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Title: A PHARMACEUTICAL COMPOSITION COMPRISING CORDYCEPIN FOR THE TREATMENT AND PREVENTION OF OBESITY

Abstract: Disclosed is a pharmaceutical composition for the treatment and prevention of obesity, comprising cordycepin as an active ingredient. Cordycepin suppresses the expression of C/EBPα, PPARγ, and anti-leptin, the essential factors involved in adipogenesis and lipogenesis, inhibiting the differentiation of fibroblast cells (3T3-L1 cells) into adipocytes and the biosynthesis of triglyceride, and is therefore a useful agent in the treatment and prevention of obesity as well as the relief and amelioration of the symptoms. Also, because Dong-Chung-Ha-Cho is already permitted as food, the cordycepin extracted therefrom is free of toxicity and side effects.
Published:

with international search report
A PHARMACEUTICAL COMPOSITION COMPRISING CORDYCEPIN FOR THE TREATMENT AND PREVENTION OF OBESITY

Technical Field

The present invention relates to a pharmaceutical composition for the treatment and prevention of obesity, comprising cordycepin as an active ingredient. More specifically, the present invention relates to a pharmaceutical composition comprising cordycepin that suppresses the expression levels of and C/EBP\(\alpha\), PPAR\(\gamma\) and anti-leptin, thereby inhibiting the differentiation of fibroblast cells (3T3-L1 cells) into adipocytes and the biosynthesis of triglyacylglycerol in the cell.

Background Art

Obesity refers to a body condition in which natural energy reserves, stored in the fatty tissues of humans, are excessively increased in proportional to body weight. With more rapid, complex and varied changes in lifestyle than ever, modern people are prone to become obese due to bad lifestyle habits, imbalance of ingested nutrients, exercise deficiency, and excessive drinking.

Under the increasing influence of Western culture, Asians, who have been accustomed to vegetables and carbohydrate as their main diet, are changing in lifestyle
with regard to diet, eating habit and residence, in the direction of increasing morbidity from obesity.

Obesity has been shown to cause various diseases, particularly hypertension, heart disease, diabetes mellitus, etc., and to increase the onset of chronic diseases, thus leading to a significant reduction in the lifespan of obese persons.

As is the case with many medical conditions, the caloric imbalance that results in obesity often develops from a combination of genetic and environmental factors. Particularly, obesity is greatly influenced by heredity. For example, when both parents are fat, their children are predisposed to obesity with a possibility of 50% or higher. When either parent is obese, their children are reported to be predisposed to obesity with a possibility of about 25%.

Examples of other factors causing obesity include excessive caloric intake, insufficient exercise, low energy expenditure, etc.

The mainstay of treatment for obesity is lifestyle change, such as adopting an energy-limited diet, increasing exercise, increasing energy-consuming behavior, etc. Other therapies for obesity are drugs and surgery.

It is not easy for a person to change his or her lifestyle. Weight loss can be achieved by changing lifestyles, but to a limited degree. Typically, chemical therapy is adopted in combination with lifestyle change.

Drugs have long been used for the treatment of obesity.
Drugs for the treatment of obesity that target materials involved in the homeostatic control of energy in the body are clinically restricted because of their side effects.

There are two drugs that have received FDA permission for long-term use in the treatment of obesity; sibutramine, functioning as a serotonin-norepinephrine reuptake-inhibitor effective in the treatment of obesity, and orlistat, a lipase inhibitor is being used in the treatment of obesity.

Sibutramine, however, produces significant side effects, such as blood pressure increase, insomnia, xerostomia, vertigo, etc., and cannot be applied to patients suffering from cardiovascular diseases or uncontrolled hypertension. When orlistat is taken, the persons may suffer from diarrhea, fatty stool and constipation. In addition, orlistat is limitedly used because its efficacy is low in persons whose diets contain relatively small amounts of fat, such as for Asians.

Thus, active research has been conducted to develop naturally occurring physiologically active substances which can be applied to the treatment of obesity.

<table>
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<th>Anti-obesity drugs</th>
<th>Side effect</th>
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<td>Thyroid hormone (1890)</td>
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<tr>
<td>Amphetamine (1930)</td>
<td>Addictive</td>
</tr>
<tr>
<td>Digitalis, diuretics (1960)</td>
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<td>Aminorex (1970)</td>
<td>Pulmonary hypertension caused</td>
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<tr>
<td>Fenfluramine (1990)</td>
<td>Valvular dysfunction caused in</td>
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"Dong-Chung-Ha-Cho" (literally insect in winter but plant in summer) is a kind of medicinal fungus produced as a result of the parasitism of *Cordyceps mlllitaris* in insects. In high temperature and moisture conditions, the fungus infects living insects, proliferates therein to kill the host insects, and forms fruiting bodies on the surface of the host insects. Primarily, "Dong-Chung-Ha-Cho" refers to *Cordyceps sinensis*, a parasite on larvae of the Hepialidae family, but generally refers to all fungi attacking arthropods, such as spiders, at present.

Taxonomically, Dong-Chung-Ha-Cho belongs to the phylum *Ascomycota*, the order *Clavicipitales*, and the family *Clavicipitaceae* and is classified into three species: *Cordyceps*, *Podonectria* and *Torrubiella*. *Cordyceps* is the most popular Dong-Chung-Ha-Cho (Kobayasi Y., Trans Mycol. Soc. Japan, 23. 329-364, 1982). Approximately 800 kinds of Dong-Chung-Ha-Cho have been known to be distributed over the world thus far. Of them, 78 kinds have been collected and

<table>
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<th>35% of persons administered in combination with other therapy, sale prohibited in 1997.</th>
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<td>Sibutramine (present)</td>
<td>Blood pressure increase, insomnia, xerostomia and vertigo. Prohibited to the patients with cardiovascular disease.</td>
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<tr>
<td>Orlistat (present)</td>
<td>Diarrhea, fatty stool, fecal incontinence.</td>
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identified as with low host specificity and therefore utilizes various insect as a host. Generally, Dong-Chung-Ha-Cho is named after the insect hosts on which they grow. Naturally occurring Dong-Chung-Ha-Cho is too rare to acquire. In practice, various kinds of Dong-Chung-Ha-Cho are artificially cultured and are intensively studied with regard to the components and medical effects thereof.

*Cordyceps sinensis* is one of the most valuable herb medicines in the world. Wild-type *Cordyceps sinensis* is very rare and difficult to culture artificially. Chinese scientists developed artificially culturable Cs-4 strains as alternatives to *Cordyceps*. *Cordyceps sinensis* is known to have various medicinal effects including the ability to scavenge oxygen-free radicals, delay aging, stimulate endocrine, improve sexual functions, potentiate the respiratory system and the liver, and cancers and renal diseases (Zhu. J. S., J. Altern. Complement. Med., 4. 289-303 1998). *Cordyceps militaris* is another species of Cordyceps and widely used in Korea and China.

Cordycepin (3'-deoxyadenosine) is assumed to be the effective ingredient of *Cordyceps sinensis*. Long ago, Cunningham et al. isolated an inhibitor against the growth of *Bacillus subtilis* from *Cordyceps militaris*. This inhibitor was identified as cordycepin, (Kaczka, E. A., et al., *Biochim. Biophys. Res. Commun.*, 14, pp. 456-457, 1964). Following the identification of cordycepin, various biological effects thereof are under study. For example, it has been proved that
cordycepin plays an inhibitory role in bacteria, malaria, herpes, tumor, and leukemia.

Meanwhile, cordycepin has come to be known as a polyadenylation inhibitor, showing cytotoxicity against cancer cells. Recently, cordycepin was clinically tested as a therapeutic for leukemia (Eiichi N., et al., Biochemical Phar., Vol. 59, pp. 273-281, 2000).

However, cordycepin has never been reported or suggested to have an antiobesity effect.

Leading to the present invention, intensive and thorough research on treatment for obesity, conducted by the present inventors, resulted in the finding that cordycepin isolated from *Cordyceps militaris* inhibits the differentiation of 3T3-L1 cells into adipose cells, as well as the synthesis of triacylglycerol. This result indicates that cordycepin can be used as anti-obestic agent.

**Disclosure of the Invention**

It is therefore an object of the present invention to provide a pharmaceutical composition for the treatment and prevention of obesity.

In order to achieve the above object, there is provided a pharmaceutical composition for the treatment and prevention of obesity, comprising cordycepin as an active ingredient.
**Brief Description of the Drawings**

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 is an HPLC profile of the active ingredient according to the present invention;

FIG. 2 is a $^1$H NMR spectrum of the active material according to the present invention;

FIG. 3 is a $^{13}$C NMR spectrum of the active material according to the present invention;

FIG. 4 is a graph showing the dose-dependent inhibitory effect of cordycepin on the differentiation of 3T3-L1 cells into adipose cells (NC: negative control (DMEM); PC: positive control (MSD and MFD); C4: cordycepin 4 µM; C16: cordycepin 16 µM; C32: cordycepin 32 µM);

FIG. 5 is a graph showing the dose-dependent effect of cordycepin on the synthesis of triacylglycerol in 3T3-L1 cells (NC: negative control (DMEM); PC: positive control (MSD and MFD); C4: cordycepin 4 µM; C16: cordycepin 16 µM; C32: cordycepin 32 µM);

FIG. 6 shows the inhibitory activity of cordycepin and/or adenosine against the differentiation of 3T3-L1 cells into adipose cells (NC: negative control (DMEM); PC: positive control (MSD and MFD); C32: cordycepin 32 µM; A32: adenosine 32 µM; CA: cordycepin 32 µM + Adenosine 32 µM);
FIG. 7 is a photograph showing the effect of adenosine on cordycepin-induced inhibitory activity against triacylglycerol synthesis (NC: negative control (DMEM); PC: positive control (MSD and MFD); C32: cordycepin 32 µM; A32: adenosine 32 µM; CA: cordycepin 32 µM + Adenosine 32 µM); and

FIG. 8 is a photograph of Western blots showing the effects of cordycepin on the protein expression of C/EBPα, PPARγ, and anti-leptin in 3T3-L1 cells (NC: negative control (DMEM); PC: positive control (MSD and MFD); C32: cordycepin 32 µM; A32: adenosine 32 µM; CA: cordycepin 32 µM + Adenosine 32 µM).

Best Mode for Carrying Out the Invention

Below, a detailed description is given in the present invention.

In accordance with the present invention, this provides a cordycepin-based pharmaceutical composition for the treatment and prevention of obesity.

Cordycepin, an active ingredient in the present invention, may be a commercially available form, or may be purified from Cordyceps militaris. However, it is encouraged to purify cordycepin from the mycelial cake or fruiting body of Cordyceps militaris.

In the present invention, cordycepin is found to have an effective inhibitory effect on the protein expression of
C/EBPα, PPARγ, and anti-leptin, which are involved in adipogenesis and lipogenesis, thereby suppressing differentiation of fibroblast cells (3T3-L1 cells) into adipose cells. Also, cordycepin is proved to compete with adenosine, the precursor of ATP, NAD+, NADP+, CoA and FAD, therefore can serve as an effective inhibitor against the synthesis of triacylglycerol (TAG).

Also, cordycepin shows a dose-dependent inhibitory effect, both on the differentiation of fibroblast cells into adipocytes and on the biosynthesis of triacylglycerol.

Depending on the usage thereof, the composition of the present invention may comprise a pharmaceutically available vehicle or expedient, and may be formulated into dosage forms suitable for administration alone or in combination with other drugs. A thickening agent, a high in content of fiber additive, a capsulation agent, and a lipid may be useful as expedients in the present invention. Examples of these expedients are well known in the art.

The dose of the pharmaceutical composition of the present invention is dependent on the severity of disease, patient's state, age, sex, body condition, body weight, diet, excretion rate, administration time and route, etc.

In a preferable embodiment of the present invention, the active ingredient of the pharmaceutical composition is administered orally or non-orally in a single dose or in multiple doses within a daily total amount from 0.01 to 100 µg per kg of body weight. However, the dosage does not limit the
present invention by no means.

A better understanding of the present invention may be obtained through the following examples, which are set forth to illustrate, but are not to be construed as the limit of the present invention.

**EXAMPLE 1: Isolation and Identification of Active Ingredient**

*Cordyceps militaris* were purchased from Kyoungdong Herbal Medicinal Market, Seoul, Korea. After being dried, the *Cordyceps militaris* were grounded into powder and mixed with 10 ml/g of 80% ethanol. The suspension was extracted three times in a water bath with reflux system at 80°C for 3 hours, and dried in vaccuo to give a crude ethanol extract.

The crude ethanol extract was dissolved in distilled water and mixed with butanol at a ratio of 1:1 (v/v) and subjected to distribution chromatograph between the two different phases (3 times). The butanol extract was pooled and concentrated in vaccuo.

The residue thus obtained was purified by silica gel flash chromatography eluting with the solvent mixture of chloroform/methanol (7:3). The active fraction was condensed in a vacuum to completion, dissolved in a small amount of methanol at a concentration of 20 µg/ml, and filtered through a Millipore filter (0.45 nm). Final purification was achieved by HPLC (column: ODS 20 x 250 mm; 15% methanol; diethylamine buffer pH 4.0; 10 ml/min) to afford an active fraction.
With reference to FIGS. 1 to 3, the HPLC profile, $^1$H and $^{13}$C NMR spectra of the active substance according to the present invention are shown, respectively.

From the data, the active substance according to the present invention was identified to be 3'-deoxyadenosine (cordycepin), represented by the following chemical formula 1.

[Chemical Formula 1]

EXPERIMENTAL EXAMPLE 1: Inhibitory Effect of Cordycepin on the Differentiation of Fibroblasts into Adipocytes

Fibroblast cells (3T3-L1 cells) were cultured in 10 cm-
culture dishes containing animal cell culture media (refer to Table 2, below) at 37°C in a 5% CO$_2$ incubator, with the media replaced with fresh media every two or three days.

3T3-L1 cell line was purchased from the American Type Culture Collection (ATCC CL-173). The cells were grown and differentiated according to the protocol of Frost and Lane (Frost and Lane, 1985): 3T3-L1 preadipocytes were cultured in
100 mm plates containing a submerged medium for proliferation (DMEM supplemented with 10% BCS, 100 U/ml penicillin, and 100 µg/ml streptomycin) in an atmosphere of 5% CO2 at 37°C. When the cell become confluent, the cells were harvested, splitted in a 6-well plate to a cell number of 4.0x10^5 cells/ml and subsequently cultured until reaching confluence again. Two days after confluence, the cells were stimulated to differentiate into adipocytes by incubating for 2 days in the medium for stimulating differentiation (MSD, DMEM containing 1 µM DEX, 0.5 mM IBMX, and 10 µg/ml insulin), and maintained in the differentiated state in the medium for differentiation (MFD containing 10% BCS and 5 µg/ml insulin) for 7 days. MSD was freshly replaced every 48 hours and the comparison of the each medium composition used in this example was shown in Table 2.

Cordycepin was dissolved in the appropriate warm medium and added at the final concentration of 4, 16, and 32 µM.

<table>
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<tr>
<th>Media</th>
<th>Glucose</th>
<th>BCS</th>
<th>FBS</th>
<th>Sodium bicarbonate</th>
<th>Insulin</th>
<th>Dex</th>
<th>IBMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>13.4 g/l</td>
<td>10%</td>
<td>-</td>
<td>3.7 g/l</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MSD</td>
<td>13.4 g/l</td>
<td>-</td>
<td>10%</td>
<td>3.7 g/l</td>
<td>5 µg/ml</td>
<td>1 µM</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>MFD</td>
<td>13.4 g/l</td>
<td>-</td>
<td>10%</td>
<td>3.7 g/l</td>
<td>5 µg/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DMEM: Dulbecco's modified Eagle's medium
BCS: Bovine calf serum
FBS: Fetal Bovine serum
DEX: Dexamethasone, IBMX: l-methyl-3-isobutylxanthine.
Cells obtained in Example 1-1 were washed twice with PBS (pH 7.4), fixed with 10% formalin in PBS for 1 hour at room temperature and washed three times with distilled water. The cells were stained with filtered 0.5% oil red 0 for 10 min, and washed again with distilled water three times. The pictures of the stained cells were taken using an Olympus (CKX-41, Tokyo, Japan) microscope. The density of stained oil red 0 was measured using a spectrometer (DU-70, Beckman, Fullerton, CA) at 518 nm after the dye was extracted with propanol.

The results are given in FIG. 4. As shown in FIG. 4, cordycepin apparently inhibits the differentiation of 3T3-L1 cells into adipose cells in a dose-dependent manner. It was found that the differentiation of 3T3-L1 cells into adipocytes was almost completely suppressed in the presence of 32 µM of cordycepin.

**EXPERIMENTAL EXAMPLE 2: Inhibitory Effect on Triacylglycerol Synthesis**

A measurement was made to find the effect of cordycepin on the synthesis of triacylglycerol (TAG). First, the cells in each group were detached using a Tris-EDTA buffer and disrupted with an ultrasonicator. A calibration curve for a standard solution was made according to the protocol of a BCA
protein assay kit. The cell lysates were diluted in the kit reagent and warmed at 37°C for 30 min. Then, within 10 min, the solutions were measured for absorbance at 562 nm to quantify protein. Separately, according to the protocol of a BCS triacylglycerol kit, the cell lysates were diluted in the kit reagent and warmed at 37°C for 10 min. Then, within 60 min, the absorbance of the solutions was measured at 550 nm against a reagent blank. The amount of intracellular triacylglycerol per unit weight of protein (µg/nig) was calculated according to a calculation formula.

As can be clearly understood from FIG. 5, cordycepin inhibits the synthesis of triacylglycerol in 3T3-L1 cells in a dose-dependent manner.

EXPERIMENTAL EXAMPLE 3: Effect of adenosine on the cordycepin-induced inhibition of differentiation and TAG synthesis

For the preliminary investigation of the mode of action of cordycepin on differentiation and TAG synthesis, adenosine, one of several cordycepin homologues, was added to the submerged medium for differentiation at the time of initiating differentiation at the same molar concentration as cordycepin (32 µM).

As shown in Fig. 6 and 7, the inhibitory effect of cordycepin on differentiation and TAG synthesis of 3T3-L1 cells was suppressed by adenosine.

This result suggests that cordycepin inhibits TAG
synthesis by competing with adenosine, which is the precursor of ATP, NAD+, NADP+, CoA, and FAD.

EXPERIMENTAL EXAMPLE 4: Western Blot Analysis

Western blot analysis was performed in order to examine the effect of cordycepin on the expression of C/EBPα, PPARγ, and anti-leptin, the essential factors involved in adipogenesis and lipogenesis.

3T3-L1 cells cultured in 6-well dishes were rinsed three times with ice-cold PBS, scraped into 0.5 ml of PBS, and centrifuged at 14,000 rpm for 1 min. Cell pellets were resuspended in 0.2 ml ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP 40, 0.25% deoxycholate) and protease inhibitors (Complete Mini Protease Inhibitor Tablets, Roche, Indianapolis, IN). Suspensions were sheared through a 20-gauge needle, incubated on ice for 30 min and centrifuged at 14,000 rpm at 4°C for 20 min. Supernatants were collected and the protein concentration was determined using a BCA assay kit. Cell lysates were mixed with an equal volume of Laemmli SDS loading buffer (Bio-Rad Laboratories, Hercules, CA), equal amounts of lysate protein (30 mg) were loaded onto SDS-PAGE, and electrophoresis was carried out before the protein was transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked for 1 h in PBS containing 0.05% Tween-20 (USB Corp., Cleveland, USA) and 5% nonfat dry milk. The membranes were incubated with
antibodies against C/EBP\(\alpha\), PPAR\(\gamma\) (Santa Cruz, CA) and anti-leptin (Sigma) for 1 hour, followed by three 10 min washes in PBS/Tween-20. The blots were subsequently treated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 hour at room temperature, and washed with PBS-T three times for 10 min. To visualize reaction complexes, an enhanced chemiluminescence system (ECL, Amersham International pic, Little Chalfont Buckinghamshire, England) was used, and reaction complexes were exposed to X-ray film.

As shown in FIG. 8, the expression levels of C/EBP\(\alpha\) and anti-leptin proteins in the NC cultured only in DMEM were almost negligible while the expression level of PPAR\(\gamma\) in NC was significantly lower compared with that in PC. Cordycepin inhibited the expression levels of C/EBP\(\alpha\), PPAR\(\gamma\), and anti-leptin. In case of adenosine, it showed no significant effect on the expression levels of PPAR\(\alpha\) and anti-leptin in the differentiated 3T3-L1 cells, whereas it inhibited the C/EBP\(\gamma\) level.

Taken together, the data obtained in the above examples demonstrate that cordycepin suppresses adipocyte differentiation through PPAR\(\gamma\)- and C/EBP\(\alpha\)-mediated adipogenesis pathway, thus acting as an inhibitor against obesity.

Although the preferred embodiments of the present
invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

**Industrial Applicability**

As described above, cordycepin suppresses the expression of C/EBPα, PPARγ, and anti-leptin, the essential factors involved in adipogenesis and lipogenesis, thereby inhibiting the differentiation of fibroblast cells (3T3-L1 cells) into adipose cells and the synthesis of triacylglycerol. Therefore, cordycepin can be useful in the treatment and prevention of obesity as well as the relief and amelioration of symptoms.

Also, because Dong-Chung-Ha-Cho (Cordyceps militaris) is already permitted as food, the cordycepin extracted therefrom is free of toxicity and side effects. Consequently, the present invention can be used as an active ingredient of a pharmaceutical composition for the treatment and prevention of obesity and as an active ingredient of health food.
Claims

1. A pharmaceutical composition for the treatment and prevention of obesity, comprising cordycepin as an active ingredient.

2. The pharmaceutical composition as set forth in claim 1, wherein the cordycepin is isolated and purified from Cordyceps militaris.

3. The pharmaceutical composition as set forth in claim 1, wherein the composition is administered orally or non-orally.

4. The pharmaceutical composition as set forth in claim 1, wherein the cordycepin inhibits the differentiation of fibroblast cells into adipocytes and the biosynthesis of triglyceride glycerol.

5. The pharmaceutical composition as set forth in one of claims 1 to 4, wherein the cordycopin suppresses adipocyte differentiation through PPARγ- and C/EBPα-mediated adipogenesis pathway, thus acting as an inhibitor against obesity.
1H of Cordycepin(3'-deoxyadenosine,C10H13N5O3) in DMSO

FIG. 2
13C of Cordycepin (3'-deoxyadenosin, C10H13N5O3) in DMSO

FIG. 3
**FIG. 4**

**FIG. 5**

SUBSTITUTE SHEET (RULE 26)
FIG. 6
FIG. 8
A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/7076(2006.01)

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)
IPC 8 as above

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)
eKIPASS(KIPO internal), PUPMED

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>KR 102005063164 A (KIM, K J et al ) 28 JUNE 2002 See the whole document</td>
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<td>JP 13131 196 A (MITSUBISHI CHEMICALS CO ) 15 MAY 2001 See the whole document</td>
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[ ] Further documents are listed in the continuation of Box C
[ ] See patent family annex

Date of the actual completion of the international search
07 JANUARY 2008 (07.01.2008)

Date of mailing of the international search report
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Name and mailing address of the ISA/KR
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Facsimile No 82-42-472-7140

Authorized officer
KIM, EUN HEE
Telephone No 82-42-481-5609
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