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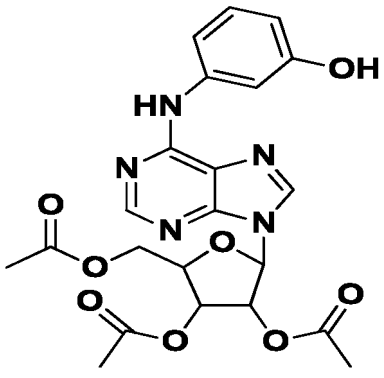
(54) TRIACETYL-3-HYDROXYPHENYLADENOSINE AND APPLICATION IN PREPARING PHARMACEUTICAL DRUG FOR PREVENTING OR TREATING NON-ALCOHOLIC FATTY LIVER DISEASE

(57) The present invention provides an application of triacetyl-3-hydroxyphenyladenosine represented by formula (I) in preventing or treating non-alcoholic fatty liver disease. The triacetyl-3-hydroxyphenyladenosine can significantly reduce the levels of serum AST, ALT and TG, significantly improve liver functions, and alleviate liver steatosis. The invention provides significant curative effects for preventing or treating non-alcoholic fatty liver and has limited toxic side effects.



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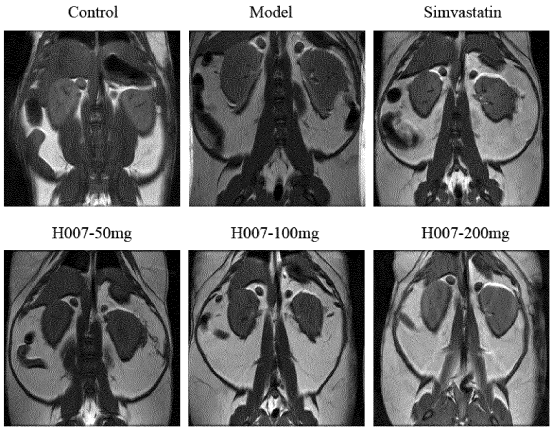


Fig. 5

Description

TECHNICAL FIELD

[0001] The invention relates to an application of triacetyl-3-hydroxyphenyladenosine and a pharmaceutical composition containing the same in the preparation of a pharmaceutical drug for preventing or treating non-alcoholic fatty liver disease, which belongs to the technical field of medicine.

BACKGROUND

[0002] Non-alcoholic fatty liver disease (NAFLD) refers to a group of clinical pathological syndromes characterized by liver parenchymal cell damage and fat accumulation caused by other than excessive alcohol consumption and other definite pathogenesis of liver damage, which is a metabolically-stressed liver damage closely related to insulin resistance (IR) and genetic susceptibility, and the pathological changes of which are similar to those of alcoholic liver disease, but the patient has no history of excessive drinking. Its components include non-alcoholic simple fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), non-alcoholic fatty liver fibrosis, non-alcoholic fatty liver cirrhosis, and related hepatic carcinoma (HCC). With the development of our country's economy, the content and time of people's diet have changed a lot, and the incidence of non-alcoholic fatty liver disease has been continuously rising, and trends in young person, which has become a common disease that seriously threatens the physical and psychological health of human beings.

[0003] The exact pathogenesis of NAFLD is still unclear, and it is generally accepted as the "two hit" theory, the first hit in the two phases of the theory is that a decrease in the amount of decomposition and excessive intake of high-fat diet results in lipid deposition and the formation of simple fatty liver. In the two hit, IR can weaken and destroy the regulation of insulin on fat metabolism, increase lipid lysis, increase non-esterified free fatty acid (FFA) concentration, and promote liver uptake of FFA in the blood. Oxidative stress and lipid peroxidation injury play an important role in the formation and development of fatty liver, which is an important factor in the further development of fatty liver by the second hit. Mitochondrion is a respiratory organ of cells, the increased generation of reactive oxygen species (ROS) damages the mitochondrion, further accelerating lipid accumulation in the liver. In addition, the free radicals produced by oxidative stress cause the damage reaction of lipid peroxidation (LPO), form a series of lipid radicals and degradation products - malondialdehyde (MDA), at the same time, destroy the structure and function of the biomembrane, increase the permeability of the cell membrane, cause the cytochrome C to flow out, initiate the apoptosis program, and finally lead to liver fibrosis, liver cirrhosis, and even to hepatic carcinoma. At present, there is still a lack of specific drugs. Commonly used lipid-lowering drugs such as statins and fibrates have poor efficacy and great toxic and side effect.

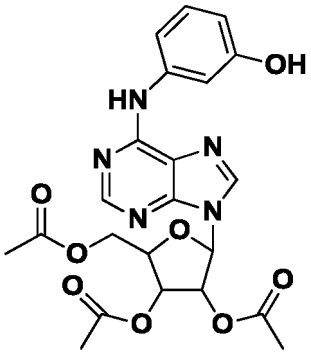
[0004] Triacetyl-3-hydroxyphenyladenosine (Patent No. ZL200980101131.6, Publication No. CN101874036B, Notice Date 2012.01.25) is a new structural type compound with significant lipid regulating activity screened in cordycepin derivatives by the Institute of Materia Medica, Chinese Academy of Medical Sciences, and has the characteristics of small toxic and side effects and good pharmacokinetics, etc., which is currently in the pre-clinical research stage. There is no report on the application of this compound in the prevention or treatment of non-alcoholic fatty liver disease.

SUMMARY OF THE INVENTION

[0005] The technical problem solved by the present invention is to provide an application of a compound triacetyl-3-hydroxyphenyladenosine and a pharmaceutical composition thereof in the preparation of a pharmaceutical drug for preventing or treating non-alcoholic fatty liver disease.

[0006] In order to solve the technical problem of the present invention, the following technical solution is provided:

a first aspect of the technical solution of the present invention is to provide an application of triacetyl-3-hydroxyphenyladenosine represented by formula (I) and a pharmaceutically acceptable salt thereof in the preparation of a pharmaceutical drug for preventing or treating non-alcoholic fatty liver disease,



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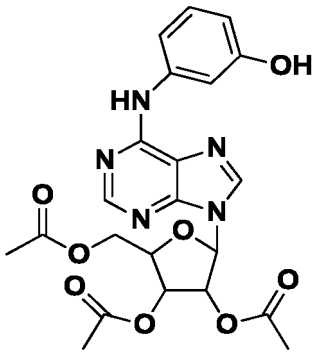
the non-alcoholic fatty liver disease is fatty liver disease caused by a high-calorie diet,
the treatment of non-alcoholic fatty liver disease with the triacetyl-3-hydroxyphenyladenosine of the present invention is that it can significantly reduce the levels of serum AST, ALT and TG, significantly improve liver function of a golden hamster with fatty liver disease, reduce the degree of fatty liver, thereby preventing or treating non-alcoholic fatty liver disease. A second aspect of the technical solution of the present invention is to provide an application of a pharmaceutical composition in the preparation of a pharmaceutical drug for preventing or treating non-alcoholic fatty liver disease, characterized in that, the pharmaceutical composition comprises triacetyl-3-hydroxyphenyladenosine represented by formula (I) and a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier,



(I),

the pharmaceutical composition includes a tablet, a capsule, a pill and an injection, a sustained release formulation, a controlled release formulation or various microparticle drug delivery systems. The pharmaceutical composition can be prepared according to methods known in the art. Any dosage form suitable for human or animal use may be made by combining the compound of the present invention with one or more pharmaceutically acceptable solid or liquid excipients and/or adjuvants. The content of the compound of the present invention in the pharmaceutical composition thereof is usually from 0.1 to 95% by weight.

[0007] The compound or the pharmaceutical composition containing the same of the present invention may be administered in unit dosage form, and the administration route may be intestinal or parenteral, such as oral, intravenous, intramuscular, subcutaneous, nasal, oral mucosa, eyes, lung and respiratory tract, skin, vagina, rectum, and the like.

[0008] The administration dosage form may be liquid, solid or semi-solid dosage forms. The liquid dosage forms may be solutions (including true solutions and colloidal solutions), emulsions (including o/w type, w/o type, and double emulsions), suspensions, injections (including water injections, powder injections and infusions), eye drops, nose drops, lotions, and liniments, etc.; the solid dosage forms may be tablets (including common tablets, enteric-coated tablets, lozenges, dispersible tablets, chewable tablets, effervescent tablets, orally disintegrating tablets), capsules (including hard capsules, soft capsules, enteric-coated capsules), granules, powders, mini-pills, dripping pills, suppositories, films, patches, (power) aerosols, sprays, and the like; and the semi-solid dosage forms may be ointments, gels, pastes, and the like. The preferred dosage form of the pharmaceutical composition is selected from the group consisting of tablets,

capsules, pills, and injections.

[0009] The compound of the present invention can be prepared into ordinary preparations, also made into sustained release preparations, controlled release preparations, targeting preparations, and various microparticle drug delivery systems.

[0010] In order to prepare the compound of the present invention into tablets, various excipients known in the art can be widely used, including diluents, binders, humectants, disintegrants, lubricants, and glidants. The diluent may be starch, dextrin, sucrose, glucose, lactose, mannitol, sorbitol, xylitol, microcrystalline cellulose, calcium sulfate, calcium hydrogen phosphate, calcium carbonate, and the like; the humectant may be water, ethanol, isopropanol, and the like; the binder may be starch slurry, dextrin, syrup, honey, glucose solution, microcrystalline cellulose, mucilago acaciae, gelatin slurry, sodium carboxymethylcellulose, methylcellulose, hydroxypropyl methyl cellulose, ethyl cellulose, acrylic resin, carbomer, polyvinylpyrrolidone, polyethylene glycol, and the like; the disintegrant may be dry starch, microcrystalline cellulose, low-substituted hydroxypropyl cellulose, crospolyvinylpyrrolidone, croscarmellose sodium, sodium carboxymethyl starch, sodium hydrogen carbonate and citric acid, polyoxyethylene sorbitol fatty acid ester, sodium dodecyl sulfate, and the like; and the lubricant and the glidant may be talc, silica, stearate, tartaric acid, liquid paraffin, polyethylene glycol, and the like.

[0011] The tablets may also be further prepared into coated tablets, such as a sugar-coated tablets, film-coated tablets, enteric coated tablets, or double-layer tablets and multilayer tablets.

[0012] In order to formulate the dosing unit into capsules, the active ingredient, the compound of the present invention, may be mixed with a diluent, a glidant, and the mixture may be placed directly in a hard or soft capsule. The active ingredient, the compound of the present invention, may also be first prepared into granules or mini-pills with a diluent, binder, or disintegrant, and then placed in a hard or soft capsule. A wide variety of diluents, binders, humectants, disintegrants, glidants for the preparation of tablets of the compound of the present invention may also be used in the preparation of capsules of the compound of the present invention.

[0013] In order to prepare the compound of the present invention into injections, water, ethanol, isopropanol, propylene glycol, or a mixture thereof may be used as a solvent and added with an appropriate amount of solubilizers, solubilizers, PH adjusting agents, osmotic pressure regulators commonly used in the art. The solubilizer or glidant may be poloxamer, lecithin, hydroxypropyl-beta-cyclodextrin, and the like; the PH adjusting agent may be phosphate, acetate, hydrochloric acid, sodium hydroxide, and the like; and the osmotic pressure regulator may be sodium chloride, mannitol, glucose, phosphate, acetate, and the like. For preparing freeze-dried powder injections, mannitol, glucose, and the like may also be added as a proppant.

[0014] In addition, colorants, preservatives, perfumes, corrigents, or other additives may also be added to the pharmaceutical preparation as needed.

[0015] In order to achieve the purpose of medication and enhance the therapeutic effect, the pharmaceutical or pharmaceutical composition of the present invention may be administered by any known method of administration.

[0016] The dosage of the pharmaceutical composition of the compound of the present invention may vary depending on the nature and severity of the disease to be prevented or treated, the individual condition of the patient or animal, the route of administration and the dosage form, and the like. In general, suitable daily dosage for the compound of the invention may range from 0.001 to 150 mg/kg body weight, preferably 0.1 to 100 mg/kg body weight, more preferably 1 to 60 mg/kg body weight, and most preferably 2 to 30 mg/kg body weight. The above dosages can be administered in one dosage unit or divided into several dosage units, depending on the clinician's clinical experience and dosage regimen including the use of other treatment means.

[0017] The compound or composition of the present invention may be administered alone or in combination with other therapeutic drugs or symptomatic drugs. When the compound of the present invention has a synergistic effect with other therapeutic drugs, its dosage should be adjusted according to actual conditions.

Beneficial technical effect

[0018] The present invention has confirmed the significant effect of triacetyl-3-hydroxyphenyladenosine in the prevention or treatment of non-alcoholic fatty liver disease using pharmacodynamics research methods, which provides a new preventive or therapeutic drug, triacetyl-3-hydroxyphenyladenosine, for this chronic disease with complicated pathogenesis and poor therapeutic effect, with obvious curative effect, little toxic and side effects and safe use, and provides scientific basis for clinical application in the prevention or treatment of non-alcoholic fatty liver disease.

DESCRIPTION OF THE DRAWINGS

[0019] In order to make the content of the present invention more clearly understood, the present invention is further described in detail in the following with reference to specific embodiments of the present invention and with reference to the accompanying drawings, wherein,

FIG. 1 is a comparison of body weight and liver coefficient of golden hamsters of each group in the experimental example of the present invention;

FIG. 2 is a comparison of the determination results of content of cholesterol, triglyceride, high-density lipoprotein, low-density lipoprotein, and free fatty acid in the serum of golden hamsters in the experimental example of the present invention;

FIG. 3 is a comparison of ALT and AST enzyme activity of golden hamsters in the experimental example of the present invention;

FIG. 4 is a comparison of the determination results of lipid contents in liver tissues of golden hamsters of each group in the experimental example of the present invention;

FIG. 5 is a nuclear magnetic imaging diagram of golden hamsters in the experimental example of the present invention;

FIG. 6 is a comparison of HE pathological staining results of liver tissues of golden hamsters in various experimental groups of the present invention;

FIG. 7 is a comparison of the Oil Red O staining results of liver tissues of golden hamsters in various experimental groups of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0020] The following examples are intended to further illustrate the present invention, but are not meant to limit the present invention in any way.

Example 1: Application of Triacetyl-3-Hydroxyphenyladenosine (IMM-H007) in the Treatment of Non-Alcoholic Fatty Liver Disease

I. Experimental Materials

1. Reagents

[0021] OCT frozen section embedding agent, SAKURA Tissue-Tek®; pentobarbital sodium, SIGMA; PEG6000, SIGMA; glycine, SIGMA; paraformaldehyde, Sinopharm Chemical ReagentCo., Ltd; Oil Red O, SIGMA; HE staining solution, Taiwan Baso Corporation; total cholesterol (TC) detection kit, Sekisui Medical Technology (China) LTD; total triglyceride detection kit, BioSino Bio-Technology & Science Inc.; free fatty acid detection kit, Sekisui Medical Technology (China) LTD; glutamic-pyruvic transaminase (AST/ALT) detection kit, Nanjing Jiancheng Bioengineering Institute; glutamic oxalacetic transaminase (AST/GOT) detection kit, Nanjing Jiancheng Bioengineering Institute.

2. Instruments

[0022] Multi-purpose low-temperature high-speed centrifuge, Eppendorff, Germany; paraffin microtome, Leica, Germany; frozen microtome, Leica, Germany; En Vision multimode reader, PerkinElmer, Inc., USA; small animal anaesthesia machine, Matrx Products, USA; small animal magnetic resonance imaging machine, Bruker PharmaScan 70T/16 US, Germany.

3. Experimental Animals

[0023] 6 to 8-week-old Syrian golden hamsters (LVG hamster, imported from Charles River Laboratories), 20, weighing 90-120 g, male, SPF grade, purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and license number: SCXK (Beijing) 2012-0001.

II. Experimental Methods

1. Animal Grouping and Rearing

[0024] After 5 days of adaptive feeding, animals were randomly divided into a normal control group (n=15), a high-fat diet group (n=15), an IMMH007 low-dose group (25 mg/kg, n=15), an IMMH007 medium-dose group (50 mg/kg, n=15), and an IMMH007 high-dose group (100 mg/kg, n=15), and given intragastric administration twice daily. The animals were reared in the Animal Experiment Center II of the Institute of Materia Medica, Chinese Academy of Medical Sciences, and the rearing conditions were SPF grade, temperature of 21±2°C, relative humidity of 50±5%, light cycle of 12/112, and 5 per cage. The normal group was given normal basal diet, and the high-fat diet group was given high-fat diet (79.8%

basal diet added with 20% lard and 0.2% cholesterol), and the animals were allowed to eat and drink freely. The feed was commissioned by Beijing HFK Bioscience Co., Ltd. Body weight was recorded every 2 weeks during the experiment.

2. Observation Indicators and Measurement Methods

2.1 Serum Biochemical Indicators

[0025] The animals were fasted for 12 hours, and 0.5 ml blood was collected from the angular vein and allowed to stand for 60 min, and centrifuged at 6000 g for 10 min, and the supernatant was aspirated as much as possible, and then centrifuged at 6000 g for 10 min. The absorbance values were measured according to the instructions of the TC, TG, AST, and ALT kits, and the concentration of each index was calculated. 50 ul serum was mixed with 50 ul PEG6000 in a ratio of 1:1, vortexed uniformly, allowed to stand for 10 min, and centrifuged at 1900 g for 20 min at room temperature, and the supernatant was carefully pipetted, stored at 4°C, and measured for HDL-C according to the instructions of the TC kit. The plasma LDL level was calculated by subtracting HDL-C and 0.2-fold TG levels from plasma total cholesterol TC, ie, LDL=TC-HLD-0.2TG.

[0026] The animals were fasted overnight before the end of the experiment, anesthetized by intraperitoneal injection of 3% pentobarbital sodium, the abdominal cavity was exposed, and the liver was rapidly separated after blood was taken from the aorta abdominalis. One liver lobe was preserved, and two pieces of 1×1 cm^3 pieces were cut at a fixed site, one piece was embedded in OCT, quickly frozen in liquid nitrogen, and stored in liquid nitrogen or at -80°C; and the other piece was placed in a 4% paraformaldehyde stationary liquid and stored at 4°C.

2.2 Magnetic Resonance Imaging

[0027] After being fasted for 12 hours, the animals were anesthetized with isoflurane, fixed on their heads, and supine and fixed on the rat's coil, the head entered first, and the center of the abdomen was positioned.

[0028] T2-weighted imaging (T2WI) of the fast spin echo sequence: TR/TE=200/3 ms, FA=30°. Field of view (FOV) = 3.6 × 3.6, matrix = 256 × 256, and number of excitations = 2 times.

2.2 Analysis of Lipid Content in Liver Tissue

[0029] 100 mg liver tissue was accurately weighed, and added with a triglyceride detection lysate, the tissue was homogenized by a homogenizer in an ice bath to no significant tissue mass, placed on ice for 5min, transferred to a 1.5ml centrifuge tube, and centrifuged at 14000g for 10 min at 4°C, the supernatant was transferred and a portion of the supernatant was taken for protein quantification, and detected for its lipid content according to the instructions of the TC and TG detection kits.

2.3 Pathological Staining of Liver Tissue

2.3.1 Preparation of Paraffin Sections

[0030] The liver fixed with paraformaldehyde stationary liquid was rinsed with tap water and dehydrated in the following steps, with 70% ethanol overnight, 80% ethanol overnight, 90% ethanol I for 30 min, 90% ethanol II for 30 min, 95% ethanol I for 60 min, 95% ethanol II for 60 min, 100% ethanol I for 60 min, and 100% ethanol II for 60 min, and the dehydration time for normal tissues can be appropriately extended. After dehydration, the tissue was transparentized with Super-safety and environmental-protection transparent agent, Super-safety and environmental-protection transparent agent I for 60 min, Super-safety and environmental-protection transparent agent II for 60 min, and Super-safety and environmental-protection transparent agent III for 60 min, and the transparentization time for normal tissues can be appropriately extended. The tissue was immersed in wax at 65°C, paraffin I for 50 min, paraffin II for 50 min, and paraffin III for 50 min, embedded, sliced at a thickness of 7 μm, exposed at 45°C, and baked overnight at 50°C.

2.3.2 Preparation of Frozen Sections

[0031] Before the liver tissue was sectioned, the temperature of a freezer of a microtome was set to -19°C and the sample head was set to -21°C. The liver stored in liquid nitrogen or -80°C was preliminarily equilibrated at -20°C, and then the tissue was placed on a sample stand of the microtome for temperature equilibration. After the tissue block was trimmed, the tissue block was serially sectioned to a thickness of 7 μm and applied to a clean polylysine-coated slide.

2.3.3 Oil Red O Staining

[0032] The frozen section was fixed in 4% paraformaldehyde physiological solution for 10 min, rinsed with tap water for 2 min, rinsed with 60% isopropanol for several seconds, stained with 0.5% Oil Red O working droplets for 10-15 min in a light-proof staining box, separated by 60% isopropanol for several seconds, washed gently with tap water, counter-stained with hematoxylin for 3-5 min, differentiated with 1% hydrochloric acid in water, rinsed with tap water for 2 min and returned to blue, sealed with glycerol gelatine and observed under a microscope.

2.3.4 HE Staining

[0033] The paraffin section was dehydrated in the following steps with Super-safety and environmental-protection transparent agent I for 5 min, Super-safety and environmental-protection transparent agent II for 5 min, Super-safety and environmental-protection transparent agent for 5 min, 100% ethanol I for 3 min, 100% ethanol II for 3 min, 95% ethanol I for 3 min, 95% ethanol II for 3 min, and 80% ethanol for 3 min, and rinsed with tap water for 1 min, stained with hematoxylin for 5 min, washed with tap water for 1 min, differentiated with 1% hydrochloric acid in ethanol for several seconds, rinsed with tap water and returned to blue, rinsed in 80% ethanol for several seconds, stained with eosin for 10 seconds, toned with 80% ethanol and 95% ethanol, dehydrated, ie, with 95% ethanol, 100% ethanol I, 100% ethanol II, Super-safety and environmental-protection transparent agent I, Super-safety and environmental-protection transparent agent II, and Super-safety and environmental-protection transparent agent III 60 for 2 min each, sealed with ultra-clean high-grade mounting glue and observed under a microscope.

3. Data Analysis

[0034] The data were expressed as mean value ± standard error, and all data were statistically analysed by ONEWAY-ANOVA using Graphpad Prism 5.0 software; the images were compared and analysed.

III. Experimental Results

3.1 Effect of IMM-H007 on Body Weight and Liver Coefficient of Golden Hamster with non-alcoholic fatty liver disease Induced by High-fat Diet

[0035] From Table 1 and FIG. 1, it can be seen that compared with golden hamsters in the normal group, the body weight of the golden hamster in the model group increased significantly, and their liver coefficient increased; and compared with the model group, both body weight and liver coefficient decreased after treatment with administration of IMM-H007. However, after treatment with administration of simvastatin, the liver coefficient of golden hamsters did not decrease but increased significantly.

Table 1. Effect of IMM-H007 on Body Weight and Liver Coefficient of Golden Hamster with Chronic Fatty Liver

Group(n=10)	Dose mg/kg	Body Weight	Liver Organ Coefficient
Normal Group	-	150±9	0.0282±0.002
Model Group	-	180±9***	0.0340±0.002***
Simvastatin Group	3	177±9	0.0433±0.002###
IMM-H007	50	170±11#	0.0313±0.002##
	100	177±9	0.0291±0.003###
	200	166±12#	0.0298±0.002###

***P<0.001, compared with normal group; ###P<0.001, ##P<0.01,#P<0.05 compared with model group

3.2 Effect of IMM-H007 on Serum Lipid Indexes in Serum of Golden Hamster with non-alcoholic fatty liver disease Induced by High-fat Diet

[0036] Compared with the golden hamsters in the normal group, the TC, TG, LDL-C, HDL-C and FFA in the serum of the golden hamsters in the model group were significantly increased; and compared with the model group, the TC, TG, LDL-C, HDL-C, and FFA were significantly reduced after administration of IMM-H007 (Table 2 and FIG. 2).

Table 2. Effect of IMM-H007 on Serum Lipid Indexes in Serum of Golden Hamster with non-alcoholic fatty liver disease Induced by High-fat Diet

Group(n=10)	Dose mg/kg	TC(mmol/L)	TG(mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	FFA μEq/L
Normal Group	-	3.3±0.17	1.4±0.3	1.3±0.3	1.8±0.2	1444.8±173.8
Model Group	-	14.0±6.2***	11.5±6.0***	8.1±4.9***	3.6±1.0***	3468.4±1058.1***
Simvastatin Group	3	9.4±2.1#	3.1±1.8###	5.1±2.3	3.3±0.7	2013.6±457.6###
IMM-H007	50	7.9±1.0##	4.7±1.9##	4.1±1.2#	2.8±0.4#	2324.6±783.4#
	100	7.0±1.0##	4.3±1.4##	4.0±0.7#	2.8±0.2#	1889.5±386.5###
	200	6.7±1.0##	2.7±0.8###	3.3±0.5##	2.6±0.5##	1497.6±326.2###

***P<0.001, compared with normal group; ###P<0.001, ##P<0.01,#P<0.05 compared with model group

3.3 Effect of IMM-H007 on Liver Function of Golden Hamster with non-alcoholic fatty liver disease Induced by High-fat Diet

[0037] As can be seen from Table 3, the levels of glutamic-pyruvic transaminase and glutamic oxalacetic transaminase in the serum of the golden hamsters in the model group were significantly increased, and the serum ALT and AST were significantly decreased after treatment with IMM-H007. However, after treatment with administration of simvastatin, there was no decrease in glutamic-pyruvic transaminase but significant increase (Table 3 and FIG. 3); while there was no significant difference between the level of glutamic oxalacetic transaminase and that of the model group, suggesting that IMM-H007 has a good hepatic protective effect, but simvastatin shows the effect of damaging the liver.

Table 3. Effect of IMM-H007 on ALT, AST levels in Serum of Golden Hamster with non-alcoholic fatty liver disease Induced by High-fat Diet

Group(n=10)	Dose mg/kg	GPT(U/L)	GOT(U/L)
Normal Group	-	14.7±3.3	5.3±1.4
Model Group	-	43.7±17.2***	12.2±3.6***
Simvastatin Group	3	65.2±22.5#	12.1±4.0
IMM-H007	50	21.1±5.2##	9.4±1.7#
	100	20.9±8.0##	8.4±2.3#
	200	12.4±3.1###	7.2±2.1##

***P<0.001, compared with normal group; ###P<0.001, ##P<0.01,#P<0.05 compared with model group

3.4 Effect of IMM-H007 on Hepatic Lipid Changes in Golden Hamster with non-alcoholic fatty liver disease Induced by High-fat Diet

[0038] From Table 4 and FIG. 4, it can be seen that compared with the golden hamsters in the normal group, TC and TG in the liver of the golden hamsters in the model group were significantly increased; and compared with the model group, TC and TG were significantly reduced after simvastatin and IMM-H007 were given.

Table 4. Effect of IMM-H007 on Hepatic Lipid Changes in Golden Hamster with non-alcoholic fatty liver disease Induced by High-fat Diet

Group(n=10)	Dose mg/kg	TG(mol/g protein)	TC(mol/g protein)
Normal Group	-	2.0±0.3	1.8±0.6
Model Group	-	4.6±0.8***	4.9±1.3**
Simvastatin Group	3	3.0±0.8##	2.7±0.9##

(continued)

Group(n=10)	Dose mg/kg	TG(mol/g protein)	TC(mol/g protein)
IMM-H007	50	3.6±0.1$^{\#}$	2.8±0.8$^{\#}$
	100	3.3±0.1$^{\#\#}$	2.4±0.4$^{\#\#}$
	200	2.8±0.1$^{\#\#\#}$	1.8±0.8$^{\#\#}$

***P<0.001, compared with normal group; $^{\#\#\#}$P<0.001, $^{\#\#}$P<0.01,$^{\#}$P<0.05 compared with model group

3.5 Liver MRI Analysis

[0039] The results of MR examination showed that the subcutaneous and abdominal adipose in the golden hamster of the model group was significantly increased compared with the normal group, and subcutaneous and abdominal adipose was reduced after administration of simvastatin and IMM-H007 (FIG. 5).

3.6 Pathological Observation of Liver Tissue

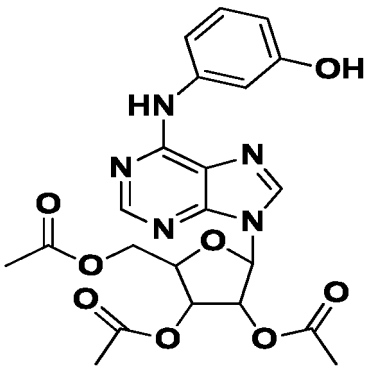
[0040] From the results of HE staining shown in FIG. 6 , in the normal animals, the liver cells were arranged radially around the central vein, and the collagenous fibers were regularly distributed in the central vein and other blood vessel walls; in the animals of the model group, the liver cells appeared vacuolated, and collagenous fibers appeared between the liver cells with irregular distribution; and in the animals of the simvastatin group, vacuoles appeared in the liver cells, and collagenous fibers appeared between the liver cells and showed an irregular distribution. In each dose group of IMM-H007, the liver cells were arranged radially around the central vein, and collagenous fibers were regularly distributed in the central vein and the hepatic lobule border zone.

[0041] From the results of Oil Red O staining (FIG. 7), in the normal animals, the liver cells were arranged radially around the central vein and contained less intracellular neutral fat; in the animals of the high-fat diet group, there was a lot of fat deposition and vacuolization in the liver cells; and in the animals of the simvastatin group, there was fat deposition and large necrosis in the liver cells. In the animals of each dose group of IMM-H007, fat deposition was also observed in the liver cells, but it was significantly reduced compared with the model group.

[0042] In summary, triacetyl-3-hydroxyphenyladenosine (IMM-H007) can significantly reduce the blood lipid level of the golden hamsters with non-alcoholic fatty liver disease, significantly reduce AST and ALT levels, and significantly improve liver function, suggesting that IMM -H007 can be used to prepare a pharmaceutical drug for preventing or treating non-alcoholic fatty liver disease.

Claims

1. An application of triacetyl-3-hydroxyphenyladenosine as shown in formula (I) and a pharmaceutically acceptable salt thereof in the preparation of a pharmaceutical drug for preventing or treating non-alcoholic fatty liver disease,

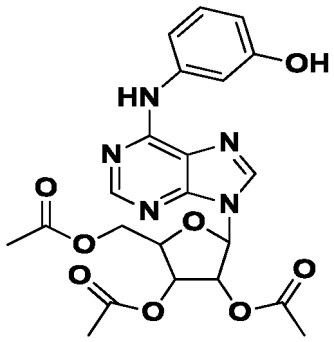


(I).

2. The application according to claim 1, **characterized in that** the non-alcoholic fatty liver disease is a fatty liver disease

caused by a high-calorie diet.

3. An application of a pharmaceutical composition for the preparation of a pharmaceutical drug for preventing or treating non-alcoholic fatty liver disease, **characterized in that** the pharmaceutical composition comprises triacetyl-3-hydroxy-phenyladenosine as shown in formula (I) and a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier,



(I).

4. The application according to claim 3, **characterized in that** the pharmaceutical composition is a tablet, a capsule, a pill or an injection.

5. The application according to claim 3, **characterized in that** the pharmaceutical composition is a sustained release preparation, a controlled release preparation or various microparticle drug delivery systems.

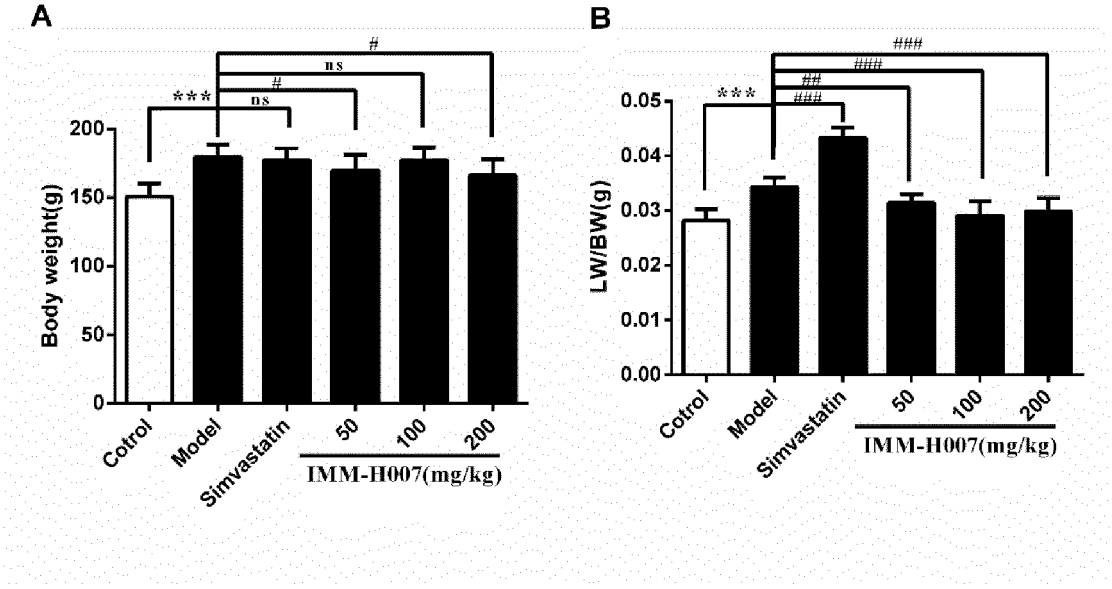

A
B
Body weight(g)
LW/BW(g)
Cotrol
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)

Fig. 1

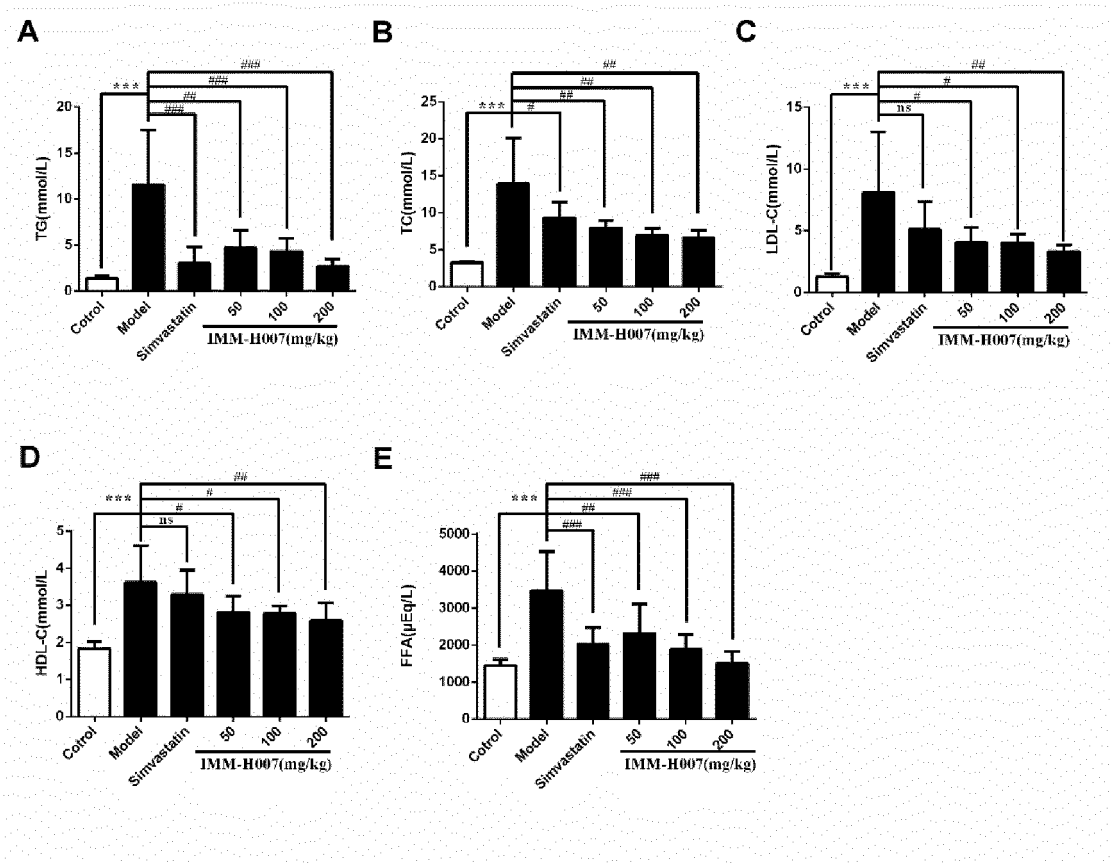

A
B
C
D
E
TG(mmol/L)
TC(mmol/L)
LDL-C(mmol/L)
HDL-C(mmol/L
FFA(μEq/L)
Cotrol
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)

Fig. 2

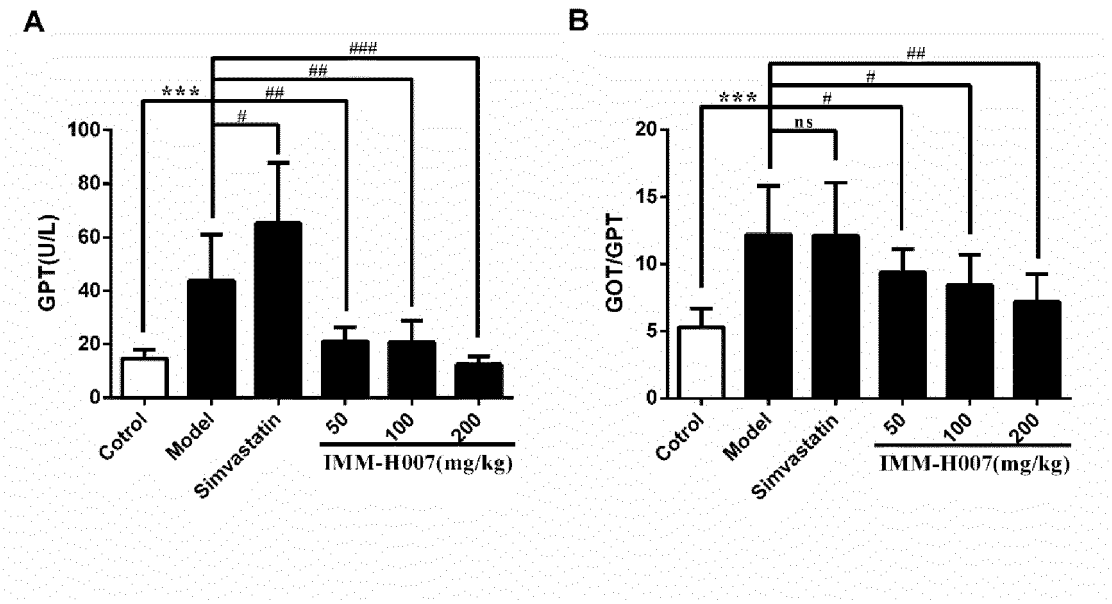

A
B
GPT(U/L)
GOT/GPT
***
##
#
###
ns
Cotrol
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)

Fig. 3

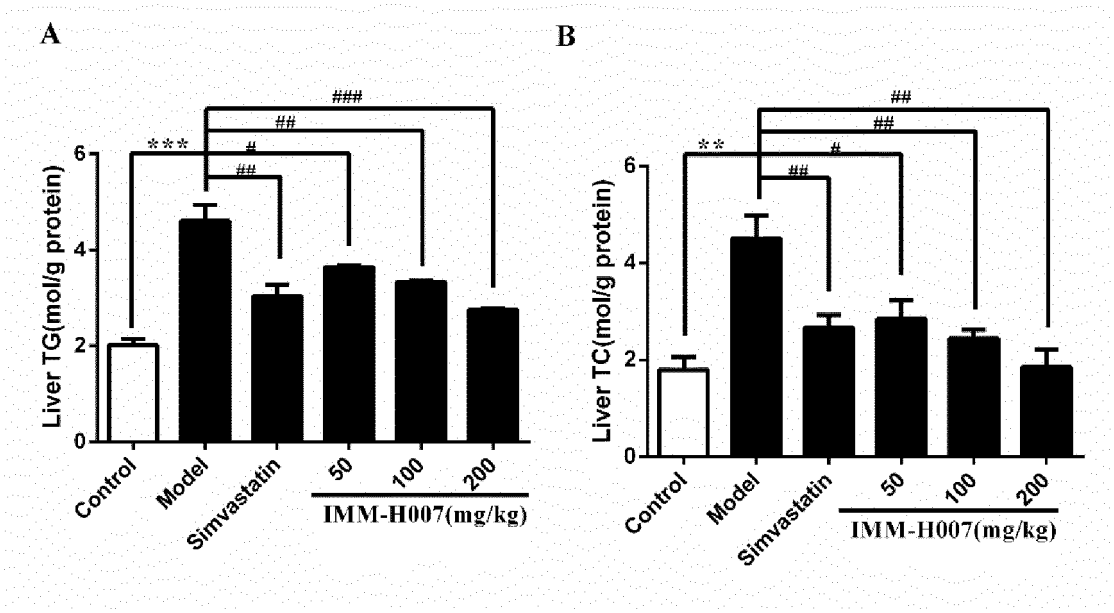

A
B
Liver TG(mol/g protein)
Liver TC(mol/g protein)
***
**
##
#
###
Control
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)

Fig. 4

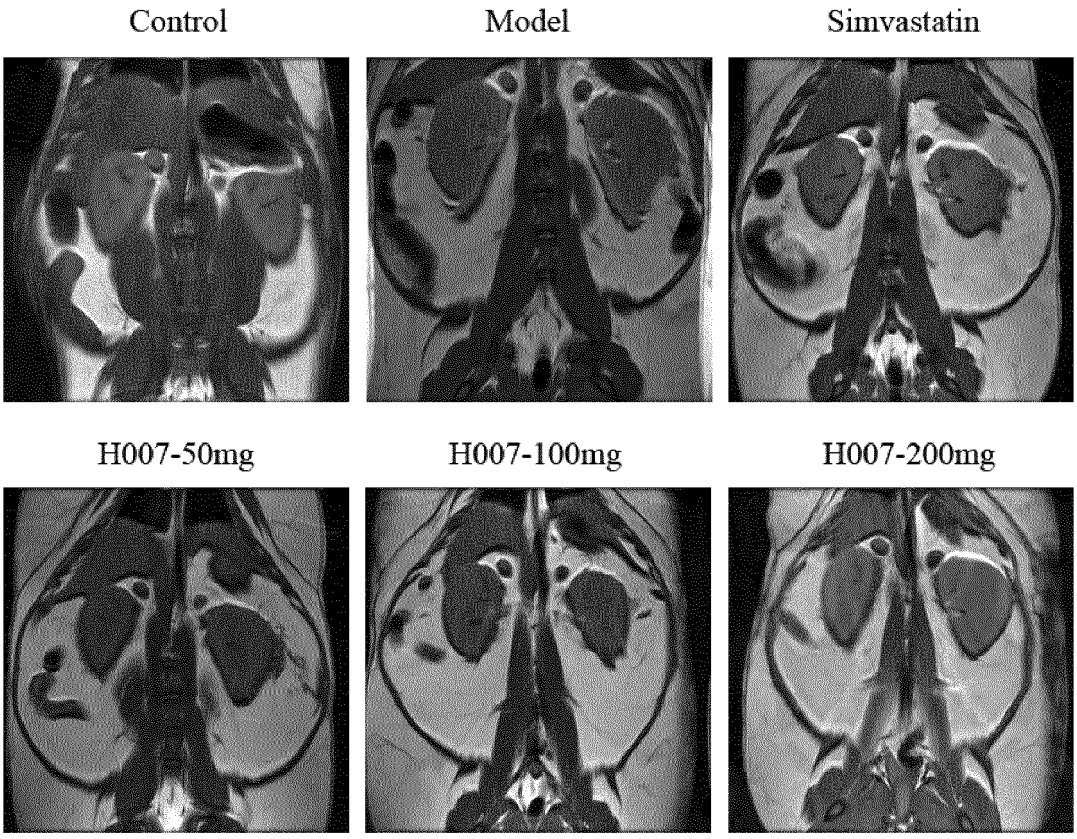


Fig. 5

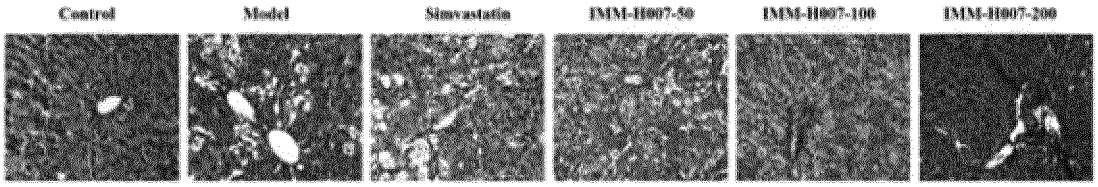


Fig. 6

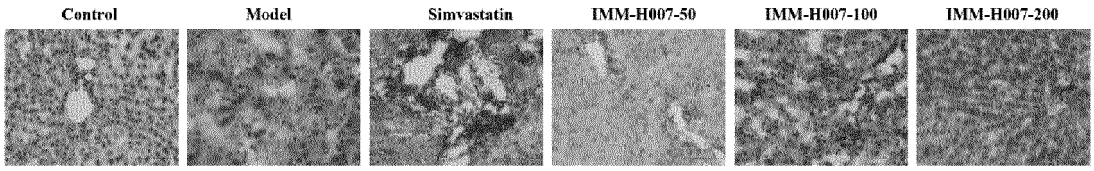


Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2016/112623

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/7076 (2006.01) i; A61P 1/16 (2006.01) i
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CHINA ACADEMIC JOURNALS FULL-TEXT DATABASE, CHINESE PHARMACEUTICAL PATENT DATABASE, CNABS, CNTXT, WPI, EPODOC, STN, DUXIU ACADEMIC SEARCH: triacetyl-3-hydroxyphenyl gland, hydroxyl phenyl, adenosine, non-alcoholic fatty liver disease, NAFLD, 1221412-23-2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	CN 105663152 A (INSTITUTE OF MATERIA MEDICA, CHINESE ACADEMY OF MEDICAL SCIENCE), 15 June 2016 (15.06.2016), claims 1-2 and 4-6	1-5
X	CN 101874036 A (INSTITUTE OF MATERIA MEDICA, CHINESE ACADEMY OF MEDICAL SCIENCE), 27 October 2010 (27.10.2010), description, paragraphs 0002, 0008 and 0011-0020, and claims 7 and 9	1-5
A	CN 104546887 A (INSTITUTE OF MATERIA MEDICA, CHINESE ACADEMY OF MEDICAL SCIENCE), 29 April 2015 (29.04.2015), the whole document	1-5
A	CN 102125580 A (TAISHAN MEDICAL UNIVERSITY), 20 July 2011 (20.07.2011), the whole document	1-5

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 February 2017 (09.02.2017)	**24 March 2017 (24.03.2017)**
Name and mailing address of the ISA/CN: State Intellectual Property Office of the P. R. China No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088, China Facsimile No.: (86-10) 62019451	Authorized officer **WU, Tiesheng** Telephone No.: (86-10) **010-62413770**

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2016/112623

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 105663152 A	15 June 2016	None	
CN 101874036 A	27 October 2010	EP 2407474 B1	14 August 2013
		CN 101874036 B	25 January 2012
		JP 2012519714 A	30 August 2012
		IL 215080 A	31 July 2014
		KR 101380140 B1	01 April 2014
		DK 2407474 T3	25 November 2013
		EP 2407474 A4	11 July 2012
		ES 2435620 T3	20 December 2013
		CN 101712709 A	26 May 2010
		EP 2407474 A1	18 January 2012
		US 2012053143 A1	01 March 2012
		IL 215080 D0	30 November 2011
		US 8435962 B2	07 May 2013
		KR 20110117257 A	26 October 2011
		JP 5698682 B2	08 April 2015
		WO 2010040286 A1	15 April 2010
		IN 270393 B	25 December 2015
CN 104546887 A	29 April 2015	None	
CN 102125580 A	20 July 2011	None	

Form PCT/ISA/210 (patent family annex) (July 2009)

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- CN ZL200980101131 **[0004]**
- CN 101874036 B **[0004]**

(12) 按照专利合作条约所公布的国际申请

(19) 世界知识产权组织
国 际 局

(43) 国际公布日
2017 年 7 月 6 日 (06.07.2017)

WIPO | PCT

(10) 国际公布号
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A61K 31/7076 (2006.01) *A61P 1/16* (2006.01)

(21) 国际申请号: PCT/CN2016/112623

(22) 国际申请日: 2016 年 12 月 28 日 (28.12.2016)

(25) 申请语言: 中文

(26) 公布语言: 中文

(30) 优先权:
201511034199.0 2015 年 12 月 31 日 (31.12.2015) CN

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(72) 发明人: **朱海波 (ZHU, Haibo)**; 中国江苏省淮安市清浦工业园区朝阳路 168 号江苏天士力帝益药业有限公司, Jiangsu 223003 (CN)。 **史会杰 (SHI, Huijie)**; 中国江苏省淮安市清浦工业园区朝阳路 168 号江苏天士力帝益药业有限公司, Jiangsu 223003 (CN)。

(74) 代理人: **北京君尚知识产权代理事务所（普通合伙） (BEIJING JOYSHINE INTELLECTUAL PROPERTY OFFICE)**; 中国北京市海淀区北四环西路 68 号左岸工社 1316-1317 室, Beijing 100080 (CN)。

(81) 指定国 (除另有指明，要求每一种可提供的国家保护): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK,

[见续页]

(54) **Title**: TRIACETYL-3-HYDROXYPHENYLADENOSINE AND APPLICATION IN PREPARING PHARMACEUTICAL DRUG FOR PREVENTING OR TREATING NON-ALCOHOLIC FATTY LIVER DISEASE

(54) **发明名称**: 三乙酰基-3-羟基苯基腺苷在制备预防或者治疗非酒精性脂肪肝药物中的应用

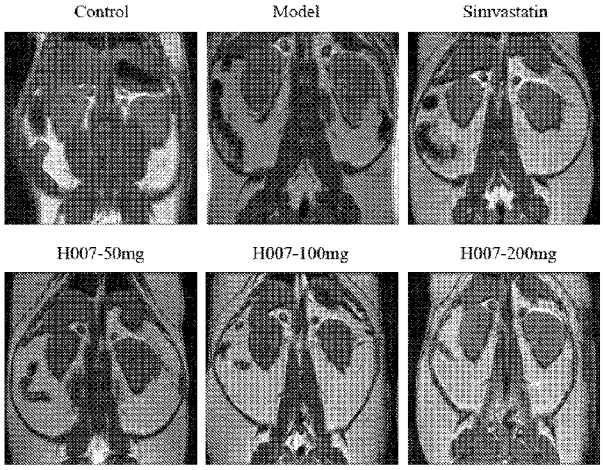
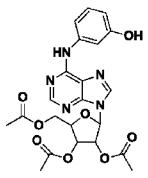


图 5



（I）

(57) **Abstract**: The present invention provides a triacetyl-3-hydroxyphenyladenosine represented by formula (I) for preventing or treating non-alcoholic fatty liver. The triacetyl-3-hydroxyphenyladenosine can significantly reduce levels of serum AST, ALT, and TG, significantly improve liver functions, and alleviate liver steatosis. The invention provides significant curative effects for preventing or treating non-alcoholic fatty liver and has limited toxic side effects.

(57) **摘要**: 式（I）所示的三乙酰基-3-羟基苯基腺苷在预防或者治疗非酒精性脂肪肝中的应用，所述三乙酰基-3-羟基苯基腺苷能够明显降低血清 AST, ALT 及 TG 水平，显著改善肝功能，减轻脂肪肝程度，在预防或者治疗非酒精性脂肪肝方面具有显著疗效、毒副作用小。

WO 2017/114413 A1

SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW。

(84) **指定国** (除另有指明，要求每一种可提供的地区保护): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), 欧亚 (AM, AZ, BY, KG, KZ, RU, TJ, TM), 欧洲 (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG)。

根据细则 4.17 的声明:

— 关于申请人有权要求在先申请的优先权(细则 4.17(iii))

本国际公布:

— 包括国际检索报告(条约第 21 条(3))。

三乙酰基-3-羟基苯基腺苷在制备预防或者治疗非酒精性脂肪肝药物中的应用

技术领域

本发明涉及三乙酰基-3-羟基苯基腺苷及含有其的药物组合物在制备预防或者治疗非酒精性脂肪肝药物中的应用，属于医药技术领域。

背景技术

非酒精性脂肪肝（non-alcoholic fatty liver disease, NAFLD）是指除外过量饮酒和其他明确的损伤肝的病因所致的以肝实质细胞损伤和脂肪蓄积为特征的一组临床病理综合征，是一种与胰岛素抵抗（insulin resistance, IR）和遗传易感密切相关的代谢应激性肝损伤，其病理学改变与酒精性肝病相似，但患者无过量饮酒史。其组成包括非酒精性单纯性脂肪肝（NAFL）、非酒精性脂肪性肝炎（NASH）、非酒精性脂肪性肝纤维化、非酒精性脂肪性肝硬化及其相关的肝癌（HCC）等。随着我国经济发展，人们饮食的内容和时间有较大的改变，非酒精性脂肪肝发病率不断提高，并呈低龄化趋势，成为严重威胁人类身心健康的常见疾病。

NAFLD 的确切发病机制目前尚不清楚，较为公认的是“二次打击”学说，在该学说的两个阶段中首次打击是由于高脂饮食摄入过多而分解量下降，造成脂质沉积，形成单纯性脂肪肝。在二次打击中 IR 可减弱和破坏胰岛素对脂肪代谢的调节作用，增加脂质溶解，提高游离脂肪酸（nonesterified free fatty acid, FFA）浓度，促进肝脏对血中 FFA 的摄取。氧化应激与脂质过氧化损伤在脂肪肝的形成和发展过程中起重要作用，是脂肪肝受到第二次打击进一步发展的重要因素。线粒体是细胞的呼吸器官，活性氧（reactive oxygen species, ROS）生成增多损伤线粒体，进一步加重肝脏的脂质蓄积。并且氧应激产生的自由基导致脂质过氧化（lipid peroxidation, LPO）的损害反应，形成一系列脂质自由基及降解产物-丙二醛（malondialdehyde, MDA），同事破坏生物膜的结构与功能，使细胞膜通透性增加，细胞色素 C 流出，启动细胞凋亡程序，最后导致肝纤维化、肝硬化，甚至进展为肝癌。目前仍缺乏特效药物，常用的他汀类、贝特类等降血脂药物，疗效欠佳，毒副作用较大。

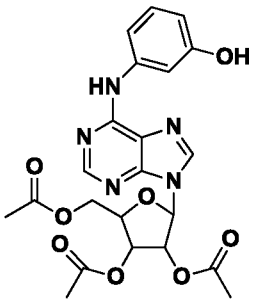
三乙酰基-3-羟基苯基腺苷（专利号 ZL200980101131.6， 公告号 CN101874036B，公告日 2012.01.25）是中国医学科学院药物研究所在虫草素衍生物中筛选出具有显著调血脂活性的新结构类型化合物，并且毒副作用小、药代动力学良好等特征，目前处于临床前研究阶段。目前尚无该化合物在预防或者治疗非酒精性脂肪肝中应用的报道。

发明内容

本发明解决的技术问题是提供化合物三乙酰基-3-羟基苯基腺苷及其药物组合物在制备预防或治疗非酒精性脂肪肝药物中的应用。

为解决本发明的技术问题，提供如下技术方案：

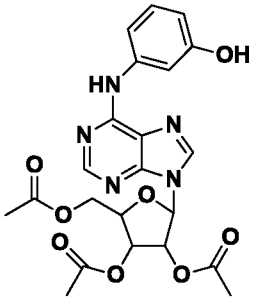
本发明技术方案的第一方面是提供了式（I）所示的三乙酰基-3-羟基苯基腺苷及其药学上可接受的盐在制备预防或者治疗非酒精性脂肪肝的药物中的应用



（Ⅰ），

所述的非酒精性脂肪肝为高能量饮食引起的脂肪肝，

本发明所述的三乙酰基-3-羟基苯基腺苷治疗非酒精性脂肪肝是其能够明显降低血清AST、ALT及TG水平，显著改善脂肪肝金黄地鼠肝功能，减轻脂肪肝程度，从而预防或者治疗非酒精性脂肪肝。本发明技术方案的第二方面是提供一种药物组合物在制备预防或治疗非酒精性脂肪肝药物中的应用，其特征在于，所述的药物组合物包含式（Ⅰ）所述的三乙酰基-3-羟基-苯基腺苷及其药学上可接受的盐和药学上可接受的载体，



（Ⅰ），

所述的药物组合物包括片剂、胶囊、丸剂和注射剂、缓释制剂、控释制剂或各种微粒给药系统。该药物组合物可根据本领域公知的方法制备。可通过将本发明化合物与一种或多种药学上可接受的固体或液体赋形剂和/辅剂结合，制成适于人或动物使用的任何剂型。本发明化合物在其药物组合物中的含量通常为0.1-95重量%。

本发明化合物或含有它的药物组合物可以单位剂量形式给药，给药途径可为肠道或非肠

道，如口服、静脉注射、肌肉注射、皮下注射、鼻腔、口腔黏膜、眼、肺和呼吸道、皮肤、阴道、直肠等。

给药剂型可以使液体剂型、固体剂型或半固体剂型。液体剂型可以是溶液剂（包括真溶液和胶体溶液）、乳剂（包括 o/w 型、w/o 型和复乳）、混悬剂、注射剂（包括水针剂、粉针剂和输液）、滴眼剂、滴鼻剂、洗剂和搽剂等；固体剂型可以是片剂（包括普通片、肠溶片、含片、分散片、咀嚼片、泡腾片、口腔崩解片）、胶囊剂（包括硬胶囊、软胶囊、肠溶胶囊）、颗粒剂、散剂、微丸、滴丸、栓剂、膜剂、贴片、气（粉）雾剂、喷雾剂等；半固体剂型可以是软膏剂、凝胶剂、糊剂等。优选的药物组合物的剂型选自片剂、胶囊、丸剂、注射剂。

本发明化合物可以制成普通制剂、也制成是缓释制剂、控释制剂、靶向制剂及各种微粒给药系统。

为了将本发明化合物制成片剂、可以广泛使用本领域公知的各种赋形剂，包括稀释剂、黏合剂、润湿剂、崩解剂、润滑剂、助流剂。稀释剂可以是淀粉、糊精、蔗糖、葡萄糖、乳糖、甘露醇、山梨醇、木糖醇、微晶纤维素、硫酸钙、磷酸氢钙、碳酸钙等；湿润剂可以是水、乙醇、异丙醇等；粘合剂可以是淀粉浆、糊精、糖浆、蜂蜜、葡萄糖溶液、微晶纤维素、阿拉伯胶浆、明胶浆、羧甲基纤维素钠、甲基纤维素、羟丙基甲基纤维素乙基纤维素、丙烯酸树脂、卡波姆、聚乙烯吡咯烷酮、聚乙二醇等；崩解剂可以是干淀粉、微晶纤维素、低取代羟丙基纤维素、交联聚乙烯吡咯烷酮、交联羧甲基纤维素钠、羧甲基淀粉钠、碳酸氢钠与枸橼酸、聚氧乙烯山梨糖醇脂肪酸酯、十二烷基磺酸钠等；润滑剂和助流剂可以是滑石粉、二氧化硅、硬脂酸盐、酒石酸、液体石蜡、聚乙二醇等。

还可以将片剂进一步制成包衣片，例如糖包衣片、薄膜包衣片、肠溶包衣片，或双层片和多层片。

为了将给药单元制成胶囊剂，可以将有效成分本发明化合物先与稀释剂、助流剂混合，将混合物直接置于硬胶囊或软胶囊中。也可将有效成分本发明化合物先与稀释剂、黏合剂、崩解剂制成颗粒或微丸，再置于硬胶囊或软胶囊中。用于制备本发明化合物片剂的各种稀释剂、黏合剂、润湿剂、崩解剂、助流剂品种也可用于制备本发明化合物的胶囊剂。

为将本发明化合物制成注射剂，可以用水、乙醇、异丙醇、丙二醇或它们的混合物做溶剂加入适量本领域常用的增溶剂、助溶剂、PH 调剂剂、渗透压调节剂。增溶剂或助流剂可以是泊洛沙姆、卵磷脂、羟丙基-β-环糊精等；PH 调剂剂可以是磷酸盐、醋酸盐、盐酸、氢氧化钠等；渗透压调节剂可以是氯化钠、甘露醇、葡萄糖、磷酸盐、醋酸盐等。如制备冻干粉针剂，还可以加入甘露醇、葡萄糖等作为支撑剂。

此外，如需要，也可以向药物制剂中添加着色剂、防腐剂、香料、矫味剂或其它添加剂。

为达到用药目的，增强治疗效果，本发明的药物或者药物组合物可用任何公知的给药方法给药。

本发明化合物药物组合物的给药剂量依照所要预防或治疗疾病的性质和严重程度，患者或动物的个体情况，给药途径和剂型等可以有大范围的变化。一般来讲，本发明化合物的每天的合适剂量范围为 0.001-150mg/kg 体重，优选为 0.1-100mg/kg 体重，更优选为 1-60mg/kg 体重，最优选为 2-30mg/kg 体重。上述剂量可以一个剂量单位或分成几个剂量单位给药，这取决于医生的临床经验以及包括运用其它治疗手段的给药方案。

本发明的化合物或组合物可单独服用，或与其它治疗药物或对症药物合并使用。当本发明的化合物与其它治疗药物存在协同作用时，应根据实际情况调整它的剂量。

有益的技术效果

本发明采用药效学研究方法证实了三乙酰基-3-羟基苯基腺苷在预防或者治疗非酒精性脂肪肝的显著作用，为这一病机复杂、治疗效果欠佳的慢性疾病提供了一种新的预防或者治疗药物--三乙酰基-3-羟基苯基腺苷，其疗效显著，毒副作用小，使用安全，为其在预防或者治疗非酒精性脂肪肝方面的临床应用提供科学依据。

附图说明

为了使本发明的内容更容易被清楚的理解，下面根据本发明的具体实施例并结合附图，对本发明作进一步详细的说明，其中，

图 1 为本发明所述实验例中金黄地鼠各组体重和肝脏系数比较；

图 2 为本发明所述实验例中金黄地鼠血清中胆固醇、甘油三酯、高密度脂蛋白、低密度脂蛋白和游离脂肪酸含量测定结果比较；

图 3 为本发明所述实验例中金黄地鼠 ALT 及 AST 酶活力比较；

图 4 为本发明所述实验例中各组金黄地鼠肝组织脂质含量测定结果比较；

图 5 为本发明所述实验例中金黄地鼠肝脏核磁成像图；

图 6 为本发明所述实验例中各组金黄地鼠肝组织 HE 病理染色结果比较；

图 7 为本发明所述实验例中各组金黄地鼠肝组织油红 O 染色情况结果比较。

具体实施方式

下面的实施例用来进一步说明本发明，但这并不意味着对本发明的任何限制。

实施例 1：三乙酰基-3-羟基苯基腺苷（IMM-H007）在治疗非酒精性脂肪肝中的应用

一、实验材料

1. 试剂

OCT 冰冻切片包埋剂，美国樱花；戊巴比妥钠，sigma 公司；PEG6000，sigma 公司；甘氨酸，sigma 公司；多聚甲醛，国药试剂有限公司；油红 O，sigma 公司；HE 染色液，台湾贝索公司；总胆固醇（TC）检测试剂盒，日本积水医疗科技（中国）有限公司；总甘油三酯检测试剂盒，中生北控生物科技股份有限公司；游离脂肪酸检测试剂盒，日本积水医疗科技（中国）有限公司。谷丙转氨酶（AST/ALT）测试盒，南京建成生物工程研究所；谷草转氨酶（AST/GOT）测试盒，南京建成生物工程研究所；

2. 仪器

多用途低温高速离心机，德国 Eppendorff；石蜡切片机，德国莱卡；冰冻切片机，德国莱卡；En Vision 多功能酶标仪，美国 PerkinElmer 股份有限公司；小动物麻醉机，美国 Matrx 产品；小动物磁共振成像仪，德国 Bruker PharmaScan 70T/16 US。

3. 实验动物

6-8 周龄叙利亚金黄地鼠（LVG hamster，源引进于 Charles River Laboratories）20 只，体重 90-120g，雄性，SPF 级，购自北京维通利华实验动物技术有限公司，许可证编号：SCXK（京）2012-0001。

二、实验方法

1. 动物分组及饲养

动物适应性喂养 5 天后按体重随机分为正常对照组（n=15）、高脂饲料组（n=15）、IMMH007 低剂量组（25mg/kg，n=15）、IMMH007 中剂量组（50mg/kg，n=15）、IMMH007 高剂量组（100mg/kg，n=15），每天灌胃给药 2 次。动物饲养于中国医学科学院药物研究所动物实验中心二部，饲养条件为 SPF 级，温度 21±2℃，相对湿度 50±5%，光照周期为 12/112，5 只每笼。正常组给予正常基础饲料，高脂饲料组给予高脂饲料（79.8%基础饲料加 20%猪油和 0.2%胆固醇），动物自由摄食、饮水。饲料委托北京华阜康生物科技有限公司生产。实验中每 2 周记录体重一次。

2. 观察指标及测定方法

2.1 血清生化指标

动物禁食 12 小时，内眦静脉取血 0.5ml，静置 60min，6000g 离心 10min，尽可能多的吸取上清，然后 6000g 离心 10min，按照 TC、TG、AST 和 ALT 试剂盒说明书测定吸光度值，

计算各指标浓度。取 50ul 血清与 50ul PEG6000 1:1 混合，涡旋均匀，静置 10min，1900g 常温离心 20min，小心吸取上清，4℃保存，按照 TC 试剂盒说明书测定 HDL-C。血浆 LDL 水平是通过将血浆总胆固醇 TC 减去 HDL-C 和 0.2 倍 TG 水平，即 LDL=TC-HLD-0.2TG。

实验终点前隔夜禁食，3%戊巴比妥钠腹腔注射麻醉，暴露腹腔，腹主动脉取血后迅速分离肝脏。留取肝一叶，于固定部位切取两块 1×1cm^3 小块后，一块用 OCT 包埋，投入液氮速冻，液氮中或-80℃保存；另一块投入 4%多聚甲醛固定液中，4℃保存。

2.2 磁共振成像

动物禁食 12 小时后，异氟烷麻醉，固定头部，仰卧固定于大鼠线圈上，头先进，定位于腹部中心。

快速自旋回波序列的 T2 加权像（T2-weighted imaging, T2WI）：TR/TE=200/3ms，FA=30°。视野（FOV）=3.6×3.6，矩阵=256×256，激励次数=2 次。

2.2 肝组织脂质含量分析

准确称取 100mg 肝脏组织，加入甘油三酯检测裂解液，匀浆器将组织在冰浴中匀浆至无明显组织块。冰上放置 5min，转移至 1.5ml 离心管中，14000g 4 ℃**离心** 10min，转移上清，取部分上清进行蛋白定量，按照 TC、TG 检测试剂盒说明书检测脂质含量。

2.3 肝组织病理染色

2.3.1 石蜡切片的制备

将多聚甲醛固定液固定好的肝脏用自来水冲洗干净，按一下步骤脱水，70%乙醇过夜，80%乙醇过夜，90%乙醇Ⅰ30min,90%乙醇Ⅱ30min，95%乙醇Ⅰ60min, 95%乙醇Ⅱ60min，100%乙醇Ⅰ60min，100%乙醇Ⅱ60min，正常组织可适当延长脱水时间。组织脱水后用超安透明，超安Ⅰ60min，超安Ⅱ60min，超安Ⅲ60min，正常对照组肝脏可适当延长透明时间。65℃浸蜡，石蜡Ⅰ50min，石蜡Ⅱ50min，石蜡Ⅲ50min，包埋，切片厚度为 7um，45℃展片，50℃烤片过夜。

2.3.2 冰冻切片的制备

肝脏组织切片前将切片机冷冻箱温度设为-19℃，样品头设为-21℃。存于液氮或-80℃的肝脏先于-20℃环境平衡温度，再将组织放入切片机样品台上进行温度平衡。组织块修快后连续切片，切片厚度为 7um，贴于干净的多聚赖氨酸包被载玻片上。

2.3.3 油红 O 染色

冰冻切片于 4%多聚甲醛生理溶液固定 10min，自来水冲洗 2min，60%异丙醇润洗数秒，于避光染色盒内用 0.5%油红 O 工作液滴染 10-15min，60%异丙醇分色数秒，自来水轻柔冲洗，

苏木素复染 3-5min，自来水轻柔冲洗，1%盐酸水分化，自来水冲洗 2min 返蓝，甘油明胶封片，镜下观察。

2.3.4 HE 染色

石蜡切片按以下步骤脱水，超安Ⅰ5min，超安Ⅱ5min，超安Ⅲ5min，100%乙醇Ⅰ3min，100%乙醇Ⅱ3min，95%乙醇Ⅰ3min, 95%乙醇Ⅱ3min，80%乙醇 3min，自来水冲洗 1min。苏木素染色 5min，自来水冲洗 1min，1%盐酸乙醇分化数秒，自来水冲洗返蓝，放入 80%乙醇润洗数秒，伊红染色 10 秒，80%乙醇和 95%乙醇调色，脱水，即 95%乙醇、100%乙醇Ⅰ、100%乙醇Ⅱ、超安Ⅰ、超安Ⅱ、超安Ⅲ60 各 2min，超净高级封片胶封片，镜下观察。

3. 数据分析

数据以平均值±标准误来表示，所有数据采用 Graphpad Prism5.0 软件进行 ONEWAY-ANOVA 统计分析；对图像进行对比分析。

三、实验结果

3.1 IMM-H007 对高脂饮食诱导的非酒精性脂肪肝金黄地鼠的体重和肝脏系数的影响

由表 1 和图 1 可见，与正常组金黄地鼠比较，模型组金黄地鼠体重明显增加，肝脏系数增加；与模型组比较，给予 IMM-H007 治疗后，体重和肝脏系数均有下降。但是给予辛伐他汀治疗后，金黄地鼠肝脏系数未见下降，反而显著升高。

表 1. IMM-H007 对慢性脂肪肝金黄地鼠体重及肝脏系数的影响

Group（n=10）	Dose mg/kg	体重	肝脏脏器系数
正常组	—	150±9	0.0282±0.002
模型组	—	180±9***	0.0340±0.002***
辛伐他汀组	3	177±9	0.0433±0.002$^{\#\#\#}$
	50	170±11$^{\#}$	0.0313±0.002$^{\#\#}$
IMM-H007	100	177±9	0.0291±0.003$^{\#\#\#}$
	200	166±12$^{\#}$	0.0298±0.002$^{\#\#\#}$

***P<0.001，与正常组比较；$^{\#\#\#}$P<0.001,$^{\#\#}$P<0.01,$^{\#}$P<0.05 与模型组比较

3.2 IMM-H007 对高脂饮食诱导的非酒精性脂肪肝金黄地鼠血清中血脂指标的影响

与正常组金黄地鼠相比，模型组金黄地鼠血清 TC、TG、LDL-C、HDL-C 及 FFA 均显著升高；与模型组比较，给予 IMM-H007 后，TC、TG、LDL-C、HDL-C 及 FFA 均显著降低（表 2 和图 2）。

表 2. IMM-H007 对高脂饮食诱导的非酒精性脂肪肝金黄地鼠血清中血脂指标的影响

Group（n=10）	Dose mg/kg	TC(mmol/L)	TG(mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	FFA μEq/L

正常组	—	3.3±0.17	1.4±0.3	1.3±0.3	1.8±0.2	1444.8±173.8
模型组	—	14.0±6.2***	11.5±6.0***	8.1±4.9***	3.6±1.0***	3468.4±1058.1***
辛伐他汀组	3	9.4±2.1#	3.1±1.8###	5.1±2.3	3.3±0.7	2013.6±457.6###
IMM-H007	50	7.9±1.0##	4.7±1.9##	4.1±1.2#	2.8±0.4#	2324.6±783.4#
	100	7.0±1.0##	4.3±1.4##	4.0±0.7#	2.8±0.2#	1889.5±386.5###
	200	6.7±1.0##	2.7±0.8###	3.3±0.5##	2.6±0.5##	1497.6±326.2###

***P<0.001，与正常组比较；###P<0.001,##P<0.01,#P<0.05 与模型组比较

3.3 IMM-H007 对高脂饮食诱导的非酒精性脂肪肝金黄地鼠肝功能的影响

由表 3 可知，模型组金黄地鼠血清谷丙转氨酶、谷草转氨酶水平显著升高，经 IMM-H007 治疗后，血清 ALT 和 AST 均显著下降。但是给予辛伐他汀治疗后谷丙转氨酶未见降低，反而显著升高（表 3 和图 3）；而谷草转氨酶水平与模型组无显著性差异，提示 IMM-H007 具有良好的肝保护作用，而辛伐他汀显示出肝损伤作用。

表 3. IMM-H007 对高脂饮食诱导的非酒精性脂肪肝金黄地鼠血清中 ALT、AST 水平的影响

Group（n=10）	Dose mg/kg	GPT(U/L)	GOT(U/L)
正常组	—	14.7±3.3	5.3±1.4
模型组	—	43.7±17.2***	12.2±3.6***
辛伐他汀组	3	65.2±22.5#	12.1±4.0
	50	21.1±5.2##	9.4±1.7#
IMM-H007	100	20.9±8.0##	8.4±2.3#
	200	12.4±3.1###	7.2±2.1##

***P<0.001，与正常组比较；###P<0.001,##P<0.01,#P<0.05 与模型组比较

3.4 IMM-H007 对高脂饮食诱导的非酒精性脂肪肝金黄地鼠肝脏脂质变化的影响

由表 4 和图 4 可见，与正常组金黄地鼠相比，模型组金黄地鼠肝脏中 TC、TG 显著升高；与模型组比较，给予辛伐他汀和 IMM-H007 后，TC、TG 均显著降低。

表 4. IMM-H007 对高脂饮食诱导的非酒精性脂肪肝金黄地鼠肝脏脂质变化的影响

Group（n=10）	Dose mg/kg	TG(mol/g protein)	TC(mol/g protein)
正常组	—	2.0±0.3	1.8±0.6
模型组	—	4.6±0.8***	4.9±1.3**
辛伐他汀组	3	3.0±0.8##	2.7±0.9##
	50	3.6±0.1#	2.8±0.8#
IMM-H007	100	3.3±0.1##	2.4±0.4##
	200	2.8±0.1###	1.8±0.8##

***P<0.001，与正常组比较；###P<0.001,##P<0.01,#P<0.05 与模型组比较

3.5 肝脏 MRI 分析

MR 检查结果显示，与正常组比较，模型组金黄地鼠皮下及腹腔脂肪显著增加，给予辛伐他汀和 IMM-H007 后可减少皮下及腹腔脂肪（图 5）。

3.6 肝脏组织病理学观察

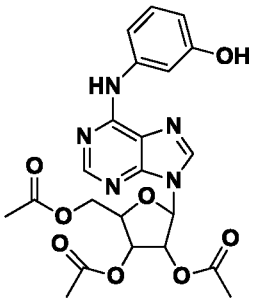
图 6 所示 HE 染色结果发现，正常动物肝细胞以中央静脉为中心向周围呈放射状排列，胶原纤维规律性分布在中央静脉和其他血管壁；模型组动物肝细胞出现空泡化，肝细胞间出现胶原纤维，且分布无规律性；辛伐他汀组动物肝细胞内有空泡出现，肝细胞间出现胶原纤维，且呈现不规律性分布。IMM-H007 各剂量组肝细胞以中央静脉为中心向周围呈放射性排列，胶原纤维呈规律性分布在中央静脉和肝小叶边界区。

油红 O 染色结果发现（图 7），正常组动物肝细胞以中央静脉为中心向周围呈放射性状排列，胞内中性脂肪含量较少；高脂膳食组动物肝细胞内有大量脂肪沉积，出现空泡化；辛伐他汀组动物肝细胞内有脂肪沉积，大片坏死灶。IMM-H007 各剂量组动物肝细胞内也可见脂肪沉积，但是与模型组比较，明显减少。

综上所述，三乙酰基-3-羟基苯基腺苷（IMM-H007）可显著降低非酒精性脂肪肝金黄地鼠的血脂水平，明显降低 AST、ALT 水平，显著改善肝功能。提示 IMM-H007 可用于制备预防或者治疗非酒精性脂肪肝的药物。

权 利 要 求 书

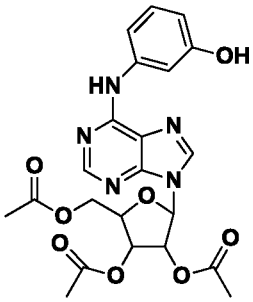
1. 如式（Ⅰ）所示的三乙酰基-3-羟基苯基腺苷及其药学上可接受的盐在制备预防或者治疗非酒精性脂肪肝的药物中的应用，



（Ⅰ）。

2. 根据权利要求 1 的应用，其特征在于，所述的非酒精性脂肪肝为高能量饮食引起的脂肪肝 。

3. 一种药物组合物在制备预防或治疗非酒精性脂肪肝药物中的应用，其特征在于，所述的药物组合物包含式（Ⅰ）所述的三乙酰基-3-羟基-苯基腺苷及其药学上可接受的盐和药学上可接受的载体，



（Ⅰ）。

4. 根据权利要求 3 的应用，其特征在于，所述的药物组合物为片剂、胶囊、丸剂或注射剂。

5. 根据权利要求 3 的应用，其特征在于，所述的药物组合物为缓释制剂、控释制剂或各种微粒给药系统。

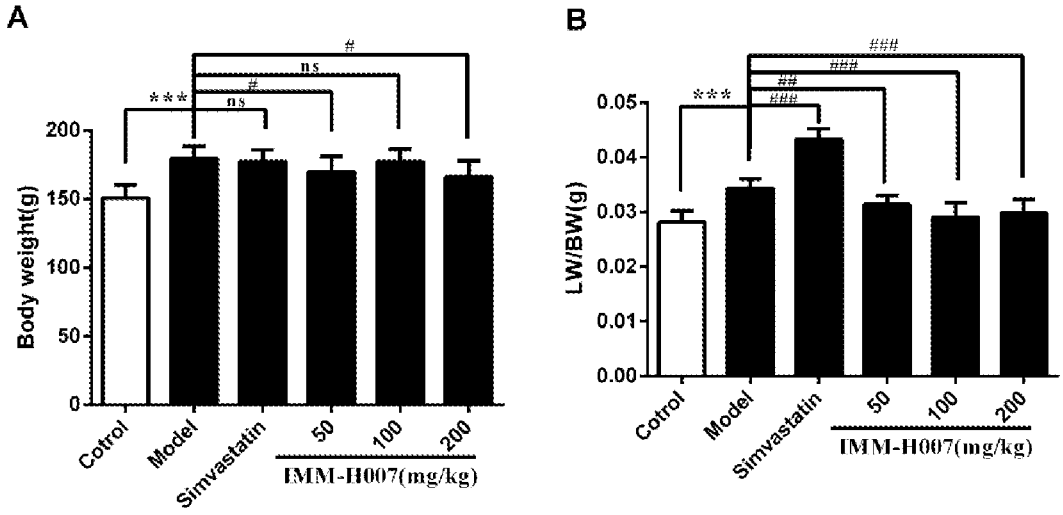

A
B
Body weight(g)
LW/BW(g)
Cotrol
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)

图 1

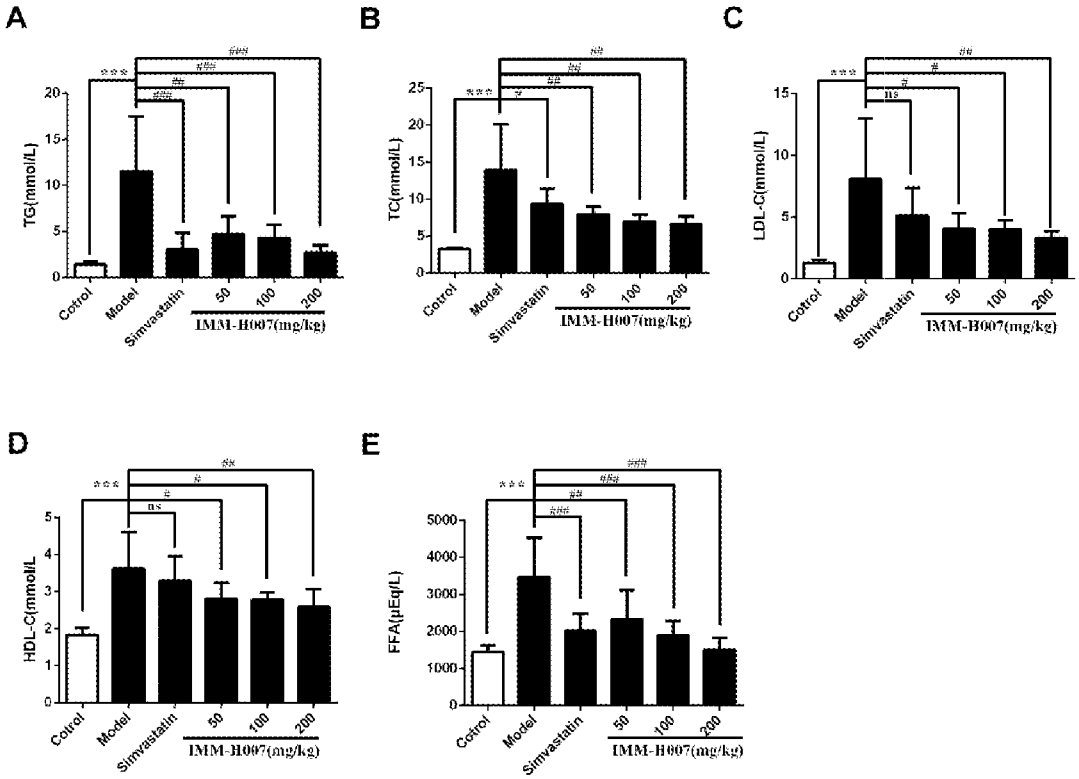

A
B
C
D
E
TG(mmol/L)
TC(mmol/L)
LDL-C(mmol/L)
HDL-C(mmol/L
FFA(μEq/L)
Cotrol
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)

图 2

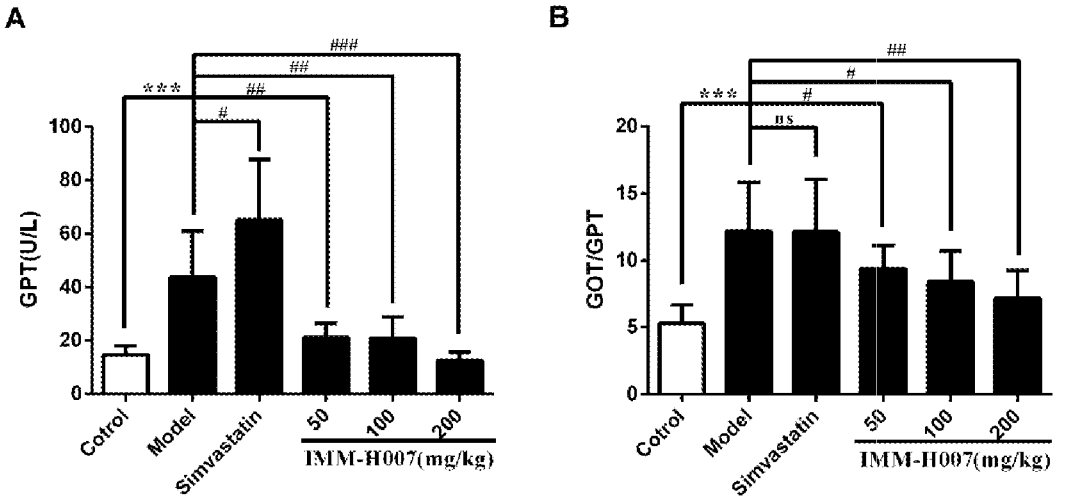

A
B
GPT(U/L)
GOT/GPT
Cotrol
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)
***
**
ns
#
##
###

图 3

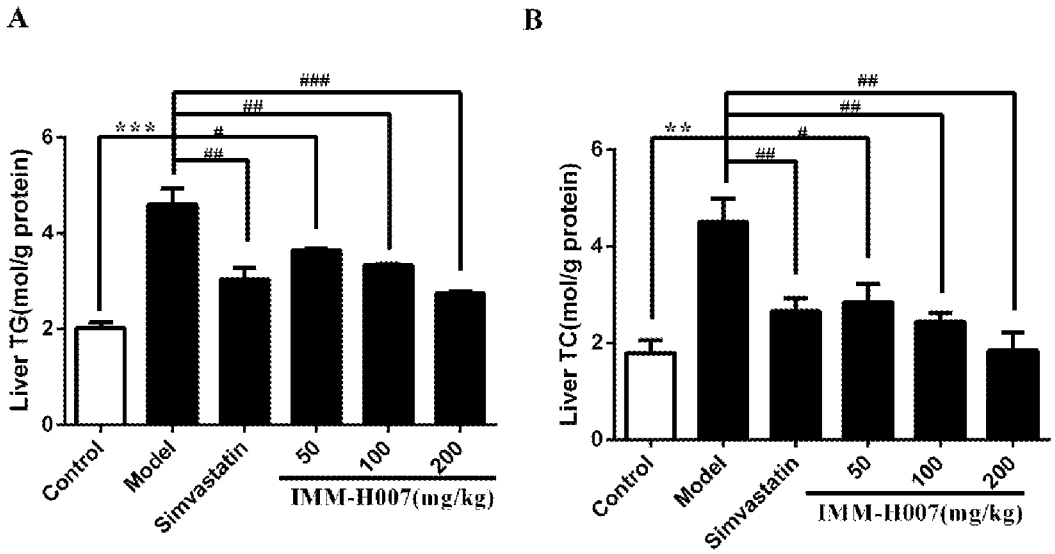

A
B
Liver TG(mol/g protein)
Liver TC(mol/g protein)
Control
Model
Simvastatin
50
100
200
IMM-H007(mg/kg)
***
**
#
##
###

图 4

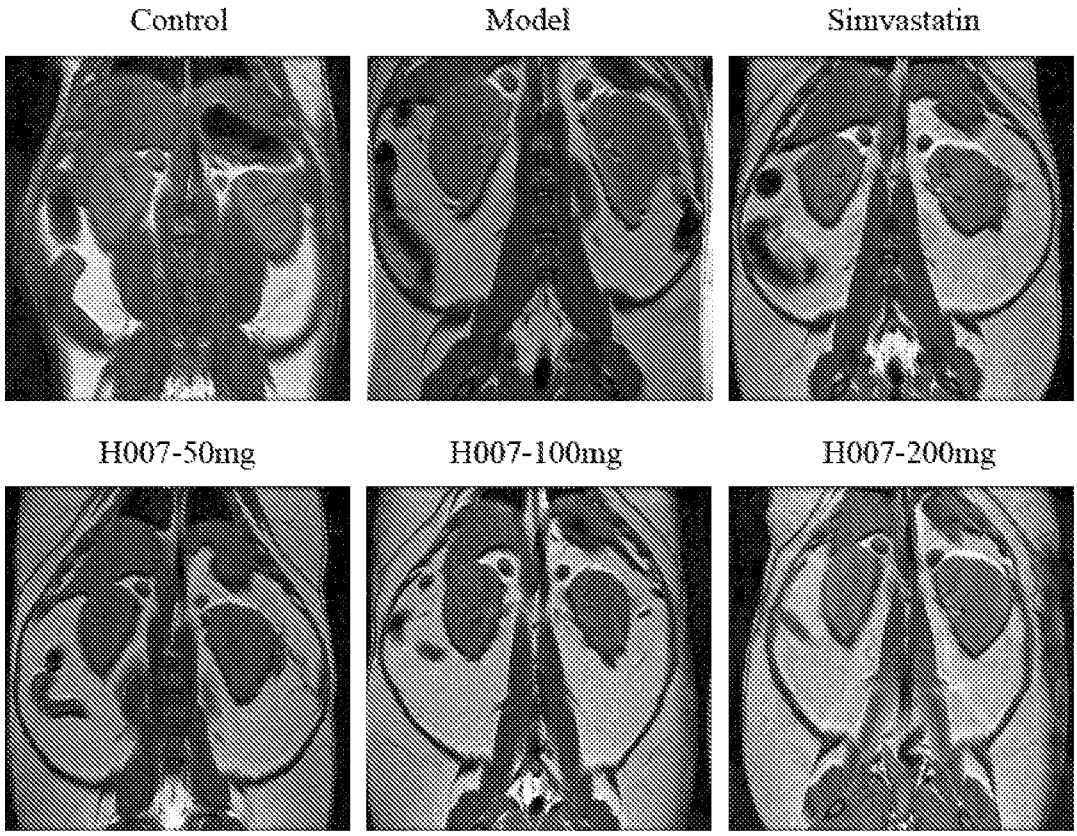

Control
Model
Simvastatin
H007-50mg
H007-100mg
H007-200mg

图 5

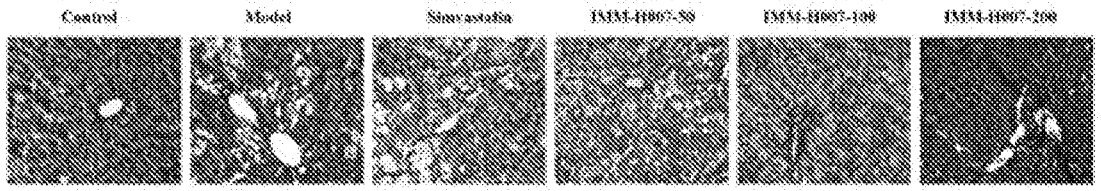

Control
Model
Simvastatin
IMM-H007-50
IMM-H007-100
IMM-H007-200

图 6

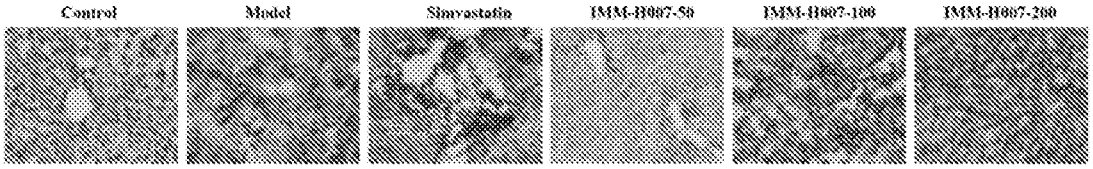

Control
Model
Simvastatin
IMM-H007-50
IMM-H007-100
IMM-H007-200

图 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2016/112623

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/7076 (2006.01) i; A61P 1/16 (2006.01) i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K, A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CHINA ACADEMIC JOURNALS FULL-TEXT DATABASE, CHINESE PHARMACEUTICAL PATENT DATABASE, CNABS, CNTXT, WPI, EPODOC, STN, DUXIU ACADEMIC SEARCH: triacetyl-3-hydroxyphenyl gland, hydroxyl phenyl, adenosine, non-alcoholic fatty liver disease, NAFLD, 1221412-23-2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	CN 105663152 A (INSTITUTE OF MATERIA MEDICA, CHINESE ACADEMY OF MEDICAL SCIENCE), 15 June 2016 (15.06.2016), claims 1-2 and 4-6	1-5
X	CN 101874036 A (INSTITUTE OF MATERIA MEDICA, CHINESE ACADEMY OF MEDICAL SCIENCE), 27 October 2010 (27.10.2010), description, paragraphs 0002, 0008 and 0011-0020, and claims 7 and 9	1-5
A	CN 104546887 A (INSTITUTE OF MATERIA MEDICA, CHINESE ACADEMY OF MEDICAL SCIENCE), 29 April 2015 (29.04.2015), the whole document	1-5
A	CN 102125580 A (TAISHAN MEDICAL UNIVERSITY), 20 July 2011 (20.07.2011), the whole document	1-5

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 February 2017 (09.02.2017)	**24 March 2017 (24.03.2017)**
Name and mailing address of the ISA/CN: State Intellectual Property Office of the P. R. China No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088, China Facsimile No.: (86-10) 62019451	Authorized officer **WU, Tiesheng** Telephone No.: (86-10) **010-62413770**

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2016/112623

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 105663152 A	15 June 2016	None	
CN 101874036 A	27 October 2010	EP 2407474 B1	14 August 2013
		CN 101874036 B	25 January 2012
		JP 2012519714 A	30 August 2012
		IL 215080 A	31 July 2014
		KR 101380140 B1	01 April 2014
		DK 2407474 T3	25 November 2013
		EP 2407474 A4	11 July 2012
		ES 2435620 T3	20 December 2013
		CN 101712709 A	26 May 2010
		EP 2407474 A1	18 January 2012
		US 2012053143 A1	01 March 2012
		IL 215080 D0	30 November 2011
		US 8435962 B2	07 May 2013
		KR 20110117257 A	26 October 2011
		JP 5698682 B2	08 April 2015
		WO 2010040286 A1	15 April 2010
		IN 270393 B	25 December 2015
CN 104546887 A	29 April 2015	None	
CN 102125580 A	20 July 2011	None	

国际检索报告

国际申请号 PCT/CN2016/112623

A. 主题的分类

A61K 31/7076(2006.01)i；A61P 1/16(2006.01)i

按照国际专利分类(IPC)或者同时按照国家分类和IPC两种分类

B. 检索领域

检索的最低限度文献(标明分类系统和分类号)

A61K，A61P

包含在检索领域中的除最低限度文献以外的检索文献

在国际检索时查阅的电子数据库(数据库的名称，和使用的检索词（如使用））

中国期刊全文数据库，中国药物专利数据库，CNABS，CNTXT，WPI，EPODOC，STN，读秀学术搜索：三乙酰基-3-羟基苯基腺，非酒精性脂肪肝，hydroxyl phenyl，adenosine，non-alcoholic fatty liver disease，NAFLD，1221412-23-2

C. 相关文件

类 型*	引用文件，必要时，指明相关段落	相关的权利要求
PX	CN 105663152 A (中国医学科学院药物研究所) 2016年 6月 15日 (2016-06-15) 权利要求1-2，4-6	1-5
X	CN 101874036 A (中国医学科学院药物研究所) 2010年 10月 27日 (2010-10-27) 说明书第0002，0008，0011-0020段，权利要求7和9	1-5
A	CN 104546887 A (中国医学科学院药物研究所) 2015年 4月 29日 (2015-04-29) 全文	1-5
A	CN 102125580 A (泰山医学院) 2011年 7月 20日 (2011-07-20) 全文	1-5

☐ 其余文件在C栏的续页中列出。

☑ 见同族专利附件。

* 引用文件的具体类型：

“A” 认为不特别相关的表示了现有技术一般状态的文件

“E” 在国际申请日的当天或之后公布的在先申请或专利

“L” 可能对优先权要求构成怀疑的文件，或为确定另一篇引用文件的公布日而引用的或者因其他特殊理由而引用的文件（如具体说明的）

“O” 涉及口头公开、使用、展览或其他方式公开的文件

“P” 公布日先于国际申请日但迟于所要求的优先权日的文件

“T” 在申请日或优先权日之后公布，与申请不相抵触，但为了理解发明之理论或原理的在后文件

“X” 特别相关的文件，单独考虑该文件，认定要求保护的发明不是新颖的或不具有创造性

“Y” 特别相关的文件，当该文件与另一篇或者多篇该类文件结合并且这种结合对于本领域技术人员为显而易见时，要求保护的发明不具有创造性

“&” 同族专利的文件

国际检索实际完成的日期	国际检索报告邮寄日期
2017年 2月 9日	2017年 3月 24日

ISA/CN的名称和邮寄地址	受权官员
中华人民共和国国家知识产权局(ISA/CN) 中国北京市海淀区蓟门桥西土城路6号 100088	吴铁生
传真号 (86-10)62019451	电话号码 (86-10)010-62413770

国际检索报告
关于同族专利的信息

国际申请号
PCT/CN2016/112623

检索报告引用的专利文件			公布日 (年/月/日)	同族专利			公布日 (年/月/日)
CN	105663152	A	2016年 6月 15日	无			
CN	101874036	A	2010年 10月 27日	EP	2407474	B1	2013年 8月 14日
				CN	101874036	B	2012年 1月 25日
				JP	2012519714	A	2012年 8月 30日
				IL	215080	A	2014年 7月 31日
				KR	101380140	B1	2014年 4月 1日
				DK	2407474	T3	2013年 11月 25日
				EP	2407474	A4	2012年 7月 11日
				ES	2435620	T3	2013年 12月 20日
				CN	101712709	A	2010年 5月 26日
				EP	2407474	A1	2012年 1月 18日
				US	2012053143	A1	2012年 3月 1日
				IL	215080	D0	2011年 11月 30日
				US	8435962	B2	2013年 5月 7日
				KR	20110117257	A	2011年 10月 26日
				JP	5698682	B2	2015年 4月 8日
				WO	2010040286	A1	2010年 4月 15日
				IN	270393	B	2015年 12月 25日
CN	104546887	A	2015年 4月 29日	无			
CN	102125580	A	2011年 7月 20日	无			