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Original Article

The effects of compound polyoxometalates on the growth of human hepatocarcinoma cells and the immune function in H22-bearing mice

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Abstract: Objective: To explore the inhibitory effects of 14 kinds of polyoxometalates and metal-organic polyoxometalates synthesized in our laboratory on the growth of human hepatocarcinoma cells in vitro, and on the immune function and tumor growth inhibition rate in H22 tumor-bearing mice. Methods: The inhibitory effects of 14 kinds of heteropoly compounds on the growth of four human hepatocarcinoma cell lines, namely hepG2, hep3B, huh7, and PLC/PRF/5, were determined using the colorimetric method of methyl thiazolyl tetrazolium (MTT), and five kinds of polyoxometalates with an average EC50 value ≤ 0.5 umol/L were screened out. The effects of the five kinds of polyoxometalates on the tumor growth and lymphocyte transformation rate in the H22 tumor-bearing mice were further tested. Results: The MTT assay results showed that 14 kinds of polyoxometalates had different degrees of inhibition on the growth of human hepatocarcinoma cells in vitro, and the inhibition rate was positively correlated with the polyoxometalate concentration. Among the 14 kinds of polyoxometalates, five were screened out: $Ti_{0.6}H_{0.6}PW$, $Sn_{0.6}H_{0.6}PW$, $M_{0.1}H_{2.7}PW$, $(C_5H_{14}N_2O_2)_2[SiMo_{12}O_{40}]\cdot 12H_2O$, and $(C_5H_{14}N_2O_2)_2[GeMo_{12}O_{40}]\cdot 12H_2O$) have a strong inhibitory effect on the growth of human hepatocarcinoma cells in vitro. The results of the tumor growth and lymphocyte transformation rates of the H22 tumor-bearing mice showed that these five kinds of polyoxometalates can significantly improve the immune function of tumor-bearing mice and inhibit tumor growth, especially in the high-dose groups. The tumor growth inhibition rate in the high-dose (160 mg/Kg) medication in the tumor-bearing mice was equivalent to the rate in the positive control group treated with CTX (40 mg/Kg), and there was no significant toxicity caused by the high-dose medication in the tumor-bearing mice. Conclusion: The five kinds of polyoxometalates screened out have good anti-hepatoma effects and have the potential to become candidate anti-liver cancer drugs.

Keywords: Polyoxometalates, MTT assay, H22 tumor-bearing mice, lymphocyte transformation rate, tumor growth inhibition rate

Introduction

At present, a large number of studies hope to find some active anti-tumor substances with low toxicity and high efficiency through continuous drug screening. Heteropoly compounds are one such substance. Heteropoly compounds, also known as polyoxometalates (POMs), are a cluster of metal and oxygen compounds formed by the condensation of the oxygen-containing metal salts of vanadium, molybdenum, tungsten, niobium, tantalum, and other transition metals under certain conditions, which have a variety of types and compositions, and have pharmacological effects such as anti-tumor, anti-bacterial, anti-viral, and anti-diabetes [1]. In 1988, Yamase, a Japanese scholar, published a research report on a kind of polyoxometalate $(NH_3iPr)_6(Mo_7O_{24})\cdot 3H_2O$ (PM-8) with strong anti-tumor effects [2], and later applying to patent it. PM-8 has shown a more satisfactory anti-tumor effect than commercially available drugs. The research on the drug revealed that the growth inhibition rate for human colon cancer was up to 54.3%, while the inhibition rate of 5-fluorouracil was only 44.0% under the same conditions. In 1993, Yamase continued to report that PM-8 had a good inhibitory effect on Meth-A, MX-1, MM-46 (breast cancer), Co-4 (colon cancer), and OAT (lung cancer) without

cytotoxicity [3]. This has set off an upsurge in research on POM. In recent years, the anti-tumor properties of POM have attracted special attention. A large number of domestic studies have reported the synthesis of various new polyoxometalates for the screening of anti-tumor drugs through various modulatory techniques, such as changing polarity, redox potential, surface charge distribution, different heteroatom ratios, and acidity, etc. [4-8].

Among tumors, liver cancer is a common malignant tumor with a high degree of malignancy and a high mortality rate. Screening for anti-hepatoma drugs has always been a focus of research. The establishment of human hepatocarcinoma cell lines solves the problem of not being able to conduct liver cancer research directly in human bodies, so it is currently an ideal in vitro model. This study first investigated the in vitro inhibitory effects of 14 kinds of POMs on four kinds of commonly-used human hepatocarcinoma cells, and then it screened out five kinds of POMs with good anti-tumor activity. In addition, an H22 tumor-bearing mouse model was established to further compare the effects of five kinds of POMs on tumor growth and immune function in tumor-bearing mice.

Materials and methods

POMs

POMs were synthesized in our laboratory. There were 11 kinds of POMs and three kinds of metal-organic POMs. The details were as follows: a. Six kinds of POMs of different metal-substituted $Mx/nH_{0.6}PW$:$Zr_{0.6}H_{0.6}PW$, $Al_{0.8}H_{0.6}PW$, $Zn_{1.2}H_{0.6}PW$, $Fe_{0.8}H_{0.6}PW$, $Ti_{0.6}H_{0.6}PW$ and $Sn_{0.6}H_{0.6}PW$. b. Five kinds of POMs of $Mx/3H3-xPW$ with different heteroatom ratios: MPW, $M_{0.8}H_{0.6}PW$, $M_{0.5}H_{1.5}PW$, $M_{0.3}H_{2.1}PW$ and $M_{0.1}H_{2.7}PW$. c. Three kinds of metal-organic POMs of $((GlyH)_2H_3PW_{12}$, $(GlyH)_2H_5PW_{12})$:$(C_5H_{13}N_2O_2)_2(H_3O)[PMo_{12}O_{40}]\cdot 8H_2O$, $(C_5H_{14}N_2O_2)_2[SiMo_{12}O_{40}]\cdot 12H_2O$ and $(C_5H_{14}N_2O_2)_2[GeMo_{12}O_{40}]\cdot 12H_2O$)

Human hepatocarcinoma cells

The human hepatocarcinoma cell lines were provided by the ATCC cell bank, including hepG2, hep3B, huh7, and PLC/PRF/5.

Experimental animals

A total of 70 SPF Kunming mice and H22 tumor-bearing mice were provided by the Experimental Animal Center of Dalian Academy of Medical Sciences. The Kunming mice were 5-8 weeks old, weighed (20±2) g, and the ratio of males to females was 3:5. This study was approved by the ethics committee of Harbin University of Commerce.

Methods

Culture of human hepatocarcinoma cells: The hepG2, hep3B, huh7, and PLC/PRF/5 cell lines were cultured in DMEM medium containing 15% FBS, 1 million units of penicillin, and 800,000 units of streptomycin, in a 37°C, 5% CO_2 incubator. During passage or inoculation, the cells were washed with pH 7.2 PBS and digested with 0.15% trypsin containing 0.02% EDTA. After stopping the digestion, the adherent cells were washed with 15% FBS-containing medium, and a cell suspension with a cell concentration of $1*10^5$ cells/ml was prepared.

Measurement of growth inhibition rate of human hepatocarcinoma cells using MTT assays [9]: The above cell suspension was inoculated in a 96-well plate at 100 ul per well (containing 1000 tumor cells). After culturing overnight, the medium in the experimental groups was replaced with a DMEM medium supplemented with different POMs. Four concentration levels were set for each POM, and three repetitive wells were set for each concentration. The negative control was cultured normally using a medium without POMs. All the groups were cultured for 3 days after the initial administration.

100 ul MTT solution (0.5 mg/ml) was added to each well, and the wells were incubated at 37°C for 4 hours, and then 200 ul DMSO/well was added, mixed well and incubated at 37°C for 1 hour. The OD value was measured at the reference wavelength of 490 nm and the detection wavelength of 570 nm with a type 680 microplate reader. The average of three replicates of each POM at each concentration level was used to calculate the inhibition rate of each POM on the various human hepatocarcinoma cells at each concentration level. The EC50 value was calculated using GraphPad Prism 7.0 software. The following experiments

using the tumor-bearing mice were performed on the POMs with an average EC50 value ≤ 0.5 umol/L for four kinds of human hepatocarcinoma cells.

Modelling, grouping, and administration of the H22 tumor-bearing mice: H22 cell suspension with a concentration of $1*10^7$ cells/ml was prepared from the well-grown ascites H22 tumor-bearing mice which were sacrificed using cervical dislocation. Seventy Kunming mice were inoculated subcutaneously (intraperitoneally) in the right forelimb axilla with 200 ul, $2*10^6$ H22 tumor cells to establish an H22 tumor-bearing mouse model [10]. The administration was started on the second day after the tumor bearing. Seventy tumor-bearing mice were divided into the polyoxometalates experimental group (n=60), the positive control group (n=5), and the model blank groups (n=5). Sixty tumor-bearing mice in the experimental group were treated with five kinds of polyoxometalates, each of which was administered with a subcutaneous injection next to the tumor with a high dosage (160 mg/Kg), medium dosage (80 mg/Kg), and low dosage (40 mg/Kg), and four mice were used for each dosage of each polyoxometalate every other day. Five tumor-bearing mice in the positive control group were treated with cyclophosphamide (CTX, 40.0 mg/kg, q.o.d). The CTX dosage is equivalent to 10 times the human dosage. Five tumor-bearing mice in the model blank group were treated with normal saline (20 mg/kg, qd.). The fur, feeding, and defecation, the activity, the mental state, and other general conditions of the tumor-bearing mice during the 14 days of administration were observed. The daily changes in body weight were recorded, and the dosages were adjusted accordingly.

Determination of the tumor growth inhibition rate and the lymphocyte transformation rate of the H22 tumor-bearing mice: Two days after the last administration, the mice were sacrificed by cervical dislocation, and the subcutaneous lump bodies were dissected and weighed to calculate the tumor growth inhibition rate. Tumor growth inhibition rate = (Average tumor mass of the model blank group - Average tumor mass of the experimental group)/Average tumor mass of the model blank group * 100%.

In addition, after the spleens were dissected, a splenic single cell suspension with a cell concentration of $5*10^5$/ml was prepared using the collagenase digestion method. The cell suspension was added to a 96-well plate at 200 ul/well of each spleen cell suspension. Nine repetitive wells were set for each cell suspension, and ConA (5 ug/ml) was added to six of the wells, and the other three wells were used as controls without ConA. The plates were placed in a 5%, 37°C CO_2 incubator for 2 days, and 10 ul 5 mg/ml MTT solution was added to each well, and the wells incubated in the incubator for 6 h, and then 150 ul DMSO was added, mixed well and incubated for 30 minutes. The OD values were measured at the detection wavelength of 570 nm with a microplate reader. Lymphocyte transformation rate = (Average OD of experimental wells - Average OD of blank wells)/Average OD of blank wells * 100%.

Statistical analysis

SPSS 22.0 was used to process the experimental data. The measurement data were expressed as the mean ± standard deviation (mean ± SD), and analyzed using t tests or F tests. $P<0.05$ indicated that a difference was statistically significant.

Results

Growth inhibition of the human hepatocarcinoma cells in vitro

The results of the inhibition experiments on the growth of human hepatocarcinoma cells in vitro showed that these 14 kinds of POMs had a certain inhibitory effect on the growth of the four kinds of human hepatocarcinoma cells, and the concentration of POM was positively correlated with the inhibitory effect. At the POM concentration of 1 umol/L, the highest inhibitory rate could reach 91%. Five kinds of POMs were screened out, namely $Ti_{0.6}H_{0.6}PW$, $Sn_{0.6}H_{0.6}PW$, $M_{0.1}H_{2.7}PW$, $(C_5H_{14}N_2O_2)_2[SiMo_{12}O_{40}]\cdot 12H_2O$, and $(C_5H_{14}N_2O_2)_2[GeMo_{12}O_{40}]\cdot 12H_2O$), and they showed good growth inhibition effects in vitro on the four kinds of hepatocarcinoma cell lines, including hepG2 (**Figure 1A**), hep3B (**Figure 1B**), huh7 (**Figure 1C**), PLC/PRF/5 (**Figure 1D**), with an average EC50 value ≤ 0.5 umol/L (**Table 1**).

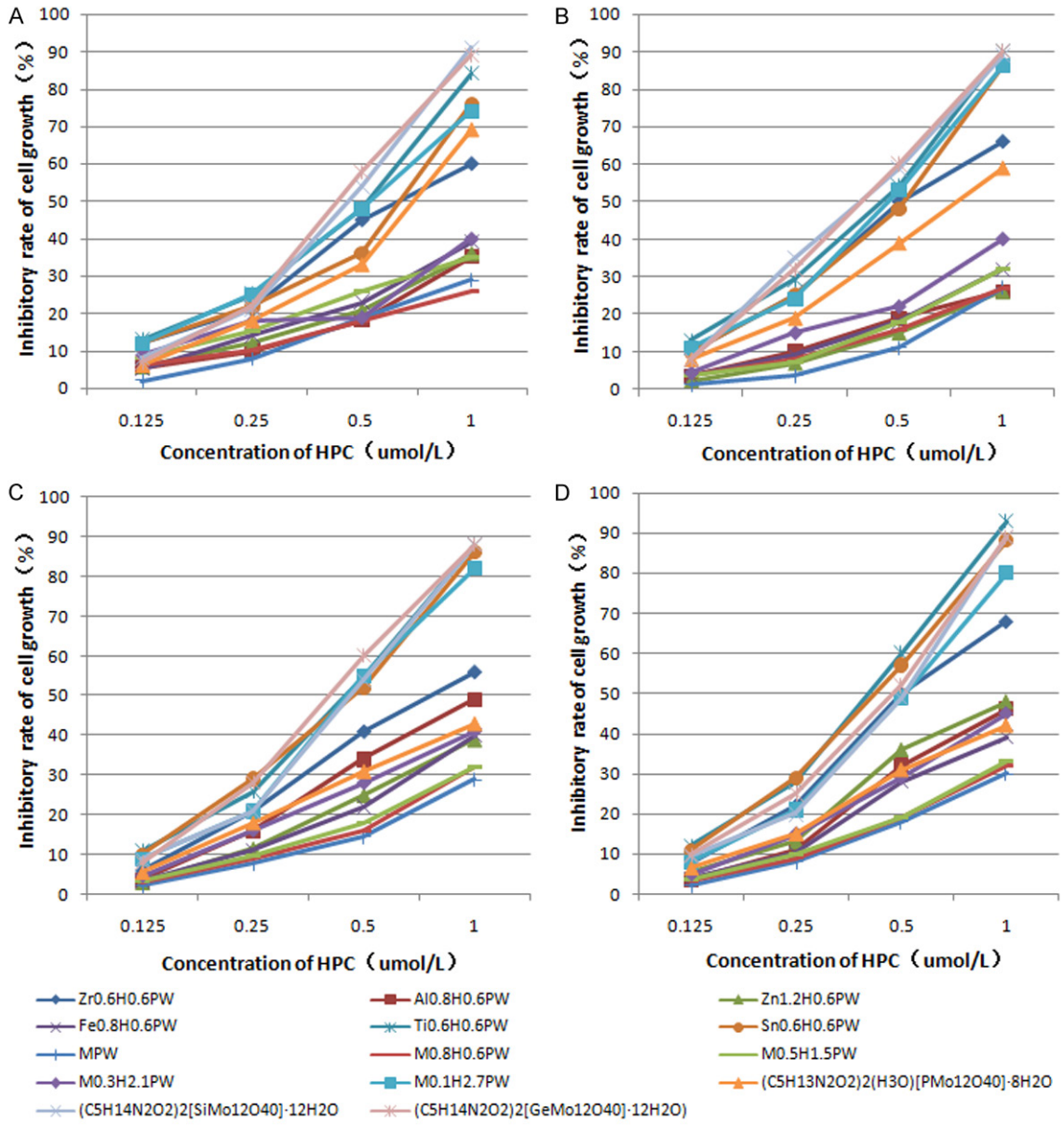

Figure 1. The inhibition rates of the 14 kinds of polyoxometalates on the growth of human hepatocarcinoma cells: hepG2 (A), hep3B (B), huh7 (C) and PLC/PRF/5 (D) in vitro.

General conditions of the tumor-bearing mice during the medication

The mice in the model blank group and the CTX positive control group were showed significant decreases in their activity and food intake, lethargy and thinness. The mice in the positive group suffered hair loss, and the mice in the model blank group had messy and dull fur. The tumors in the positive group were significantly smaller than the tumors in the model blank group. The mice in the experimental group were in a good mental condition, with smooth and shiny coats. In particular, the mice in the medium and high-dose groups had good activities, normal food intake, and a slight increase in weight. The mice in the low-dose medication groups had moderate activity and diet with no visible weight loss. The experimental results showed that the high, medium and low dosages did not cause any significant toxicity in the mice.

Table 1. EC50 values of the five kinds of polyoxometalates initially screened using MTT assays (umol/L)

Polyoxometalates	$Ti_{0.6}H_{0.6}PW$	$Sn_{0.6}H_{0.6}PW$	$M_{0.1}H_{2.7}PW$	$(C_5H_{14}N_2O_2)_2[SiMo_{12}O_{40}]\cdot 12H_2O$	$(C_5H_{14}N_2O_2)_2[GeMo_{12}O_{40}]\cdot 12H_2O)$
hepG2	0.45	0.57	0.51	0.42	0.42
hep3B	0.39	0.45	0.43	0.38	0.38
huh7	0.41	0.42	0.46	0.43	0.40
PLC/PRF/5	0.37	0.40	0.49	0.45	0.42
Average EC50 value	0.405	0.46	0.47	0.42	0.405

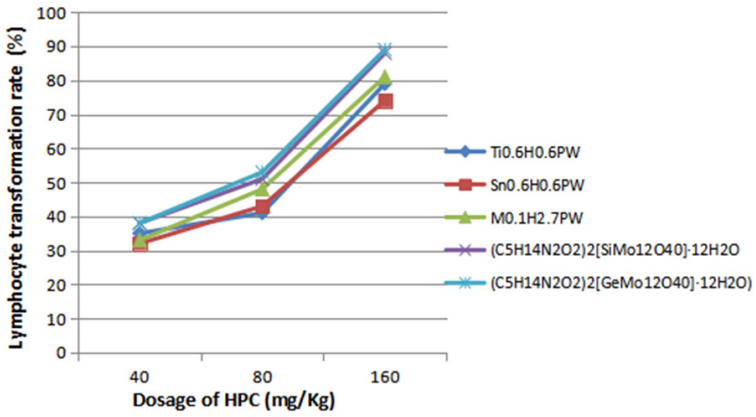

Figure 2. The effect of the different polyoxometalate concentrations on the lymphocyte transformation rates of the H22 tumor-bearing mice.

The effect on the lymphocyte transformation rate of the H22 tumor-bearing mice

Five kinds of POMs screened out by the in vitro inhibitory experiment of the hepatocarcinoma cells were used to further test their effects on the lymphocyte transformation rate of the H22 tumor-bearing mice. Compared with the positive control group and the model blank group, the lymphocyte transformation rates in the high, medium, and low-dose POM medication groups all increased significantly, among which the lymphocyte transformation rate in the high-dose medication group increased significantly ($P<0.01$), and the highest lymphocyte transformation rate reached 89%. The lymphocyte transformation rate in the medium and low-dose POM medication groups was also significantly improved ($P<0.05$). The lymphocyte transformation rate in the high-dose medication group was significantly higher than the transformation rate in the medium and low-dose groups in terms of the same POM ($P<0.05$). There was no significant difference in the lymphocyte transformation rate among the five kinds of POMs at the same dosage ($P>0.05$) (**Figure 2**). The lymphocyte transformation rate in the CTX group was slightly lower than it was in the model group, but there was no significant difference between them.

The inhibition of tumor growth in the H22 tumor-bearing mice

Compared with the tumor-bearing mice in the model group, the tumor masses in the high, medium, and low-dose POM medication groups and the CTX group were reduced, and the tumor inhibitory rates were significantly increased ($P<0.01$). There was no significant difference in the tumor inhibitory rates between the high-dose POM medication group (160 mg/Kg) and the CTX group (40 mg/Kg) ($P>0.05$). The tumor inhibitory rates in the high-dose POM medication group and the CTX control group were significantly higher than they were in the medium and low-dose POM medication groups ($P<0.01$). There were no significant differences in the growth inhibition rates of the tumor-bearing mice treated with the same dosage of five kinds of POMs ($P>0.05$) (**Table 2**).

Discussion

The heteropoly compounds are numerous in number and have diverse structures, and their anti-tumor properties are particularly concerning. In order to formulate and use the anti-tumor properties of heteropoly compounds, in the process of exploring their spatial structure and pharmacological effects, it is possible to conduct chemical transformations and modification such as replacing metal atoms on the polyacid skeleton or introducing various inorganic and organic groups to make changes in their molecular properties and spatial structures, thereby changing their pharmacological

Table 2. The Tumor inhibitory rate of polyoxometalates on tumor growth in the H22 tumor-bearing mice (%)

Types	Dosage (mg/Kg)			*P* value	
	40	80	160	Comparison of the high-dose POM group and medium and low-dose POM groups	Comparison of the high-dose POM groups and CTX group
$Ti_{0.6}H_{0.6}PW$	39.7	52.5	83.5	0.0033	0.078
$Sn_{0.6}H_{0.6}PW$	36.6	49.3	80.3		
$M_{0.1}H_{2.7}PW$	30.5	44.7	78.8		
$(C_5H_{14}N_2O_2)_2[SiMo_{12}O_{40}]\cdot 12H_2O$	37.0	52.4	82.5		
$(C_5H_{14}N_2O_2)_2[GeMo_{12}O_{40}]\cdot 12H_2O)$	40.2	52.9	83.1		
CTX	86.0	-	-	-	-
P value (Comparison of the same dosage of different POMs)	0.056	0.063	0.059	-	-

The anti-tumor rate of five kinds of POMs in the high-dose (160 mg/Kg) treatment of tumor-bearing mice is equivalent to the effect of CTX (40 mg/Kg) treatment ($P>0.05$). The tumor inhibition rate of the mice treated with high-dose POMs was significantly higher than it was in the mice treated with medium and low-doses ($P<0.01$). There was no significant difference in the inhibitory rates of tumor growth of the mice treated with the same dosage of five kinds of POMs ($P>0.05$).

activity and toxicity. In this study, a total of 14 kinds of different heteropoly compounds with different metal-substituted, different heteroatom ratios, and metal-organic hybridization were synthesized by adjusting the preparation process, which were applied for the first time in vitro anti-tumor experiments to verify the anti-tumor effect of these compounds. First, the MTT assay was used as a preliminary screening test to determine the inhibitory effects of 14 kinds of POMs on the growth of four kinds of hepatocarcinoma cells in vitro, from which five kinds of POMs with an average EC50 value of ≤ 0.5 umol/L of four kinds of tumor cells were screened out. The H22 tumor-bearing mouse model was established to further test the effects of the five kinds of POMs selected on tumor growth and lymphocyte transformation rate in tumor-bearing mice, and the inhibition rate of tumor growth was calculated. Preliminary screening of drugs by in vitro experiments can simplify the test operation, save labor and material resources, and have good reproducibility and good correlation with in vivo tests, which will greatly improve the efficiency of drug screening. This screening model has been applied in many studies [11].

The lymphocyte transformation rate is one of the indicators used to determine the body' immune function and can reflect the body's level of cellular immunity. The T-type immune response induced by the proliferation and activation of T cells is considered to play an important role in anti-tumor immunity [12]. The results of the lymphocyte transformation rate of tumor-bearing mice showed that five kinds of POMs can significantly increase the lymphocyte transformation rate when administered at high, medium, and low dosages compared with the CTX group and the model group. In particular, the highest lymphocyte transformation rate in the high-dose POM group reached 89%, indicating that these POMs can significantly enhance the responsiveness of mouse splenocytes to ConA. The non-specific mitogen ConA can induce mouse spleen cells to increase in size, enhance their cell metabolism, promote intracellular protein and nucleic acid synthesis, and transform into lymphoblasts. Although POMs' mechanism of action is not yet clear, through a variety of detection techniques and experiments, studies have proved that POMs penetrate cell membranes, enhance the immune function of mouse T cells, promote T cell proliferation and activation, and improve NK cell activity, thereby improving the body's cellular immune function, inducing tumor cell apoptosis, and inhibiting the growth of ascites [13, 14]. The test results of the tumor inhibition rate in the tumor-bearing mice also revealed that five kinds of POMs showed a significant tumor inhibition effect, and the inhibitory degree was positively correlated with the drug concentration, showing a clear dose-effect relationship. In particular, the effect of high-dose POM at 160 mg/Kg is equivalent to that of CTX at 40 mg/Kg, showing good anti-tumor properties. Compared with the CTX control mice, the mice in the POM administration group saw no significant impact on their daily behavior, spirits, food intake, or body weight, indicating that the high-dose POM administration had no obvious toxicity in the tumor-bearing mice.

The pharmacokinetics and mechanisms of POMs are still poorly studied. It is not well understood why different polyacid structures, different hybrid materials, different group modifications and metal substitutions, and different atom ratios can so greatly affect the POMs' anti-tumor effects. A large number of scholars have synthesized a variety of new POMs through various preparation methods and process adjustments, and conducted a large number of animal experiments and pharmacokinetic experiments [15-20], and tried to theoretically explain the mechanism of action of polyacid drugs and explain the differences in drug activity and toxicity of different POMs against different tumors. These studies will surely promote the development of polyacid drugs to the clinical stage, and ultimately screen out highly efficient and low-toxic POM drugs.

In the preliminary work in our laboratory, 11 kinds of POMs with different metal substitutions and different heteroatom ratios, and three kinds of metal-organic POMs were prepared using the chemical modulatory process. In this study, MTT assays were first used to screen the 14 kinds of heteropoly compounds for their anti-tumor properties in vitro, and five kinds of POMs with better anti-tumor effects in vitro were screened out, then we further tested their effects on tumor growth in H22 tumor-bearing mice as well as their effects on immune function. The experimental results showed that these five kinds of POMs have good tumor-inhibiting properties on H22 tumor-bearing

mice with no significant toxicity, indicating their potential to become candidate anti-liver cancer drugs. In the next step, a more extensive study on the anti-tumor effects of these five kinds of POMs will be conducted to study the phenomenon of POMs inducing tumor cell apoptosis through cell morphology observation and cell cycle quantification, in order to more deeply explore the anti-tumor effect of POMs.

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Disclosure of conflict of interest

None.

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