of blood cells and function in liver and kidney of nude mice were normal and no pathological changes after inspection. Nude mice in AAP-3b dosage groups during the administration were normal in eating and drinking, no significant reduction in body weight, no signs in sluggish spirit, slow activity, diarrhea, and so on, indicating no adverse reactions caused by AAP-3b on the body.

(5) Effect of AAP-3b on the proliferation activity of human hepatocellular carcinoma xenografts in nude mice

One of the basic features of liver cancer is the uncontrollable cell proliferation, which is also an important factor in malignant biological behavior. In this study, the effect of AAP-3b on the proliferation of HepG2 solid tumor cells was detected by MTT assay. The results showed that AAP-3b could suppress the proliferation of tumor cells, thereby inhibiting the growth of transplanted tumors and affecting the malignant biological behavior of liver cancer. Furthermore, the higher the dose is, the higher the inhibition rate becomes (tumor inhibition rates at 17.23% and 41.51% with dosage being 50 and 200 mg / kg.bw respectively). Reducing the proliferation activity of AAP-3 cells may be one of its mechanisms for anti-hepatoma in vivo.

(6) Effects of AAP-3b on immune organs of transplanted human hepatocellular carcinoma in nude mice

Spleen index is the most basic indicator reflecting the body immunity and widely used to evaluate the body's immune system. Its changes reflect the changes in numbers of the spleen lymphocytes, T and B lymphocyte included. Up-regulation in spleen index signifies increased splenic lymphocyte proliferation, which enhances the immune capacity and prevents cancer and other diseases. The result of tests for spleen index showed that the spleen index was elevated with the increase of AAP-3b dosage. The spleen index of model control group, AAP-3b low dosage group (50 mg / kg.bw), AAP-3b high dosage group (200 mg / kg.bw) and cyclophosphamide positive control group were 3.51 ± 0.66, 4.27 ± 0.67, 5.05 ± 0.90 and 2.20 ± 0.81 respectively. Meanwhile, the weight of HepG2 solid tumors also reduced, showing that the immune enhancement of AAP-3b on the body may be one of the mechanisms for anti-liver cancer. In contrast with the model control group, spleen index in cyclophosphamide positive control group significantly decline, indicating the inhibition effect of cyclophosphamide on the immune system.

Summary

(1) HepG2 cell line was inoculated subcutaneously on the back of the right forelimb in nude mice to establish the tumor model which consisted of the model control group, AAP-3b

low dosage group (50 mg / kg.bw), AAP-3b high dosage group (200 mg / kg.bw), cyclophosphamide positive control group. Before the administration, there was no statistically significant difference among the dosage groups, positive control group and model control group (P> 0.05). During the period of administration, weight in the tumor and the mice in each group gained with time. Compared with the model control group, tumor growth in the other three groups was slower. Nude mice in the model control group were gradually sluggish in spirit and slow in action, while conditions were better for the other three groups. The comparison in weight among all the groups after the end of administration was not statistically significant（P>0.05）. Whereas, there was significant difference between the positive control group and the high dosage group in weight change (P <0.01), stating cyclophosphamide may produce toxic and side effects to the body.

(2) AAP-3b had no effect on nude mice's general condition, blood cell count, hepatic and renal function, no adverse reactions.

(3) Immunological index measurements showed that: compared with the model group, AAP-3b at 200 mg / kg.bw increased the spleen index significantly (P <0.01), indicating that the anti-tumor mechanism of AAP-3b was related to the immune enhancement.

(4) In vivo antitumor test showed that AAP-3b inhibited the tumor growth of HepG2 tumor-bearing mice at a dose of 50 mg / kg .bw, 200 mg / kg. bw with tumor inhibition rate being 16.76% and 43.06 respectively;

(5) AAP-3b potently inhibited the proliferation of human hepatoma cell line HepG2. The inhibitory rates were 17.23% and 41.51% respectively at the dose of 50 mg / kg.bw and 200 mg / kg.bw.

(6) Although AAP-3b is less effective as cyclophosphamide in inhibiting liver cancer, it spares the spleen from damage and is also weaker than cyclophosphamide in the influence on the weight of nude mice. Therefore, AAP-3b is a bioactive substance, relatively safe and harmless.

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