Description

Title of Invention: COMPOSITIONS FOR TREATING LIVER CANCER COMPRISING A VASCULAR DISRUPTING AGENT

Technical Field

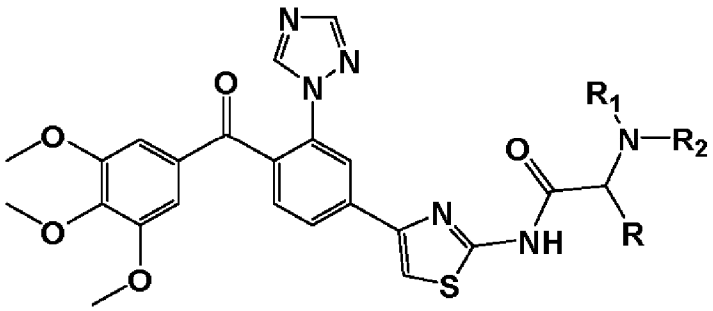
[1] The present disclosure relates to compositions for treating liver cancer, which comprises a vascular disrupting agent (VDA), and a method for treating liver cancer using the same.

Background Art

[2] Liver cancer is a malignant tumor with poor prognosis, which ranks second in Korea and third in the world, among the causes of death by cancer. Radical surgery is impossible in 70% or more of liver cancer patients, and even when liver cancer patients received radical dissection, the probability of recurrence of cancer in other areas within 5 years is 50% or more. Furthermore, the possibility for advanced liver cancer to respond to systemic anticancer therapy is very low (10% or less).

[3] Vascular disrupting agents (VDAs) aim to rapidly and selectively disrupt established tumor blood vessels by selectively destroying microtubules in the cytoskeleton of vascular endothelial cells, and may induce ischemic necrosis in central area of tumors. Thus, methods for disrupting blood vessels using vascular disrupting agents (VDAs) have recently received attention as a new anticancer strategy. Accordingly, the applicant developed compounds of the following formula I for use as vascular disrupting agents (International Patent Publication No. WO 2009-119980).

[4] Formula I

[5] 

[6] The compounds of formula I are tubulin polymerization inhibitors having double mechanisms: rapid disruption of already existing tumor blood vessels by microtubule instabilization; and apoptosis by cell cycle arrest. However, when vascular disrupting agents (VDAs), including the compounds of formula 1, are treated alone, there is a problem in that tumors may rapidly regrow from viable rims, thereby reducing the therapeutic availability of such agents.

[7] Therefore, the present inventors have conducted extensive studies to provide com-

positions and treatment methods capable of treating liver cancer, a malignant tumor, by solving the tumor regrowth problem while taking the advantage of a vascular disrupting agent as an anticancer agent.

Disclosure of Invention

Technical Problem

[8] It is an object of the present disclosure to provide a composition for treatment or adjuvant treatment of liver cancer, which comprises a vascular disrupting agent (VDA).

[9] Another object of the present disclosure is to provide a combination for treatment of liver cancer, which comprises a vascular disrupting agent (VDA) and sorafenib.

[10] Still another object of the present disclosure is to provide a composition for chemoembolization, which comprises a vascular disrupting agent (VDA).

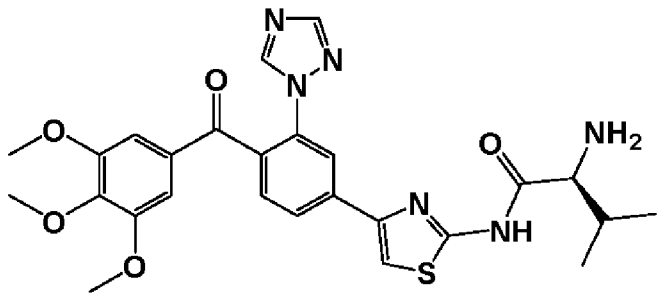
Solution to Problem

[11] The present inventors have made extensive efforts to achieve the above objects, and as a result, have developed pharmaceutical compositions for treatment or adjuvant treatment of liver cancer, which comprises a vascular disrupting agent (VDA).

[12] In the present disclosure, a compound used as the vascular disrupting agent is (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide represented by the following formula 1 or a pharmaceutically acceptable salt thereof:

[13] Formula 1

[14]



[15] In the present disclosure, the term "pharmaceutically acceptable salt" means salts that are generally used in the pharmaceutical field. Examples of pharmaceutically acceptable salts include salts formed with inorganic ions such as calcium, potassium, sodium or magnesium; salts formed with inorganic acids such as hydrochloric acid, nitric acid, phosphoric acid, hydrobromic acid, iodic acid, perchloric acid or sulfuric acid; salts formed with organic acids such as acetic acid, trifluoroacetic acid, citric acid, maleic acid, succinic acid, oxalic acid, benzoic acid, tartaric acid, fumaric acid, mandelic acid, propionic acid, lactic acid, glycolic acid, gluconic acid, galacturonic acid, glutamic acid, glutaric acid, glucuronic acid, aspartic acid, ascorbic acid, carbonic

acid or vanillic acid; salts formed with sulfonic acids such as methanesulfonic acid, ethanesulfonic acid, benzenesulfonic acid, p-toluenesulfonic acid or napthalenesulfonic acid; salts formed with amino acids such as glycine, arginine or lysine; and salts formed with amines such as trimethylamine, triethylamine, ammonia, pyridine or picoline, but the scope of salts that are used in the present disclosure is not limited to these listed salts.

[16] In the present disclosure, a salt of (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide may be a hydrochloride salt.

[17] In the present disclosure, the compositions of the present disclosure may be applied for treatment or adjuvant treatment of liver cancer. The liver cancer includes all primary liver cancer or metastatic liver cancer, but the compositions of the present disclosure may be applied to primary liver cancer. Furthermore, the liver cancer includes all hepatocellular carcinoma or cholagiocarcinoma, but the compositions of the present disclosure may be applied to hepatocellular carcinoma. In addition, the liver cancer may be early-stage liver cancer, advanced liver cancer or end stage liver cancer, and the compositions of the present disclosure may be applied to advanced liver cancer.

[18] The pharmaceutical compositions of the present disclosure may be prepared as single dosage forms or in multiple-dosage containers by formulating it with pharmaceutically acceptable carriers according to a method which can be readily carried out by a person skilled in the art.

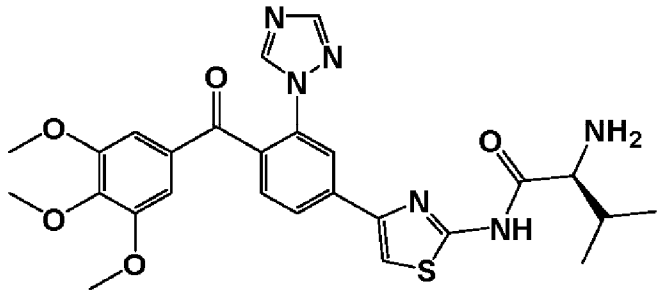
[19] The pharmaceutically acceptable carriers include, but are not limited to, lactose, dextrose, sucrose, sorbitol, mannitol, starch, gum acacia, calcium phosphate, alginate, gelatin, calcium silicate, microcrystalline cellulose, polyvinyl pyrrolidone, cellulose, water, syrup, methyl cellulose, methyl hydroxybenzoate, propyl hydroxybenzoate, talc, magnesium stearate and mineral oil. The pharmaceutical compositions may further contain, in addition to the above-described components, lubricants, wetting agents, sweetening agents, flavoring agents, emulsifying agents, suspending agents, preservatives, and the like. Suitable pharmaceutically acceptable carriers and formulations are described in detail in Remington's Pharmaceutical Sciences (19^{th} ed., 1995).

[20] The present disclosure provides a method for treatment of liver cancer, which comprises administering the pharmaceutical compositions of the present disclosure to a subject in need thereof. As used herein, the term "subject" is meant to include mammals, particularly humans.

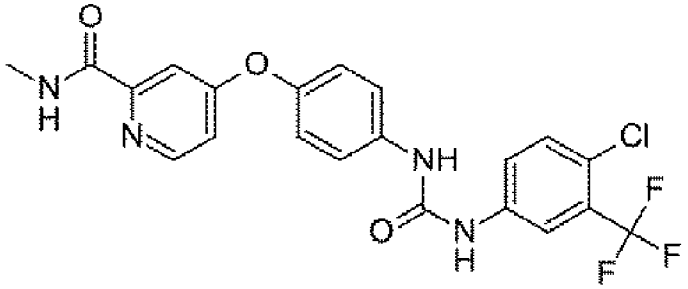
[21] According to one embodiment of the present disclosure, the present disclosure provides a combination of a vascular disrupting agent and sorafenib.

[22] Specifically, the present disclosure provides a combination for treatment of liver cancer, which comprises: (1) (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide represented by the following formula 1 or a pharmaceutically acceptable salt thereof; and (2) sorafenib represented by the following formula 2 or a pharmaceutically acceptable salt thereof.

[23] Formula 1

[24] 

[25] Formula 2

[26] 

[27] An antiangiogenic agent has the effect of inhibiting tumor angiogenesis, and thus has been widely used in anticancer strategies. Sorafenib developed by Bayer is known to have two properties: direct anticancer effect, and angiogenic inhibition. The anticancer effect of sorafenib is partially mediated by inhibition of B-RAF kinase, and appears to have the property of inhibiting angiogenesis by inhibiting VEGFR-2 and PDGFR-β. However, when an antiangiogenic agent such as sorafenib is administered alone, it inhibits only one aspect of angiogenesis, and thus the anticancer effect thereof is insufficient.

[28] However, in the combination of the present disclosure, it was shown that when the compound of formula 1 (vascular disrupting agent) and sorafenib (antiangiogenic agent), which are anticancer agents having different treatment mechanisms, were administered in combination, they exhibited a very excellent liver cancer treatment activity due to their synergistic effect. Furthermore, the combination of the present disclosure can solve the tumor recurrence problem occurring when the compound of formula 1 or sorafenib is administered alone, because there remain little or no viable tumor cells as a result of administration of the combination of the present disclosure.

[29] In the present disclosure, the pharmaceutically acceptable salt of sorafenib may be

any salt that is generally used in the pharmaceutical field, and sorafenib p-toluenesulfonate salt may be used.

[30] The combination of the present disclosure may comprise two separate formulations, and may also be composed of a single formulation.

[31] The combination of the present disclosure may be administered orally or parenterally (e.g., intravenously, subcutaneously, intraperitoneally or topically) according to the intended use. In the present disclosure, (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof may be administered orally or parenterally, preferably orally. Furthermore, sorafenib or a pharmaceutically acceptable salt thereof may be administered orally.

[32] The suitable doses of the active ingredients in the combination of the present disclosure may vary depending on the patient's body weight, age, sex, health conditions, diet, the time of administration, the mode of administration, excretion rate, the severity of the disease, and the like. In the present disclosure, the daily dose of (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide represented or a pharmaceutically acceptable salt thereof is about 1.0 to 40.0 mg, preferably about 2.5 to 25.0 mg. Furthermore, the daily dose of sorafenib or a pharmaceutically acceptable salt thereof is about 50 to 1500.0 mg, preferably about 100 to 1000 mg.

[33] In addition, the frequency of administration of the active ingredients in the combination of the present disclosure may be determined depending on the dose thereof. In the present disclosure, (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof may be administered at a frequency of once per day to once per week, and may also be administered 1 to 5 times per week depending on the route of administration. Furthermore, in the present disclosure, sorafenib or a pharmaceutically acceptable salt thereof may be administered at a frequency of twice per day to once per week, and may also be administered 1 to 7 times per week or 1 to 2 times per day.

[34] According to one embodiment of the present disclosure, when (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof in the combination of the present disclosure is administered parenterally, it may be administered once per week, and in this case, (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof may be administered once on day 1 of each week, and sorafenib or a pharmaceutically acceptable

salt thereof may be administered once per day during day 1 to day 6 of each week.

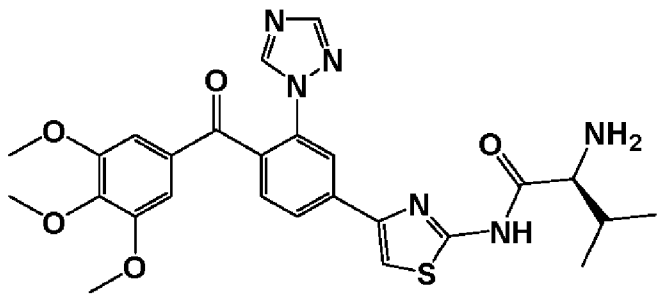
[35] According to another embodiment of the present disclosure, when (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof in the combination of the present disclosure is administered orally, it may be administered once per day on days 1, 3 and 5 of each week (three times per week), and sorafenib or a pharmaceutically acceptable salt thereof may be administered once per day (7 times per week). In addition, according to one embodiment of the present disclosure, sorafenib or a pharmaceutically acceptable salt thereof may be administered at 20 minutes to 1 hour after oral administration of (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof.

[36] The combination of the present disclosure has a very excellent liver cancer treatment activity, and when it is administered, there will remain little or no viable cancer cells, and thus the possibility of recurrence of cancer will be significantly low. Thus, the combination of the present disclosure may be effectively used for treatment of liver cancer.

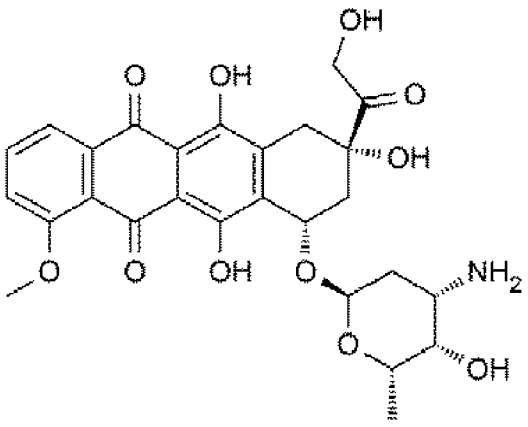
[37] According to still another embodiment of the present disclosure, the present disclosure provides a composition for chemoembolization, which comprises a vascular disrupting agent.

[38] Specifically, the present disclosure provides a composition for chemoembolization, which comprises: (1) (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide represented by the following formula 1 or a pharmaceutically acceptable salt thereof; (2) doxorubicin represented by the following formula 3 or a pharmaceutically acceptable salt thereof; and (3) lipiodol:

[39] Formula 1

[40] 

[41] Formula 3

[42] 

[43] Among image-guided therapies that are applied for treatment of liver cancer, two therapies are typically used. Of the two therapies, one is transarterial chemoembolization (TACE) that comprises injecting an anticancer agent topically into liver cancer through the hepatic artery and subjecting the hepatic artery to chemoembolization, and the other one is radiofrequency ablation (RFA).

[44] The transarterial chemoembolization (TACE) uses a strategy that maximizes a local anticancer effect against liver cancer by injecting an anticancer agent into the hepatic artery and obstructing the hepatic artery with an embolic agent. In many cases, the transarterial chemoembolization (TACE) is used as palliative therapy. Normal liver tissue receives 70% or more of blood via the portal vein, but liver cancer tissue receives 90% or more of blood through the hepatic artery. Thus, when an anticancer agent is injected through the hepatic artery in transarterial chemoembolization (TACE), the anticancer agent can be administered to liver cancer tissue at a relatively high concentration compared to normal liver tissue. Particularly, this direct injection through the hepatic artery can increase the amount of anticancer agent accumulated in liver cancer tissue by about 100-fold compared to systemic injection. Nevertheless, it is known that growth of new blood vessels around liver cancer tissue due to a response to hypoxia induced by chemoembolization is one of the major causes of recurrence.

[45] However, in the case of the composition for chemoembolization according to the present disclosure, when the compound of formula 1 (vascular disrupting agent) and doxorubicin are administered in combination topically to liver cancer cells, they will exhibit a very high liver cancer treatment activity due to their synergistic effect. In addition, there will remain little or no viable tumor cells as a result of administration of the composition of the present disclosure, and thus the composition of the present disclosure makes it possible to solve the tumor recurrence problem occurring when the compound of formula 1 or doxorubicin is administered alone.

[46] Lipiodol in the composition for chemoembolization according to the present disclosure performs various functions. Specifically, lipiodol functions to deliver the an-

ticancer agents in the composition for chemoembolization to liver cancer cells. Furthermore, lipiodol also functions as a contrast medium. In addition, lipiodol functions to embolize the hepatic artery, and thus can enhance the embolic effect of an embolic agent such as gelatin. Thus, lipiodol in the composition for chemoembolization according to the present disclosure functions to deliver the anticancer agents in the composition to liver cancer cells, functions as a contrast medium to make it possible to perform chemoembolization, and also exhibits an embolic effect to assist in treatment of liver cancer.

[47] The composition for chemoembolization according to the present disclosure may further contain an aqueous contrast medium. Doxorubicin is a hydrophilic substance that is difficult to dissolve in lipiodol. For this reason, the composition of the present disclosure preferably further contains an aqueous contrast medium.

[48] According to an embodiment of the present disclosure, (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof and doxorubicin or a pharmaceutically acceptable salt thereof in the composition of the present disclosure may be present at a weight of 5:1 to 1:5, preferably 2:1 to 1:2.

[49] According to another embodiment of the present disclosure, (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof in the composition of the present disclosure is preferably contained in an amount of 5.0 to 30.0 mg per mL of lipiodol.

[50] According to still another embodiment of the present disclosure, doxorubicin or a pharmaceutically acceptable salt thereof in the composition of the present disclosure is preferably contained in an amount of 5.0 to 50.0 mg per mL of the aqueous contrast medium.

[51] The composition for chemoembolization according to the present disclosure may be applied to various chemoembolization therapies. Thus, the composition for chemoembolization according to the present disclosure may be administered topically to an area to be embolized. Most preferably, it is administered topically to the liver via transarterial chemoembolization (TACE).

[52] Moreover, the composition for chemoembolization according to the present disclosure has a very excellent liver cancer treatment activity, and when it is administered, there will little or no viable cancer cells, and thus the possibility of recurrence of cancer will be significantly low. Accordingly, the composition for chemoembolization according to the present disclosure can be effectively used for treatment of liver cancer.

[53] After the composition for chemoembolization according to the present disclosure is

injected, an embolic agent may be injected to embolize a desired area. The embolic agent is preferably a gelatin-like substance.

[54] In the composition for chemoembolization according to the present disclosure, the preferred doses of (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof and doxorubicin or a pharmaceutically acceptable salt thereof may vary depending on various factors, including the patient's body weight, age, sex, health conditions, diet, the time of administration, the mode of administration, excretion rate, the severity of the disease, and the like. The daily dose of (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)thiazol-2-yl)-2-amino-3-methylbutanamide or a pharmaceutically acceptable salt thereof in the composition for chemoembolization according to the present disclosure is about 1.0 to 40.0 mg/kg, preferably 2.5 to 25.0 mg/kg. In addition, the daily dose of doxorubicin or a pharmaceutically acceptable salt thereof in the composition for chemoembolization according to the present disclosure is about 10.0 to 1000.0 mg/kg, preferably 20.0 to 800.0 mg/kg.

Advantageous Effects of Invention

[55] The compositions of the present disclosure exhibit excellent liver cancer treatment activity, and act such that the possibility of recurrence of cancer is low. Thus, the compositions of the present disclosure may be used for treatment or adjuvant treatment of liver cancer.

Brief Description of Drawings

[56] FIG. 1 shows the results of bioluminescence imaging (BLI) of a control group and each test groups (administered parenterally) in Hep3B xenograft mouse models.

[57] FIG. 2 is a graph comparing the relative BLI signal intensity between a control group and each test groups (administered parenterally) in Hep3B xenograft mouse models.

[58] FIG. 3 shows H&E staining and TUNEL staining images of a control group and each test groups (administered parenterally).

[59] FIG. 4 is a graph comparing apoptosis rate between a control group and each test groups (administered parenterally).

[60] FIG. 5 shows CD31 staining images of a control group and each test group (administered parenterally).

[61] FIG. 6 is a graph comparing microvessel density (MVD) between a control group and each test groups (administered parenterally).

[62] FIG. 7 shows the results of bioluminescence imaging (BLI) of a control group and each test groups (administered orally) in Hep3B xenograft mouse models.

[63] FIG. 8 is a graph comparing the relative BLI signal intensity between a control group and each test groups (administered orally) in Hep3B xenograft mouse models.

[64] FIG. 9 is a graph comparing apoptosis rate between a control group and each test groups (administered orally).

[65] FIG. 10 shows CD31 staining images of a control group and each test groups (administered orally).

[66] FIG. 11 is a graph comparing the microvessel area (MVA) between a CD31-stained control group and each CD31-stained test groups (administered orally).

[67] FIG. 12 shows images of a meca-32-staiend control group and each meca-32-stained test groups (administered orally).

[68] FIG. 13 is a graph comparing the microvessel area (MVA) between a meca-32-stained control group and each meca-32-stained test groups (administered orally).

[69] FIG. 14 is a graph comparing the viable tumor portion between test groups after injecting test substances into rabbit VX2 liver cancer models via transarterial chemoembolization (TACE) (A: group administered with lipiodol; B: group administered with lipiodol + VDA; C: group administered with lipiodol + doxorubicin; D: group administered with lipiodol + VDA + doxorubicin).

Mode for the Invention

[70] Hereinafter, the present disclosure will be described in further detail with reference to examples. It is to be understood, however, that these examples are for illustrative purposes only and are not intended to limit the scope of the present disclosure.

[71] Example 1: Evaluation of Synergistic Effect of Parenterally Administered Compound of Formula 1 (VDA) and Sorafenib

[72] 1. Test Method

[73] Preparation of Cell Line and Infection of Hep3B Cells

[74] Human hepatocellular carcinoma Hep3B cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Hep3B cells were infected with lentivirus containing firefly luciferase reporter gene, and a clone strongly expressing the reporter was isolated in order to establish a Hep3B+luc cell line.

[75] Stable expression of luciferase was confirmed by bioluminescence imaging (BLI) (PerkinElmer, Waltham, MA, USA). The Hep3B+luc cell line was maintained with Dulbecco's modified Eagle medium (DMEM, WelgeneInc, Seoul, Korea) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% antibiotics (Gibco, USA).

[76] Preparation of Animal Tumor Models

[77] Male Balb/c nude mice (6-week-old) were purchased from Orientbio (Seoul, Korea).

[78] 1×10^6 Hep3B+luc cells were inoculated into the right flank of each animal through

a 27-gauge needle syringe. When the tumor diameter exceeded 5 mm on about 14 days after inoculation, the animals were used in the test.

[79] Preparation of Active Ingredient

[80] The compound of formula 1 (VDA) was prepared by the preparation method disclosed in International Patent Publication No. WO 2009-119980, and was dissolved in 0.9% saline.

[81] Sorafenib p-toluenesulfonate salt (LC laboratories, Woburn, MA, USA) was dissolved in CremophorEL/ethanol (50:50; Sigma CremophorEL, 95% ethyl alcohol) at 4-fold, and then diluted with water on the day of use.

[82] *In* Vivo Bioluminescence Imaging

[83] Bioluminescence imaging (BLI) was performed before drug administration (day 0) and day 2, day 6, day 10 and day 14 after drug administration. Mice were anesthetized, and then imaged with an IVIS spectrum system (PerkinElmer, Waltham, MA, USA). D-luciferin (0.375 mg/ml; Promega, Madison, WI, USA) in phosphate buffered saline (PBS) was injected intraperitoneally into the mice. Bioluminescence was recorded as sum of measured photons per sec in the region of interest (ROI). For measurement of *in* vivo bioluminescence, ROIs having the same size and shape, which includes the tumor of each animal, were used. Quantification of BLI data was performed using Living Image software (version 2.50.1, Perkin Elmer, Waltham, MA, USA).

[84] Evaluation of Liver Cancer Treatment Activity

[85] The anticancer effects of the compound of formula 1 and sorafenib were evaluated in Hep3B xenograft models. Animals were randomly divided into the following groups:

[86] - Control group: vehicle (saline, administered intraperitoneally);

[87] - Group administered with the compound of formula 1 (VDA) alone: the compound of formula 1 (5 mg/kg, administered intraperitoneally once per week (on day 1));

[88] - Group administered with sorafenib alone: sorafenib (30 mg/kg, administered orally 6 times per week (once per day for day 1 to day 6));

[89] - Group administered with a combination: the compound of formula 1 (5 mg/kg, administered intraperitoneally once per week (on day 1)), and sorafenib (30 mg/kg, administered orally 6 times per week (once per day for day 1 to day 6 of each week)).

[90] All the test groups were administered with each drug for 2 weeks. Toxicity was observed by measuring the body weight of the mice, and tumor growth was measured using calipers during the test period. The tumor volume was calculated using the following equation:

[91] Tumor volume = (length × width^2) / 2

[92] Histopathological Studies

[93] At the end of the test (day 14), all the mice were sacrificed for tissue processing, immunohistochemical staining and histological analysis.

[94] In tissue processing, the test animals were anesthetized deeply, transcardially perfused with saline by a ventricular catheter, and then treated with 10% paraformaldehyde (PFA) by gravity flow for 10 minutes. The tumor tissue was collected, fixed in formalin, and embedded in paraffin. The largest portion of the tumor was sectioned to a thickness of 4 μm.

[95] In order to evaluate the degree of apoptosis of tumor cells, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using a kit (Millipore, Billerica, MA, USA). Hematoxylin & eosin (H&E) staining was performed for the representative region of the collected tumor tissue. Percent apoptosis was calculated using software (NIH-Image J, Bethesda, MD, USA).

[96] To evaluate the change of tumor blood vessels, immunohistochemical staining of CD31 was performed using primary rabbit antibody (Abcam, Cambridge, MA) against mouse CD31. Visualization was performed using Dako EnVision + System HRP labeled polymer anti-rabbit (Dako) for 30 minutes. Images were observed with an optical microscope (model DM2500, Leica, Germany).

[97] Measurement of immunostained areas was based on the Weidner method. Using a Leica microscope (model DM2500, leica, Germany), three areas were observed at low magnifications (40x and 100x). The number of microvessels in each area was measured in a 200x field (20x objective lens, 10x ocular lens). Image analysis for the number of blood vessels was performed using software (Image analysis, Leica, Germany). In this software, one cell in stained lumen was calculated as one point. The average number of microvessels in all the measured areas was determined as microvessel density (MVD).

[98] Statistical analysis

[99] Quantitative data were expressed as mean ± standard deviation. The statistical significance of difference in tumor volume, bioluminescence imaging, percent apoptosis and microvessel density between the groups was analyzed using Mann-Whitney's U-test. $p<0.05$ was considered significant. All data were calculated using commercial software (SPSS, version 21.0 for Windows, SPSS, Chicago, IL, USA).

[100] 2. Test Results

[101] Liver Cancer Treatment Activity of the Compound of Formula 1 in Hep3B Xenograft Models

[102] To evaluate the antitumor effect of administration of the compound of formula 1 (VDA) alone, xenograft mice were administered with the compound of formula 1 (VDA), and the body weight of the mice and tumor growth in the mice were observed during the test period. Mice that become weakened during the test period were not observed. The difference in body weight of the group administered with the compound of formula 1 (VDA) alone, the group administered with sorafenib alone, and the group

administered with the combination of VDA and sorafenib, between before and day 14 after the start of administration, was not significant ($p > 0.05$). The tumor growth inhibitory effect of the group administered with the combination of VDA and sorafenib was similar to that of the group administered with sorafenib alone. This suggests that the tumor growth inhibitory effect of administration of the compound of formula 1 (VDA) alone is not significant.

[103] BLI Signal Observation

[104] During the test period, the relative BLI signal of the control group continued to increase. This suggests that Hep3B-luc tumors actively grew. In the group administered with sorafenib alone, the BLI signal decreased gradually for 6 days and was significantly restored after day 6. In the group administered with the compound of formula (VDA) alone, the relative BLI signal decreased after administration of the compound of formula 1 (day 1 and day 8), and this decreased signal was continued for 2 days and significantly restored after 2 days. This suggests that the compound of formula 1 exhibits a rapid shut down effect and a cytotoxic effect in the initial stage after administration. However, in the group administered with a combination of VDA and sorafenib, the relative BLI signal significantly decreased in the initial stage, and the combination showed a tumor inhibitory effect for 10 days after the start of administration (see FIG. 1).

[105] Furthermore, the relative BLI signal intensities are consistent with the BLI signal results (see FIG. 2). Such results suggest that the compound of formula 1 and sorafenib have a synergistic effect. Specifically, it appears that the compound of formula 1 induces a rapid treatment effect and sorafenib maintains the tumor inhibitory effect.

[106] Correlation between BLI Parameter and Histological Results

[107] To verify the BLI signal results, a series of histopathological studies were conducted. On day 14 after the start of administration, percent apoptosis of tumor cells was quantified using a TUNEL assay. As a result, percent apoptosis was higher in the order of the group administered with the combination (43.0±14.0), the group administered with the compound of formula 1 alone (38.0±13.7), the group administered with sorafenib alone (18.3±6.0), and the control group (11.1±3.7). However, the apoptotic results of the group administered with the compound of formula 1 alone did not significantly differ from those of the group administered with the combination. This suggests that the effect of sorafenib against tumor cell growth is cytostatic rather than cytotoxic (see FIG. 4). Furthermore, as can be seen in microscopic images of H&E staining and TUNEL staining, in the group administered with sorafenib alone and the control group, tumor cells appeared like scattered points, whereas, in the group administered with the compound of formula 1, tumor cells appeared predominantly in apoptotic tumor cells. Furthermore, apoptosis in the group administered with the com-

bination was similar to that in the group administered with the compound of formula 1 (see FIG. 3). Such results indicate that the compound of formula 1 exhibits extensive apoptosis due to its tumor destruction effect and exhibits a tumor growth inhibitory effect due to the tumor growth inhibitory property of sorafenib.

[108] In addition, anti-CD31-stained histological samples and quantification of tumor microvessel density (MVD) were obtained on day 14 after single administration of each of the drugs. In the control group, tumor blood vessels were large and distributed throughout the tumor. However, in the test groups, blood vessel images different from that observed in the control group appeared. Specifically, in the test groups, blood vessels had a short length and were distributed at the margin of the tumor. Comparison between the test groups was based on the density of blood vessels (see FIG. 5). Quantification of the tumor MVD indicated that the microvessel density (MVD) was higher in the order of the normal saline group (3180±465), the group administered with the compound of formula 1 (2334±60), the group administered with sorafenib (1871±225), and the group administered with the combination (1122±212) (see FIG. 6). Statistical significance was observed in all the groups. Such results also indicate that the vascular disruption property of the compound of compound 1 and the anti-angiogenesis property of sorafenib exhibit a synergistic effect.

[109] Example 2: Evaluation of the Synergistic Effect of Orally Administered Compound of Formula 1 (VDA) and Sorafenib

[110] 1. Test Method

[111] Preparation of cell lines, animal tumor models and active ingredients was performed in the same manner as described in Example 1.

[112] *In Vivo* Bioluminescence Imaging

[113] Bioluminescence imaging (BLI) was performed before drug administration (day 0) and day 2, day 4, day 8, day 11, day 15, day 18 and day 20 after drug administration. Mice were anesthetized, and then imaged with an IVIS spectrum system (PerkinElmer, Waltham, MA, USA). D-luciferin (0.375 mg/ml; Promega, Madison, WI, USA) in phosphate buffered saline (PBS) was injected intraperitoneally into the mice. Bioluminescence was recorded as sum of measured photons per sec in the region of interest (ROI). For measurement of *in* vivo bioluminescence, ROIs having the same size and shape, which includes the tumor of each animal, were used. Quantification of BLI data was performed using Living Image software (version 2.50.1, Perkin Elmer, Waltham, MA, USA).

[114] Evaluation of Liver Cancer Treatment Activity

[115] The anticancer effects of the compound of formula 1 and sorafenib were evaluated in Hep3B xenograft models. Animals were randomly divided into the following groups:

[116] - Control group: vehicle (saline, administered orally);

[117] - Group administered with the compound of formula 1 (VDA) alone: the compound of formula 1 (4 mg/kg, administered orally three times week (on day 1, day 3 and day 5));

[118] - Group administered with sorafenib alone: sorafenib (15 mg/kg, administered orally 7 times per week);

[119] - Ggroup administered with a combination: the compound of formula 1 (4 mg/kg, administered orally three times per week (on day 1, day 3 and day 5)), and sorafenib (15 mg/kg, administered orally 7 times per week (once per day)).

[120] Oral administration was performed at 2 pm each day, and in the group administered with the combination, sorafenib was administered at 30 minutes after administration of the compound of formula 1.

[121] All the test groups were administered with each drug for 3 weeks. Toxicity was observed by measuring the body weight of the mice, and tumor growth was measured using calipers during the test period. The tumor volume was calculated using the following equation:

[122] Tumor volume = (length × width^2) / 2

[123] Histopathological Studies

[124] At the end of the test (day 21), all the mice were sacrificed for tissue processing, immunohistochemical staining and histological analysis.

[125] Treatment of extracted tumor tissue was performed according to the method described in Example 1.

[126] In order to evaluate the degree of apoptosis of tumor cells, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using a kit (Millipore, Billerica, MA, USA). Hematoxylin & eosin (H&E) staining was performed for the representative region of the collected tumor tissue. Percent apoptosis was calculated using software (NIH-Image J, Bethesda, MD, USA).

[127] To evaluate the change of tumor blood vessels, immunohistochemical staining was performed using primary rabbit antibody (Abcam, Cambridge, MA) against mouse CD31 and primary rat antibody (Novus Biologicals, Littleton, Colorado, USA) against meca-32. Visualization of CD31 was performed using Dako EnVision + System HRP labeled polymer anti-rabbit (Dako) for 30 minutes. Images were observed with an optical microscope (model DM2500, Leica, Germany). For visualization of meca-32, Alexa Fluor 488 anti-rat antibody was used.

[128] Microvessel areas (MVAs) were determined by immunochemical staining of CD31 and meca-32. Using a Leica microscope (model DM2500, leica, Germany), three areas were observed at low magnifications (40x and 100x). The number of microvessels in each area was measured in a 200x field (20x objective lens, 10x ocular lens). Image analysis for the number of blood vessels was performed using software (Image

analysis, Leica, Germany).

[129] Statistical analysis

[130] Quantitative data were expressed as mean ± standard deviation. The tumor volume and bioluminescence imaging of each group was evaluated using RMANOVA test, and the statistical significance of difference in percent apoptosis and microvessel area between the groups was analyzed using one-way ANOVA test. $p<0.05$ was considered significant. All data were calculated using commercial software (SPSS, version 21.0 for Windows, SPSS, Chicago, IL, USA).

[131] 2. Test Results

[132] Observation of BLI Signal

[133] During the test period, the relative BLI signal of the control group increased up to day 11, and then was maintained at that level. In the latter half of the administration period, it was observed that the signal intensity of the center of the cancer decreased. This is believed to be natural necrosis that occurred as the cancer grew (see FIG. 7).

[134] In the group administered with the compound of formula 1, it was observed that the signal intensity decreased rapidly up to day 2, and then increased. In the group administered with sorafenib, it was observed that the signal intensity decreased gradually throughout the test period. In the group administered with the combination, the signal strength decreased rapidly, and the decreased signal was maintained throughout the administration period (see FIG. 8).

[135] Such results indicate that the compound of formula 1 induces a rapid treatment effect and maintains the tumor inhibitory effect of sorafenib, like the results of Example 1. In addition, it can be seen that, even when the compound of formula 1 is administered orally, it exhibits excellent effects, like when it is administered by injection.

[136] Correlation between BLI Parameter and Histological Results

[137] To verify the BLI signal results, a series of histopathological studies were conducted. On day 21 after the start of administration, percent apoptosis of tumor cells was quantified using a TUNEL assay. As a result, percent apoptosis was higher in the order of the group administered with the combination (34.18±5.99), the group administered with the compound of formula 1 alone (16.24 ± 6.16), the group administered with sorafenib alone (10.36 ± 3.62), and the control group (3.74 ± 2.08) (see FIG. 9).

[138] In addition, anti-CD31-stained histological samples (FIG. 10) and quantification of the tumor MVA therein (FIG. 11), and meca-32-stained histological samples (FIG. 12) and quantification of the tumor MVA therein (FIG. 13) were obtained on day 21 after the start of each drug. MVA analysis performed using CD31 indicated that the control group, the group administered with the compound of formula 1 alone, and the group administered with sorafenib alone, showed tumor MOVs of 16339 ± 3614, 14452 ± 2709, and 14934 ± 3154, respectively, whereas the group administered with the com-

bination showed a tumor MVA of 7161 ± 4522, which was significantly lower than those of the other groups (see FIG. 11).

[139] MVA analysis performed using meca-32 indicated that the tumor MVD was higher in the order of the control group (17183 ± 5508), the group administered with sorafenib alone (14897 ± 3556), the group administered with the compound of formula 1 alone (12871 ± 3433), and the group administered with the combination (7631 ± 2872) (see FIG. 13).

[140] Such results also indicate that the vascular disruption property of the compound of compound 1 and the anti-angiogenesis property of sorafenib exhibit a synergistic effect.

[141] In addition, a difference in the morphology of blood vessels between the groups was observed. Specifically, in the group administered with the compound of formula 1, the number of blood vessels decreased, and the vascular lumens were narrowed (see FIGS. 10 and 12). This is good example showing the vascular disruption activity of the compound of formula 1. Furthermore, it is believed that administration of the compound of formula 1 in combination with sorafenib having anti-angiogenesis activity exhibits a synergistic effect by changing the morphology and number of blood vessels in liver cancer.

[142] Example 3: Evaluation of the Liver Cancer Treatment Effect and Hepatotoxicity of Composition for Chemoembolization According to the Present disclosure

[143] 1. Test Method

[144] Rabbit VX2 carcinoma liver tumor models established as representative animal models of liver cancer were used. The abdomens of male New Zealand White rabbits (weighing 3 kg) were incised, and the left lobe of the liver was exposed. Then, a VX2 carcinoma tumor chip prepared to have a size of 1 mm^3 was implanted into the surface of the liver by a syringe needle. At 2 to 3 weeks after tumor implantation, non-contrast computed tomography (CT) and perfusion MR imaging were performed to determine whether or not a tumor would be produced.

[145] Establishment of Test Groups

[146] Animals were randomly divided into the following groups A to D, each consisting of 10 animals:

[147] - Test group A: a group administered with lipiodol (0.2 mL of lipiodol + 0.05 mL of contrast medium [GE Healthcare Visipaque 270]);

[148] - Test group B: a group administered with lipiodol and the compound of formula 1 (VDA) (0.2 mL of lipiodol + 1.26 mg of the compound of formula 1 (VDA) + 0.05 mL of contrast medium [GE Healthcare Visipaque 270]);

[149] - Test group C: a group administered with lipiodol and doxorubicin (0.2 mL of lipiodol + 0.05 mL of contrast media [GE Healthcare Visipaque 270] and 1 mg of dox-

orubicin);

[150] - Test group D: a group administered with lipiodol, the compound of formula 1 (VDA) and doxorubicin (0.2 mL of lipiodol + 1.26 mg of the compound of formula 1 (VDA) + 0.05 mL of contrast medium [GE healthcare Visipaque 270] + 1 mg of doxorubicin).

[151] Administration of Drugs

[152] A microcatheter was passed through the rabbit auricular artery and celiac trunk and advanced into the left hepatic artery, and then drugs corresponding to test groups A to D were carefully injected into the left hepatic artery. Before and after TACE, non-enhanced CT was performed to determine the presence or absence of tumors, and after TACE, lipiodol deposited in tumors was evaluated.

[153] 2. Test Results

[154] Evaluation of Liver Cancer Treatment Effect

[155] At 1 week after administration, liver tissue was extracted to make pathological slides. H&E staining and TUNEL staining were performed to determine necrotic tumor areas. The necrotic tumor area was calculated using the Image J program, and the viable tumor portion was expressed as percentages. The results are shown in Table 1 below and FIG. 14.

[156] Table 1

[157]

Test Group	Viable tumor portion (%)
A	47.06 ± 11.38
B	27.48 ± 13.64
C	14.36 ± 12.51
D	0.66 ± 1.03

[158] (mean ± standard deviation)

[159] As can be seen in Table 1 above, in the case of test group A administered with lipiodol alone having no anticancer activity, the viable tumor portion was 47.06%, and in the case of test group B administered with lipiodol in combination with the compound of formula 1 (VDA), the viable tumor portion was 27.48%, and in the case of group C administered with lipiodol in combination with doxorubicin, the viable tumor portion was only 14.36%. Such results indicate that the possibility of recurrence of cancer in test groups A to C is high. Meanwhile, in the case of test group D administered with a combination of lipiodol, the compound of formula 1 (VDA) and doxorubicin, the viable tumor portion was 0.66%, indicating that most tumors were removed. Thus, it can be seen that the possibility of recurrence of cancer in test group D is significantly lower than those in test groups A to C.

[160] In addition, the results for test groups A and C had a standard deviation of 10% or more, indicating that the reproducibility of the tumor treatment effect of the drugs was poor. However, the results for test group D had a standard deviation of only about 1%, indicating that the drugs administered to test group D reproducibly treated liver cancer.

[161] Evaluation of Toxicity

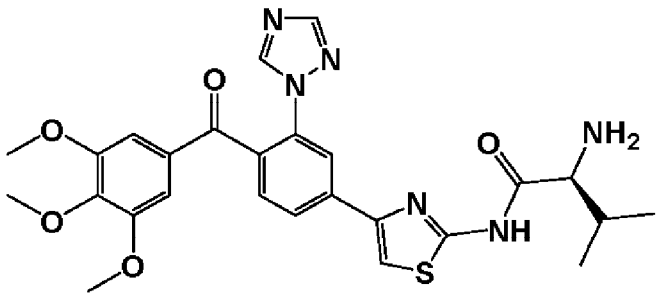
[162] Before drug injection and on days 1, 3 and 7 after drug injection, blood was sampled, and AST and ALT levels therein were measured to evaluate the degree of acute liver injury.

[163] As a result, it was shown that the degree of acute liver injury in test group D was the highest, but was restored to levels similar to those in the other test groups after 7 days. In addition, no rabbit death was observed in test group, indicating that liver injury in test group D was acceptable.

Claims

[Claim 1] A pharmaceutical composition for treatment or adjuvant treatment of liver cancer, comprising (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide represented by the following formula 1 or a pharmaceutically acceptable salt thereof:

Formula 1



[Claim 2] The pharmaceutical composition of claim 1, wherein the pharmaceutically acceptable salt of (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide is a hydrochloride salt.

[Claim 3] The pharmaceutical composition of claim 1, wherein the liver cancer is primary liver cancer.

[Claim 4] The pharmaceutical composition of claim 1, wherein the liver cancer is advanced liver cancer.

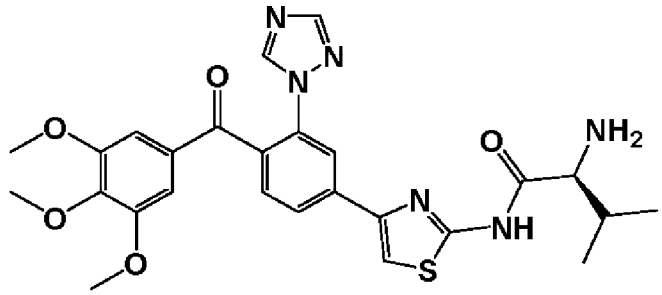
[Claim 5] The pharmaceutical composition of claim 1, wherein the liver cancer is hepatocellular carcinoma (HCC).

[Claim 6] A combination for treatment of liver cancer, comprising:

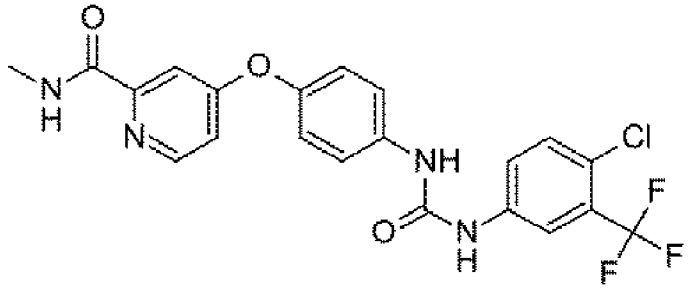
(1) (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide represented by the following formula 1 or a pharmaceutically acceptable salt thereof; and

(2) sorafenib represented by the following formula 2 or a pharmaceutically acceptable salt thereof:

Formula 1



Formula 2



[Claim 7] The combination of claim 6, wherein the pharmaceutically acceptable salt of sorafenib is a p-toluenesulfonate salt.

[Claim 8] The combination of claim 6, wherein the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt thereof is administered parenterally or orally.

[Claim 9] The combination of claim 6, wherein the sorafenib or the pharmaceutically acceptable salt thereof is administered orally.

[Claim 10] The combination of claim 6, wherein the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt is administered 1 to 5 times per week.

[Claim 11] The combination of claim 6, wherein the sorafenib or the pharmaceutically acceptable salt thereof is administered 1 to 7 times per week.

[Claim 12] The combination of claim 11, wherein the sorafenib or the pharmaceutically acceptable salt thereof is administered once or twice per day.

[Claim 13] The combination of claim 6, wherein the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt is administered 1 to 5 times per week, and the sorafenib or the pharmaceutically acceptable salt thereof is administered 1 to 7 times per week.

[Claim 14] The combination of claim 6, wherein the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl)

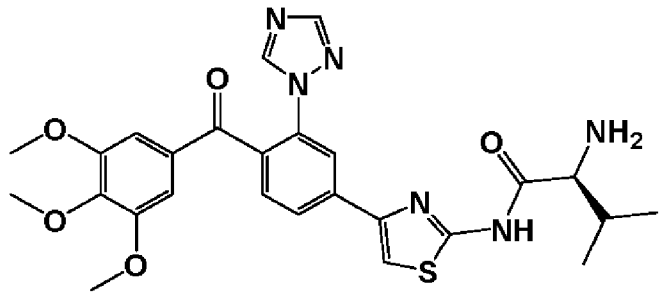
thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt thereof is administered parenterally once per week on day 1 of each week, and the sorafenib or the pharmaceutically acceptable salt thereof is administered once per day during day 1 to day 6 of each week.

[Claim 15] The combination of claim 6, wherein the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt thereof is administered orally once per day on days 1, 3 and 5 of each week (three times per week), and the sorafenib or the pharmaceutically acceptable salt thereof is administered once per day (7 times per week).

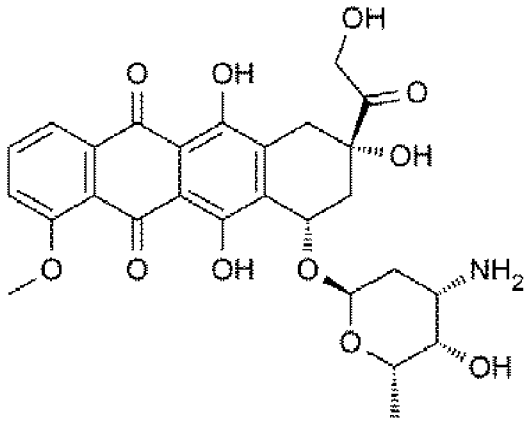
[Claim 16] The combination of claim 9, wherein the sorafenib or the pharmaceutically acceptable salt thereof is administered at 20 minutes to 1 hour after oral administration of the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt thereof.

[Claim 17] A composition for chemoembolization, comprising:
(1) (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide represented by the following formula 1 or a pharmaceutically acceptable salt thereof;
(2) doxorubicin represented by the following formula 3 or a pharmaceutically acceptable salt thereof; and
(3) lipiodol:

Formula 1



Formula 3



[Claim 18] The composition of claim 17, further comprising an aqueous contrast medium.

[Claim 19] The composition of claim 17, wherein the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt thereof and the doxorubicin or the pharmaceutically acceptable salt thereof are present at a weight ratio of 2:1 to 1:2.

[Claim 20] The composition of claim 17, wherein the (S)-N-(4-(3-(1H-1,2,4-triazol-1-yl)-4-(3,4,5-trimethoxybenzoyl)phenyl) thiazol-2-yl)-2-amino-3-methylbutanamide or the pharmaceutically acceptable salt thereof is contained in an amount of 5.0 to 30.0 mg per mL of the lipiodol.

[Claim 21] The composition of claim 17, wherein the doxorubicin or the pharmaceutically acceptable salt thereof is contained in an amount of 5.0 to 50.0 mg per mL of aqueous contrast medium.

[Claim 22] The composition of claim 17, wherein the composition is used for transarterial chemoembolization (TACE).