

PHARMACEUTICAL COMPOSITION FOR PREVENTING OR TREATING
CANCER, COMPRISING TETRAARSENIC HEXOXIDE CRYSTALLINE
POLYMORPH

5 Technical Field

The present invention relates to a pharmaceutical composition containing a crystalline polymorph of tetraarsenic hexoxide for prevention or treatment of cancer.

10

Background Art

Cancer is characterized by uncontrolled cell growth, and such abnormal cell growth forms a mass of cells called a tumor, which penetrates into surrounding
15 tissues, and, in severe cases, causes metastasis into other organs of the body. Academically, tumors are called neoplasia. Cancer affects all tissues and organs of the body at various prevalence rates.

The types of cancer may be classified according to
20 tissues and organs in which cancer cells first develop, and may be classified according to shapes and origins of cancer cells. Lung cancer, gastric cancer, colorectal cancer, cervical cancer, and the like are often referred to on the basis of the organs in which the tumor first
25 developed. Also, the types of cancer may be largely divided into connective tissue tumors, epithelial tumors, adenocarcinoma, and the like in an aspect of the origin of cancer cells. In Korea, the most common cancer is thyroid cancer, followed by gastric cancer,
30 colorectal cancer, and lung cancer, in the order of incidence.

Cancer therapies are divided into aggressive therapies of removing cancer tissues solidified in the organs or killing cancer cells and palliative therapies
35 of delaying the development of cancer cells to minimize

side effects. In the early stage of cancer development, treatment methods of removing tumors through surgery, chemotherapy, radiotherapy, and the like or killing cancer cells through chemical drugs, radiation, and the like may be selected. However, aggressive therapies have relatively serious side effects in end-stage cancer patients, and thus treatment methods of delaying the development of cancer cells to reduce side effects and improve the quality of life can be selected. In general, anticancer drugs refer to anticancer chemotherapy belonging to aggressive therapies, and include cytotoxic anticancer drugs for killing cancer cells with toxic substances, target cancer drugs of selectively acting on cancer cells and new blood vessels around tissues, and the like (Jung Geun-Young, Introduction and treatment trend of chemotherapy, BRIC View Trend Report, 2016-T20, 2016).

Most anticancer drugs are medicines that show effects by acting on rapidly growing and dividing cancer cells, but cause side effects by attacking even some normal cells showing rapid growth and division. Therapeutic agents that can completely treat cancer have not yet been developed. Therefore, with respect to cancer treatment, there is a continuing need for the development of therapeutic agents having excellent anticancer effects.

The present inventors have already received patent rights of technical features wherein tetraarsenic hexoxide purified from natural arsenolite containing arsenic through separation and purification techniques showed cancer metastasis suppressing effects in animal experiments and had excellent anticancer treatment effects when administered to end-stage cancer patients with uterine cancer, bladder cancer, lung cancer, maxillary sinus cancer, kidney cancer, and the like

(Korean Patent No. 272835, Novel use of natural chemical substance tetraarsenic hexoxide as novel antitumor drug and pharmaceutical composition thereof, Registered on 30 August 2000).

5 The present inventors, as a result of continuous research on arsenic, revealed that tetraarsenic hexoxide having 99% or more of tetraarsenic hexoxide crystalline polymorph a can be produced by a novel preparation method, different from the method disclosed in the above
10 registered patent, and a composition containing such tetraarsenic hexoxide has a remarkable effect on cancer prevention or treatment by suppressing the growth of various cancers, and completed the present invention.

15 Detailed Description of the Invention
Technical Problem

 An aspect of the present invention is to provide a pharmaceutical composition containing a crystalline polymorph of tetraarsenic hexoxide (As_4O_6) as an active
20 ingredient for prevention or treatment of cancer.

 Another aspect of the present invention is to provide a method for preparing a pharmaceutical composition containing a crystalline polymorph of tetraarsenic hexoxide (As_4O_6) as an active ingredient for
25 prevention or treatment of cancer.

Technical Solution

 The present invention is directed to a pharmaceutical composition containing tetraarsenic
30 hexoxide (As_4O_6) for prevention or treatment of cancer, wherein the tetraarsenic hexoxide includes 99% or more of tetraarsenic hexoxide crystalline polymorph a ($\text{As}_4\text{O}_6\text{-a}$).

 The tetraarsenic hexoxide of the composition may be
35 prepared by: a first step of heating sodium chloride at

100~800°C, followed by cooling; a second step of placing arsenic trioxide (As_2O_3) on the sodium chloride, followed by heating from 100°C to 1000°C in an airtight state and then cooling; a third step of separating crystals
 5 crystallized in a filter bed collecting sublimated arsenic; and a fourth step of repeating the second and third steps four to ten times using the crystals obtained in the third step instead of the arsenic trioxide in the second step, thereby obtaining
 10 tetraarsenic hexoxide crystals.

The tetraarsenic hexoxide of composition may contain less than 1% of tetraarsenic hexoxide crystalline polymorph b ($\text{As}_4\text{O}_6\text{-b}$).

The tetraarsenic hexoxide may have a purity of
 15 99.9% or more.

The $\text{As}_4\text{O}_6\text{-a}$ and $\text{As}_4\text{O}_6\text{-b}$ may have features (i) to (iii) below.

[Table 1]

Category	Crystalline polymorph a ($\text{As}_4\text{O}_6\text{-a}$)	Crystalline polymorph b ($\text{As}_4\text{O}_6\text{-b}$)
(i) Cell parameters	$a = b = c = 11.0734 \text{ \AA}$ $\alpha = \beta = \gamma = 90^\circ$ $V = 1357.82 \text{ \AA}^3$	$a = b = c = 11.0600 \text{ \AA}$ $\alpha = \beta = \gamma = 90^\circ$ $V = 1352.90 \text{ \AA}^3$
(ii) As-O bond length	1.786 $\text{\AA}$	2.011 $\text{\AA}$
(iii) O-As-O bond angle	98.36°	109.47°

20 The $\text{As}_4\text{O}_6\text{-a}$ has a crystal form, of which the X-ray powder diffraction spectrum obtained by using a light source wavelength of 1.5406 $\text{\AA}$ within a diffraction angle (2 θ) of 10° to 50° at a rate of 1°/min (scan step of 0.02°) shows peaks at 2 θ values of 13.84, 27.88, 32.32,
 25 35.3, 39.84, 42.38, 46.34, 48.6, and 49.34 (see FIG. 1). In addition, the ratio of main peaks shown at 2 θ values of 13.8 and 27.9 is 1:1.3.

The $\text{As}_4\text{O}_6\text{-b}$ has a crystal form, of which the X-ray powder diffraction spectrum obtained by using a light

source wavelength of 1.5406 Å within a diffraction angle (2θ) of 10° to 50° at a rate of 1°/min (scan step of 0.02°) shows peaks at 2θ values of 13.86, 27.92, 32.36, 35.34, 39.9, 42.44, 46.4, 48.66, and 49.4 (see FIG. 1).
5 In addition, the ratio of main peaks shown at 2θ values of 13.8 and 27.9 is 1:2.5.

The cancer may be selected from the group consisting of lung cancer, esophageal cancer, gastric cancer, colorectal cancer, prostate cancer, pancreatic
10 cancer, cervical cancer, ovarian cancer, and endometrial cancer.

Hereinafter, the present invention will be described in detail.

The present invention is directed to a
15 pharmaceutical composition containing tetraarsenic hexoxide (As_4O_6) as an active ingredient for prevention or treatment of cancer, wherein the tetraarsenic hexoxide includes 99% or more of tetraarsenic hexoxide crystalline polymorph a ($\text{As}_4\text{O}_6\text{-a}$).

20 In accordance with another aspect of the present invention, there is provided a method for preparing a pharmaceutical composition containing a crystalline polymorph of tetraarsenic hexoxide (As_4O_6) as an active ingredient for prevention or treatment of cancer, the
25 method including: a first step of heating sodium chloride at 100~800°C, followed by cooling; a second step of placing arsenic trioxide (As_2O_3) on the sodium chloride, followed by heating from 100°C to 1000°C in an airtight state and then cooling; a third step of
30 separating crystals crystallized in a filter bed collecting sublimated arsenic; and a fourth step of repeating the second and third steps four to ten times using the crystals obtained in the third step instead of the arsenic trioxide in the second step, thereby
35 obtaining tetraarsenic hexoxide crystals, wherein the

tetraarsenic hexoxide crystals obtained in the fourth step include 99% or more of tetraarsenic hexoxide crystalline polymorph a ($\text{As}_4\text{O}_6\text{-a}$).

A synthesis reactor of a kaolin material and clamps
5 capable of mounting filters thereon above the synthesis reactor are prepared. Then, sodium chloride is placed in the synthesis reactor, and heated and cooled. The reason why sodium chloride is used in the preparation method of the present invention is that when heating is carried
10 out while arsenic trioxide is placed on the sodium chloride in the second step, heat is uniformly transferred to arsenic compounds, thereby helping the sublimation of the arsenic compounds. In order to remove impurities and moisture from such sodium chloride, the
15 sodium chloride is heated at 100-800°C for 2-6 hours in the first step. In the first step, the sodium chloride is cooled at room temperature for 3-10 hours after the heating.

Then, the second step is conducted by placing
20 arsenic trioxide (As_2O_3) on the sodium chloride, followed by heating from 100°C to 1000°C in an airtight state and then cooling. Here, after the placing of arsenic trioxide, three to six filters (filter beds) capable of collecting sublimated arsenic are mounted on the clamps
25 such that the intervals between the filters are 2-6 mm. The filters used herein preferably have a basic weight of 70-100 g/m², a thickness of 0.17-0.25 mm, a filtration speed of 22-30 s/100 ml, and a retention rate of 5-10 μm.

After the mounting of the filters, an airtight
30 state was made, and then a bottom portion of the synthesis reactor is heated for 3-10 hours while the temperature is gradationally raised from 100°C to 1000°C, so that the temperature of the center portion of the highest filter bed is maintained at 150±100°C, and
35 tetraarsenic hexoxide is crystallized passing through

the filter beds. Then, cooling is carried out at room temperature for 5 hours or longer, and preferably 5-10 hours.

Then, the third step is conducted by separating
5 white crystals collected in the three to six spaced filter beds installed in a stacked type.

After a small amount of arsenic trioxide remaining on the sodium chloride in the synthesis reactor is removed, the collected white crystals are placed
10 thereon, and then the second and third steps are repeated four to ten times in the same conditions, thereby finally obtaining tetraarsenic hexoxide crystals. As a result of checking the crystal structures obtained according to the preparation method of the
15 present invention, it was verified that most of the crystals were $\text{As}_4\text{O}_6\text{-a}$, which accounted for 99% or more.

The cancer may be selected from the group consisting of lung cancer, esophageal cancer, gastric cancer, colorectal cancer, prostate cancer, pancreatic
20 cancer, cervical cancer, ovarian cancer, and endometrial cancer.

The pharmaceutical composition containing a crystalline polymorph of tetraarsenic hexoxide of the present invention can be favorably used in the
25 prevention or treatment of cancer.

The pharmaceutical composition of the present invention may be formulated in the form of: an oral formulation, such as a powder, granules, a tablet, a capsule, a suspension, an emulsion, a syrup, or an
30 aerosol; an externally applied preparation; a suppository; and a sterile injectable solution, according to usual methods, respectively. Examples of a carrier, an excipient, and a diluent that may be contained in the pharmaceutical composition may include
35 lactose, dextrose, sucrose, sorbitol, mannitol, xylitol,

erythritol, maltitol, starch, acacia rubber, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, microcrystalline cellulose, polyvinyl pyrrolidone, water, methyl hydroxybenzoate, propyl
5 hydroxybenzoate, talc, magnesium stearate, and mineral oil. The pharmaceutical composition may be formulated into preparations by using a diluent or an excipient, such as a filler, an extender, a binder, a wetting agent, a disintegrant, or a surfactant. A solid
10 preparation for oral administration includes a tablet, a pill, a powder, granules, a capsule, and the like. These solid preparations may be prepared by mixing the tetraarsenic hexoxide of the present invention with at least one excipient, for example, starch, calcium
15 carbonate, sucrose or lactose, gelatin, or the like. Also, lubricants, such as magnesium stearate and talc, may be used in addition to simple excipients. A liquid preparation for oral administration corresponds to a suspension, a liquid for internal use, an emulsion, a
20 syrup, and the like, and may include simple diluents that are frequently used, such as water and liquid paraffin, and several excipients, such as a wetting agent, a sweetener, an aromatic agent, and a preservative. A preparation for parenteral
25 administration includes a sterile aqueous solution, a non-aqueous solvent, a suspension, an emulsion, a freeze-drying agent, and a suppository. The non-aqueous solvent and the suspension may include propylene glycol, polyethylene glycol, vegetable oils such as olive oil,
30 injectable esters such as ethylolate, and the like. A base material for the suppository may include Witepsol, Macrogol, Tween 61, cacao butter, laurin butter, glycerogelatin, and the like.

The dose of the pharmaceutical composition may vary
35 depending on age, gender, and body weight of a subject

to be treated, a particular disease or pathological condition to be treated, severity of a disease or pathological condition, route of administration, and determination of a prescriber. The determination of the
5 dose based on these factors is within the level of a person skilled in the art, and the general dose is in the range of approximately 0.01-500 mg/kg/day. A more preferable dose is 0.1-100 mg/kg/day. The administration may be carried out once a day or several times in a
10 divided dose a day. The above dose is not intended to restrict the scope of the present invention in any way.

The pharmaceutical composition may be administered to mammals, such as rats, domestic animals, and humans, via various routes. All manners of administration may be
15 predicted, and for example, the administration may be carried out through oral, rectal, intravenous, intramuscular, subcutaneous, endometrial, intracerebroventricular injection.

20 Advantageous Effects

The pharmaceutical compositions for prevention or treatment of cancer of the present invention have excellent anticancer effects by containing tetraarsenic hexoxide including 99% or more of tetraarsenic hexoxide
25 crystalline polymorph a.

Brief Description of the Drawings

FIG. 1 shows X-ray powder diffraction spectra of As_4O_6 -a and As_4O_6 -b.

30 FIG. 2 shows graphs depicting the results of assessing cell proliferation inhibitory effects after the lung cancer cell line NCL-H226 was treated with Example 1 and Comparative Examples 1 to 3 and incubated for 48 hours (FIG. 2A) and 72 hours (FIG. 2B).

35 FIG. 3 shows a graph depicting the results of

assessing lung cancer cell proliferation inhibitory effects according to treatment time when the lung cancer cell lines NCL-H226 and SK-MES-1 were treated with 10 μ M Example 1.

5 FIG. 4 shows graphs depicting the results of assessing esophageal cancer cell proliferation inhibitory effects according to treatment time when the esophageal cancer cell lines KYSE-150 and TE-1 were treated with 10 μ M Example 1 (FIG. 4A) and cell proliferation inhibitory effects after TE-1 was treated with Example 1 and Comparative Examples 1 to 3 and then incubated for 72 hours (FIG. 4B).

15 FIG. 5 shows graphs depicting the results of assessing gastric cancer cell proliferation inhibitory effects according to concentration of Example 1 and treatment time when the gastric cancer cell line AGS was treated with Example 1 (FIG. 5A) and cell proliferation inhibitory effects after AGS was treated with Example 1 and Comparative Examples 1 to 3 and then incubated for 20 72 hours (FIG. 5B).

25 FIG. 6 shows graphs depicting the results of assessing colorectal cancer cell proliferation inhibitory effects according to treatment time when the colorectal cancer cell lines HT29 and HCT116 were treated with 10 μ M Example 1 (FIG. 6A) and cell proliferation inhibitory effects after HT29 was treated with Example 1 and Comparative Examples 1 to 3 and then incubated for 72 hours (FIG. 6B).

30 FIG. 7 shows graphs depicting the results of assessing cell proliferation inhibitory effects after the prostate cancer cell line PC-3 was treated with Example 1 and Comparative Examples 1 to 3 and incubated for 48 hours (FIG. 7A) and 72 hours (FIG. 7B).

35 FIG. 8 shows graphs depicting the results of assessing cell proliferation inhibitory effects after

the pancreatic cancer cell line BxPC-3 was treated with Example 1 and Comparative Examples 1 to 3 and incubated for 72 hours (FIG. 8A) and cell proliferation inhibitory effects after the pancreatic cancer cell lines BxPC-3 and PANC-1 were treated with Example 1 of different concentrations and incubated for 72 hours (FIG. 8B).

FIG. 9 shows graphs depicting the results of assessing the cell proliferation inhibition after the ovarian cancer cell line SK-OV-3 (FIG. 9A), the human cervical cancer cell line HeLa (FIG. 9B), and the human endometrial cancer cell line HEC-1B (FIG. 9C) were treated with Example 1 and Comparative Examples 1 to 3 and then incubated for 72 hours.

FIG. 10 shows a graph depicting the results of comparing, through IC_{50} values, cell proliferation inhibition degrees of Example 1 in human lung, esophageal, gastric, colorectal, prostate, pancreatic, cervical, ovarian, and endometrial cancer cell lines.

Mode for Carrying Out the Invention

Hereinafter, preferable examples of the present invention will be described in detail. However, the present invention is not limited to the examples described herein, and thus may be embodied into different forms. Rather, these examples are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

Example 1: Preparation of present tetraarsenic hexoxide

A synthesis reactor (100 mm in height and 190 mm in diameter) specially manufactured using kaolin and three to six clamps capable of mounting filters thereon were prepared. A first clamp was installed at a distance of

50 mm from the synthesis reactor, and second to sixth clamps were installed above the first clamp at intervals of 2-6 mm from the first clamp, and the dimension of each clamp was 210 mm in diameter and 10 mm in thickness.

Coarse salt weighing 400-600 g (a moisture content of 10% or less) was introduced into the synthesis reactor, and then evenly spread out and packed to a thickness of about 20 mm. The synthesis reactor was slowly heated at 100-800°C for 3 hours, and continuously heated such that the surface temperature of the salt was 290±30°C inside the reactor, thereby removing moisture and impurities. Then, cooling was carried out at room temperature for 5 hours.

Then, 100 g of a raw material, As₂O₃ (a purity of 98% or higher, prepared by YUNNAN WENSHAN JINCHI ARSENIC CO., LTD.) was placed on the coarse salt inside the synthesis reactor, and filters (filter beds) capable of collecting sublimated arsenic were mounted on the three to six clamps installed above the synthesis reactor such that the intervals between the filters were 2-6 mm. The filters used herein preferably had a basic weight of 70-100 g/m², a thickness of 0.17-0.25 mm, a filtration speed of 22-30 s/100 ml, and a retention rate of 5-10 μm.

The filters were fixed using the clamps, and then heat was applied to the bottom portion of the synthesis reactor to gradationally raise the temperature from 100°C to 1,000°C. First, the bottom portion of the synthesis reactor was heated for 1 hour such that the temperature outside the bottom portion of the synthesis reactor was about 350±100°C, and thereafter, heating was carried out such that the temperature outside the bottom portion of the synthesis reactor was about 600-650°C and about 700-1,000°C, so the temperature of the center portion of the highest filter bed was maintained at 150±100°C through

heating for a total of 5-10 hours. Then, cooling was carried out at room temperature for 5-7 hours. In this procedure, the As_2O_3 powder placed on the salt inside the synthesis reactor transformed into a gas inside the synthesis reactor, and the gas moved up, and then transformed into a liquid since the upper temperature outside the synthesis reactor was relatively low, and thereafter, the liquid was crystallized as a solid, and thus white crystals were generated on the filters.

The collected white crystals were placed on the coarse salt inside the synthesis reactor, and the heating, cooling, and crystal collecting processes were again repeated four times, thereby finally obtaining 12.0 g of the crystals. As a result of checking the structure of the obtained arsenic compound crystals, it was confirmed that most of the crystals were As_4O_6 -a while 99 wt% or more of As_4O_6 -a and less than 1 wt% of As_4O_6 -b were obtained.

It was confirmed that as for the differential scanning calorimetry (DSC) value at a temperature rise rate of 10 $^{\circ}\text{C}/\text{min}$, As_4O_6 -a showed an endothermic peak (melting point) at 282.67 $^{\circ}\text{C}$ and As_4O_6 -b showed an endothermic peak (melting point) at 286.77 $^{\circ}\text{C}$.

X-ray powder diffraction spectra of As_4O_6 -a and As_4O_6 -b are shown in FIG. 1, and diffraction data of As_4O_6 -a and As_4O_6 -b are shown in Table 2 below.

[Table 2]

As_4O_6 -a		As_4O_6 -b	
2 θ ($^{\circ}$)	Diffraction intensity	2 θ ($^{\circ}$)	Diffraction intensity
13.84	7631.01	13.86	4012.09
27.88	10000	27.92	10000
32.32	2801.74	32.36	2130.23
35.3	3369.82	35.34	2511
39.84	623.242	39.9	447.422
42.38	1551.5	42.44	1431.86

46.34	2345.2	46.4	4159.8
48.6	447.69	48.66	564.995
49.34	502.761	49.4	375.571

As confirmed in FIG. 1 and Table 2, the ratio of main peaks shown at 2θ values of 13.8 and 27.9 was 1:1.3 in As_4O_6 -a, and the ratio of main peaks shown at 2θ values of 13.8 and 27.9 was 1:2.5 in As_4O_6 -b. DSC analysis, structure determination, and X-ray diffraction analysis of the prepared compounds were carried out by the following methods.

(1) DSC analysis

Using a DSC system (SDT Q600 V20.9 Build 20), 20.0 mg of a sample was analyzed while the temperature was raised to 310°C at a temperature rise rate of 10°C/min with N_2 flowing out at 100mL/min.

(2) X-ray crystallography

Single crystals of tetraarsenic hexoxide (As_4O_6 , MW=395.6) were placed on a glass fiber and then an X-ray beam was applied thereto, to observe diffraction patterns on photographic films and the presence or absence of the organization of diffraction data, thereby determining space groups and cell parameters. Diffraction intensities were collected in the range of $10^\circ < 2\theta < 50^\circ$. The crystal structure of As_4O_6 was determined from the data by the Patterson method by using a structure determination program (SHELXTL program).

(3) X-ray diffractometry

A sample was prepared by pulverizing the obtained crystals into particles having a size of 10-30 μm (-325 mesh), filling a glass holder for X-ray diffraction analysis (20 mm x 16 mm x 1 mm) with the particles, compressing the particles by a glass slide or the like, and flattening the particles to allow a sample surface to be parallel with a holder surface. The X-ray

diffraction spectrum of the crystals was drawn using Cu $K\alpha_1$ (1.54060 Å) of XRD within a diffraction angle (2θ) of 10° to 50° at a rate of $1^\circ/\text{min}$ (scan step of 0.02°).

5 **Comparative Example 1: Preparation of tetraarsenic hexoxide**

 A synthesis reactor (100 mm in height and 190 mm in diameter) specially manufactured using kaolin and three to six clamps capable of mounting filters thereon were
10 prepared. A first clamp was installed at a distance of 50 mm from the synthesis reactor, and second to sixth clamps were installed above the first clamp at intervals of 2-6 mm from the first clamp, and the dimension of each clamp was 210 mm in diameter and 10 mm in
15 thickness.

 Coarse salt weighing 400-600 g (a moisture content of 10% or less) was introduced into the synthesis reactor, and then evenly spread out and packed to a thickness of about 20 mm. The synthesis reactor was
20 slowly heated at $100-800^\circ\text{C}$ for 3 hours, and continuously heated such that the surface temperature of the salt was $290\pm 30^\circ\text{C}$ inside the reactor, thereby removing moisture and impurities. Then, cooling was carried out at room temperature for 5 hours.

25 Then, 100 g of a raw material, As_2O_3 (a purity of 98% or higher, prepared by YUNNAN WENSHAN JINCHI ARSENIC CO., LTD.) was placed on the coarse salt inside the synthesis reactor, and filters (filter beds) capable of collecting sublimated arsenic were mounted on the three
30 to six clamps installed above the synthesis reactor such that the intervals between the filters were 2-6 mm. The filters used herein preferably had a basic weight of 70-100 g/m^2 , a thickness of 0.17-0.25 mm, a filtration speed of 22-30 s/100 ml, and a retention rate of 5-10 μm .

35 The filters were fixed using the clamps, and then

heat was applied to the bottom portion of the synthesis reactor to gradationally raise the temperature from 100°C to 1,000°C. First, the bottom portion of the synthesis reactor was heated for 1 hour such that the temperature outside the bottom portion of the synthesis reactor was about 350±100°C, and thereafter, heating was carried out such that the temperature outside the bottom portion of the synthesis reactor was about 600-650°C and about 700-1,000°C, so the temperature of the center portion of the highest filter bed was maintained at 150±100°C through heating for a total of 5-10 hours. Then, cooling was carried out at room temperature for 5-7 hours. In this procedure, the As₂O₃ powder placed on the salt inside the synthesis reactor transformed into a gas inside the synthesis reactor, and the gas moved up, and then transformed into a liquid since the upper temperature outside the synthesis reactor was relatively low, and thereafter, the liquid was crystallized as a solid, and thus white crystals were generated on the filters. 48.5 g of crystals were collected from the filters. As a result of checking the crystal structure of the collected arsenic compounds, it was confirmed that As₄O₆-b accounted for 99 wt% or more.

Comparative Examples 2 to 4: Preparation of tetraarsenic hexoxide

Comparative Examples 2 and 3 were prepared by mixing Example 1 (composition having 99% or more of crystalline polymorph As₄O₆-a) and Comparative Example 1 (composition having 99% or more of crystalline polymorph As₄O₆-b) at 4:1 and 1:1, respectively.

Test Example 1: Test on human cancer cell proliferation inhibitory effects

(1) Materials and Cell culture

Fetal bovine serum (FBS) and cell culture medium were prepared (Hyclone), and dimethyl sulfoxide (DMSO) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco LLC, USC) were prepared.

5 As human cancer cell lines, human lung cancer cell lines NCL-H226 and SK-MES-1, human esophageal cancer cell lines KYSE-150 and TE-1, human gastric cancer cell line AGS, human colorectal cancer cell lines HT29 and HCT116, human prostate cancer cell line PC-3, human
10 pancreatic cancer cell lines BxPC-3 and PANC-1, human cervical cancer cell lines SiHa and HeLa, human ovarian cancer cell line SK-OV-3, and human endometrial cancer cell lines Ishikawa, HEC-1A, and HEC-1B were obtained from the Shanghai Cell Bank of Chinese Academy of
15 Sciences. The cells were incubated in media supplemented with 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin and suitable for incubation of respective cells (RPMI-1640 (NCL-H226, TE-1, BxPC-3, Ishikawa), MEM (SK-MES-1, SiHa, HeLa, HEC-1B), Ham's F-12K (AGS), Ham's
20 F-12 (PC-3), McCoy's 5A (HT29, HCT116, SK-OV-3, HEC-1A), RPMI-1640:Ham's F-12=1:1(v:v) (KYSE-150), and DMEM (PANC-1)) in a humidified incubator with 5% CO₂ and 95% air. The media were exchanged every three days.

(2) Cell proliferation assay (MTT assay)

25 The effects of Example 1 and Comparative Examples 1 to 3 on cell proliferation were assessed using MTT assay. MTT assay is based on the ability of viable cells against MTT to produce insoluble dark blue formazan products. After the cells were suspended in the medium
30 by trypsin treatment and collected, the cells were dispensed at a density of 4×10^3 cells/well in a 96-well culture dish (Costar, Cambridge, MA, USA). After 24 hours, the cells in the media containing 10% FBS were treated with Example 1 and Comparative Examples 1 to 3,
35 at 0, 0.625, 1.25, 2.5, 5, 10, 20, 40, or 80 µM, and

then incubated. Here, stock solutions obtained by dissolving Example 1 and Comparative Examples 1 to 3 at 5×10^{-2} M in 1 M sodium hydroxide was used. For MTT assay for cell proliferation, supernatants were removed from the cells incubated for 24 hours, 48 hours, and 72 hours after the sample treatment, and 20 μ l of 5 mg/ml MTT solution was added per well, and the cells were incubated at 37°C for 4 hours to form formazan crystals. After the incubation, supernatants were again removed, followed by addition of 100 μ l of DMSO to every well, and then mixing was carried out to completely dissolve dark blue crystals. All the crystals were completely dissolved by standing at room temperature for 15 minutes, and the absorbance was measured using a micro-plate reader at a wavelength of 570nm ($A_{570\text{nm}}$).

(3) Statistical analysis

The absorbance value of the control group treated without the sample was calculated as 100, and the absorbance value of the treatment group treated with the sample, compared with that of the control group, was calibrated, and the percentage of inhibition of cell proliferation was calculated according to the following equation.

Percentage (%) of inhibition of cell proliferation
= ((mean absorbance of control group cells - mean absorbance of treatment group cells)/mean absorbance of control group cells) $\times$ 100

All data were expressed as mean $\pm$ standard error of the mean (mean $\pm$ SEM). One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used to perform multiple comparison. Statistical significance was defined as $p < 0.05$, and each test was repeated three times.

(4) Results of investigating inhibition of lung cancer cell line proliferation

The human lung cancer cell lines NCL-H226 and SK-MES-1 were treated with Example 1 and Comparative Examples 1 to 3, and incubated for 24, 48, and 72 hours, followed by MTT assay. The results obtained using NCL-H226 are shown in FIGS. 2 and 3.

All of the test results obtained by treating NCL-H226 with Example 1 and Comparative Examples 1 to 3 and then incubating the cells for 48 hours (FIG. 2A) and 72 hours (FIG. 2B) confirmed that the percentages of inhibition of the lung cancer cell line NCL-H226 proliferation were higher in the treatment with Example 1 than the treatment with Comparative Examples 1 to 3.

In addition, as a result of investigating the inhibition of cancer cell proliferation according to treatment time when the lung cancer cell lines NCL-H226 and SK-MES-1 were treated with 10 μ M Example 1 (FIG. 3), it was confirmed that the percentage of inhibition of cell proliferation was increased with the treatment time in both of NCL-H226 and SK-MES-1.

(5) Results of investigating inhibition of esophageal cancer line proliferation

The human esophageal cancer cell lines KYSE-150 and TE-1 were treated with Example 1 and Comparative Examples 1 to 3, and incubated for 24, 48, and 72 hours, followed by MTT assay. The results are shown in FIG. 4.

As a result of investigating the inhibition of cancer cell proliferation according to treatment time after KYSE-150 and TE-1 were treated with 10 μ M Example 1 (FIG. 4A), it was confirmed that in the case of the esophageal cancer cell line TE-1, the percentage of inhibition of cell proliferation was increased with the treatment time, and in the case of the esophageal cancer cell line KYSE-150, there is no great difference in percentage of inhibition of cell proliferation between the treatment for 24 hours and the treatment for 48

hours, but the percentage of inhibition of cell proliferation was significantly increased in the treatment for 72 hours.

In addition, all of the test results obtained by
5 treating TE-1 with Example 1 and Comparative Examples 1 to 3 and then incubating the cells for 72 hours (FIG. 4B) confirmed that the percentage of inhibition of the esophageal cancer cell line TE-1 proliferation was higher in the treatment with Example 1 than the
10 treatment with Comparative Examples 1 to 3.

(6) Results of investigating inhibition of gastric cancer line proliferation

The human gastric cancer cell line AGS was treated with Example 1 and Comparative Examples 1 to 3, and
15 incubated for 24, 48, and 72 hours, followed by MTT assay. The results are shown in FIG. 5.

As a result of investigating the inhibition of cancer cell proliferation according to treatment time when the gastric cancer cell line AGS was treated with
20 Example 1 of different concentrations (FIG. 5A), it was confirmed that the inhibition of cell proliferation was increased with the treatment time at each treatment concentration, and the percentage of inhibition of gastric cancer cell proliferation was high in spite of
25 the treatment for only 48 hours.

In addition, all of the test results obtained by treating AGS with Example 1 and Comparative Examples 1 to 3 and then incubating the cells for 72 hours (FIG. 5B) confirmed that the percentage of inhibition of
30 gastric cancer cell line AGS proliferation was higher in the treatment with Example 1 than the treatment with Comparative Examples 1 to 3.

(7) Results of investigating inhibition of colorectal cancer cell line proliferation

35 The human colorectal cancer cell lines HT29 and

HCT116 were treated with Example 1 and Comparative Examples 1 to 3, and incubated for 24, 48, and 72 hours, followed by MTT assay. The results are shown in FIG. 6.

As a result of investigating the inhibition of cancer cell proliferation according to treatment time when HT29 and HCT116 were treated with 10 μ M Example 1 (FIG. 6A), it was confirmed that the percentage of inhibition of cell proliferation was increased with the treatment time in both HT29 and HCT116.

In addition, all of the test results obtained by treating HT29 with Example 1 and Comparative Examples 1 to 3 and then incubating the cells for 72 hours (FIG. 6B) confirmed that the percentage of inhibition of the colorectal cancer cell line HT29 proliferation was higher in the treatment with Example 1 than the treatment with Comparative Examples 1 to 3.

(8) Results of investigating inhibition of prostate cancer cell line proliferation

The prostate cancer cell line PC-3 was treated with Example 1 and Comparative Examples 1 to 3, and incubated for 48 and 72 hours, followed by MTT assay. The results are shown in FIG. 7.

All of the test results obtained by treating PC-3 with Example 1 and Comparative Examples 1 to 3 and then incubating the cells for 48 hours (FIG. 7A) and 72 hours (FIG. 7B) confirmed that the percentage of inhibition of the prostate cancer cell line PC-3 proliferation was higher in the treatment with Example 1 than the treatment with Comparative Examples 1 to 3.

(9) Results of investigating inhibition of pancreatic cancer cell line proliferation

The human pancreatic cancer cell lines BxPC-3 and PANC-1 were treated with Example 1 and Comparative Examples 1 to 3, and incubated for 24, 48, and 72 hours, followed by MTT assay. The results are shown in FIG. 8.

All of the test results obtained by treating BxPC-3 with Example 1 and Comparative Examples 1 to 3 and then incubating the cells for 72 hours (FIG. 8A) confirmed that the percentage of inhibition of the pancreatic cancer cell line BxPC-3 proliferation was higher in the treatment with Example 1 than the treatment with Comparative Examples 1 to 3.

In addition, the test results obtained by treating the pancreatic cancer cell lines BxPC-3 and PANC-1 with Example 1 according to concentration of Example 1 and then incubating the cells for 72 hours (FIG. 8B) confirmed that the percentage of inhibition of cell proliferation was increased dependent on the concentration of Example 1 in both BxPC-3 and PANC-1.

(10) Results of investigating inhibition of ovarian, cervical, and endometrial cancer cell proliferation

The human ovarian cancer cell line SK-OV-3, the human cervical cancer cell lines SiHa and HeLa, and the human endometrial cancer cell lines Ishikawa, HEC-1A, and HEC-1B were treated with Example 1 and Comparative Examples 1 to 3, and incubated for 72 hours, followed by MTT assay. The results are shown in FIG. 9.

All of the test results obtained by treating the human ovarian cancer cell line SK-OV-3 (FIG. 9A), the human cervical cancer cell line HeLa (FIG. 9B), and the human endometrial cancer cell line HEC-1B (FIG. 9C) with Example 1 and Comparative Examples 1 to 3 and then incubating the cells for 72 hours confirmed that the percentages of inhibition of the ovarian, cervical, and endometrial cancer cell proliferation were higher in the treatment with Example 1 than the treatment with Comparative Examples 1 to 3.

(11) Comparison of cell proliferation inhibitory activity in various cancer cell lines

IC₅₀ values of Example 1 were analyzed on the basis of the test results obtained by the treatment with Example 1 for 72 hours in the test results obtained through the tests of inhibition of the human lung, esophageal, gastric, colorectal, prostate, pancreatic, ovarian, cervical, and endometrial cancer cells proliferation. The analysis results are shown in Table 3 and FIG. 10.

[Table 3]

Cancer type	Cell line	Cancer cell growth inhibition (IC ₅₀ , μ M)
Lung cancer	NCL-H226	5.12
	SK-MES-1	2.47
Esophageal cancer	KYSE-150	9.85
	TE-1	2.35
Gastric cancer	AGS	0.9
Colorectal cancer	HT29	1.27
	HCT116	5.07
Prostate cancer	PC-3	4.98
Pancreatic cancer	BxPC-3	4.98
	PANC-1	5.62
Cervical cancer	SiHa	2.8
	HeLa	1.63
Ovarian cancer	SK-OV-3	5.02
Endometrial cancer	Ishikawa	2.75
	HEC-1A	2.47
	HEC-1B	4.61

10

As can be seen from Table 3 and FIG. 10, the IC₅₀ values of Example 1, which are associated with cancer cell proliferation inhibitory activity, had low concentrations in all of the types of cancer.

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Although not shown in the present invention, the tetraarsenic hexoxide of the present invention was confirmed to inhibit the proliferation of various cancer cells, such as brain cancer, breast cancer, liver cancer, and skin cancer.

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Therefore, it can be seen that Example 1 showed excellent anticancer effects by inhibiting the growth of cancer cells in various cancers, such as lung cancer,

esophageal cancer, gastric cancer, colorectal cancer, prostate cancer, pancreatic cancer, cervical cancer, ovarian cancer, and endometrial cancer.

Claims

1. A pharmaceutical composition containing tetraarsenic hexoxide (As_4O_6) as an active ingredient for prevention or treatment of cancer, wherein the tetraarsenic hexoxide includes 99 wt% or more of tetraarsenic hexoxide crystalline polymorph a having features (i) to (iii) below:
- (i) Cell parameters:
- $a = b = c = 11.0734 \text{ \AA}$
 $\alpha = \beta = \gamma = 90^\circ$
 $V = 1357.82 \text{ \AA}^3$
- (ii) As-O bond length: $1.786 \text{ \AA}$
- (iii) O-As-O bond angle: 98.36°
2. The pharmaceutical composition of claim 1, wherein the tetraarsenic hexoxide is prepared by:
- a first step of heating sodium chloride at $100\sim 800^\circ\text{C}$, followed by cooling;
- a second step of placing arsenic trioxide (As_2O_3) on the sodium chloride, followed by heating from 100°C to 1000°C in an airtight state and then cooling;
- a third step of separating crystals crystallized in a filter bed collecting sublimated arsenic; and
- a fourth step of repeating the second and third steps four to ten times using the crystals obtained in the third step instead of the arsenic trioxide in the second step, thereby obtaining tetraarsenic hexoxide crystals.
3. The pharmaceutical composition of claim 1, wherein the tetraarsenic hexoxide has a purity of 99.9% or higher.
4. The pharmaceutical composition of claim 1,

wherein in the X-ray powder diffraction spectrum of the crystalline polymorph a, obtained by using a light source wavelength of 1.5406 Å within a diffraction angle (2θ) of 10° to 50° at a rate of 1°/min (scan step of 5 0.02°), peaks are shown at 2θ values of 13.84, 27.88, 32.32, 35.3, 39.84, 42.38, 46.34, 48.6, and 49.34.

5. The pharmaceutical composition of any one of claims 1 to 4, wherein the cancer is selected from the 10 group consisting of lung cancer, esophageal cancer, gastric cancer, colorectal cancer, prostate cancer, pancreatic cancer, cervical cancer, ovarian cancer, and endometrial cancer.