(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 2 027 283 B1

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
28.09.2011 Bulletin 2011/39

(21) Application number: **07701966.9**

(22) Date of filing: **08.01.2007**

(51) Int Cl.:
G01N 33/50 (2006.01) ***G01N 31/00*** (2006.01)
G01N 33/48 (2006.01) ***G01N 33/53*** (2006.01)
C12Q 1/00 (2006.01)

(86) International application number:
PCT/CN2007/000034

(87) International publication number:
WO 2008/086645 (24.07.2008 Gazette 2008/30)

(54) METHOD FOR SCREENING AGENT WITH ANGIOGENIC-MODULATING ACTIVITIES USING TELEOST EMBRYO

VERFAHREN ZUM SCREENING EINES AGENS MIT ANGIOGEN-MODULIERENDEN AKTIVITÄTEN UNTER VERWENDUNG EINES TELEOST-EMBRYOS

PROCÉDÉ DE CRIBLAGE D’UN AGENT AYANT DES ACTIVITÉS QUI MODULENT L’ ANGIOGENESIS EN UTILISANT UN EMBRYON TÉLÉOSTÉEN

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU LV MC NL PL PT RO SE SI SK TR

(43) Date of publication of application:
25.02.2009 Bulletin 2009/09

(73) Proprietor: **City University of Hong Kong**
Kowloon, Hong Kong (CN)

(72) Inventors:
- **CHENG, Shuk Han**
  Hong Kong (CN)
- **CHAN, Pokwok**
  Hong Kong (CN)

(74) Representative: **Comoglio, Elena et al**
Jacobacci & Partners S.p.A.
Corso Emilia 8
10152 Torino (IT)

(56) References cited:
WO-A1-99/42606 WO-A1-99/42606
CN-A- 1 866 025

- **PARNG CHUENLEI ET AL: "Zebrafish: A preclinical model for drug screening." ASSAY AND DRUG DEVELOPMENT TECHNOLOGIES, vol. 1, no. 1, November 2002 (2002-11), pages 41-48, XP002558882 ISSN: 1540-658X**
- **KIDD KAMEHA R ET AL: "Fishing for novel angiogenic therapies." BRITISH JOURNAL OF PHARMACOLOGY, vol. 140, no. 4, October 2003 (2003-10), pages 585-594, XP002558883 ISSN: 0007-1188**
- **LAWSON NATHAN D ET AL: "In vivo imaging of embryonic vascular development using transgenic zebrafish" DEVELOPMENTAL BIOLOGY, vol. 248, no. 2, 15 August 2002 (2002-08-15), pages 307-318, XP002558884 ISSN: 0012-1606**
- **WHITE R E: "High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery." ANNUAL REVIEW OF PHARMACOLOGY AND TOXICOLOGY 2000, vol. 40, 2000, pages 133-157, XP002558885 ISSN: 0362-1642**
- **Anonymous: "High-throughput screening" Wikipedia website Internet article 4 December 2006 (2006-12-04), XP002558886 Retrieved from the Internet: URL:http://en.wikipedia.org/w/index.php?ti tle=High-throughput_ screening&oldid=920919 08> [retrieved on 2009-12-04]**
- **ZHANG L. ET AL.: ’The effect of exogenous retinoic acid on the cardiovascular development of zebrafish embryos’ ACTA LABORATORIUM ANIMALIS SCIENTIA SINICA vol. 14, no. 2, June 2006, pages 84 - 88, XP008099997**

Note: Within nine months of the publication of the mention of the grant of the European patent in the European Patent Bulletin, any person may give notice to the European Patent Office of opposition to that patent, in accordance with the Implementing Regulations. Notice of opposition shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

Printed by Jouve, 75001 PARIS (FR)

(Cont. next page)

- **WEINSTEIN B.M.: 'Plumbing the mysteries of vascular development using the zebrafish' SEMINARS IN CELL & DEVELOPMENTAL BIOLOGY vol. 13, 2002, pages 515 - 522, XP008103211**

Description

Background-field of the Invention

[0001] Angiogenesis is a physiological process of formation of new blood vessels from existing blood vessels (Risau, 1997). This process is only normally occurred in growth and development, and wound healing by restoring blood flow to tissues. However, loss control in angiogenesis is a common factor in most important diseases such as cancer and rheumatoid arthritis (Liekens et al., 2001). These diseases may be resulted from or get progression when new blood vessels either grow excessively or insufficiently. Excessive angiogenesis occurs, for example, in cancer by abnormal production of excessive amount of angiogenic growth factors in order to form new blood vessels for excessive demand of nutrient. On the other hands, insufficient angiogenesis occurs, for example, in coronary artery disease, by causing tissue damage and death due to inadequate production of angiogenic growth factor for sufficient growth of blood vessels. Therefore, therapy aims at treating the loss of the control in angiogenesis is actively pursued by pharmaceutical companies all over the world.

Background Knowledge and Description of Prior Art

[0002] Even though there is a huge potential market for angiogenesis-related therapeutics, it is difficult to find a suitable assay to assess the effect of angiogenesis response (Staton et al., 2004). The most widely used assays are *in vitro* assays in which new drugs are tested in endothelial cells cultured in plastic flasks. The drawback is that it is difficult to extrapolate the observation made in an *in vitro* assay to model the complex process of angiogenesis actually occurred in *in vivo* situations (Staton et al., 2004;Taraboletti and Giavazzi, 2004). On the other hands, *in vivo* assays using animal models, for example mouse, are costly, time-consuming and difficult for quantification (Staton et al., 2004). Thus, commonly multiple *in vitro* assays are employed for the identification of lead compounds the efficacy of lead compounds on angiogenesis will subsequently be validated by more than one *in vivo* assays. However, quantification is still an important key parameter for angiogenesis assays. Criteria for an ideal quantitative angiogenesis assay (Hasan et al., 2004) are that the assay should:

1) provide quantitative measure of structure of newly formed blood vessels;
2) provide quantitative measure of functional characters of newly formed blood vessels;
3) be able to distinct the newly formed and pre-existing blood vessels;
4) allow long-term study;
5) be cost-effective, rapid, easy to use, reproducible; and
6) not cause any tissue damage;

[0003] Up to now, none of the existing *in vivo* assays really meets these requirements (Hasan et al., 2004; Taraboletti and Giavazzi, 2004). It appears that an *in vivo* drug screening assay using zebrafish as model fulfill the criteria of an ideal quantitative angiogenesis assay. Zebrafish *in vivo* assay has the advantages and convenience of *in vitro* assays (high throughput comparing to *in vitro* assays) and *in vivo* assays (being an intact organism), and thus capable to serve as bridge or filter between *in vitro* screening and subsequent *in vivo* validation (Epstein and Epstein, 2005;Goldsmith, 2004;Parng et al., 2002). Zebrafish *in vivo* assay would eliminate those lead compounds, due to the toxic effects, from the list identified from the *in vitro* assays before entering a more expensive phase of *in vivo* validation. Zebrafish *in vivo* assay has been demonstrated as a useful method to screening angiogenic drugs (reviewed by (Kidd and Weinstein, 2003)). For example, Serbedzija and his coworkers described a screening methodology which was based on the counting number of subintestinal blood vessels formed on the surface of the yolk on both side of the embryo (Serbedzija et al., 1999). Blood vessels were stained for endogenous alkaline phosphatase activity. Transgenic zebrafish lines with fluorescent blood vessels have been developed (Cross et al., 2003;Lawson and Weinstein, 2002) such that the imaging and analysis of angiogenesis can be greatly simplified. However, there is still no methodology describing fully the procedures and tools how zebrafish may be used as a quantitative tool for angiogenic drug discovery together with the corresponding toxicity test.

[0004] WO 99/42606 discloses biological screening assay systems wherein teleosts are used for screening for angiogenic modulating activity and for toxic activity of an agent in parallel assay systems.

Summary of the Invention

[0005] This invention relates to a biological method of screening as defined in the appended claims. The present 3-tier screening system fulfills the criteria for an ideal quantitative angiogenesis assay listed above and provides an effective screening assay system to identify angiogenesis modulating agent with least toxicity. The preferred model of the present invention is zebrafish or medaka embryos. The advantage of the present invention is that it can rapidly identify the potential angiogenic modulating agents at first tier and these potential angiogenic modulating agents then subject to the tiers 2 and 3 safety tests respectively. Therefore, the final candidates of the lead agents which pass all the 3 tiers will have the desirable angiogenic activity with the least toxicity.

[0006] In the first tier, the object is to identify quickly any agent capable to induce alteration in the vasculature pattern in teleost. In this tier, embryos are treated by the agent in wide range of concentration, covering 7 orders of magnitude. To visualize blood vessels in transparent embryos, color staining is applied to detect endogenous alkaline phosphatase activity predominantly in vascular endothelial cells. Pattern of intersegmental vessels is chosen as the target to be examined because the pattern is regular and the variation between individual is very little, making the interpretation of angiogenic effect much easier. The preferred stage of embryos to be examined is at least 72 hour-post fertilization (hpf). Any alteration in the pattern of intersegmental vessels in any concentration of the agent will conclude as the angiogenic modulating agent and the agent will proceed to next tier.

[0007] In the second tier, the dose response relationship of the agent will be determined. The dose response relationship describes the change in the effects, i.e. death and malformation, caused by differing levels of concentrations of the agent. Studying dose response relationship is central to determine safe and hazardous levels for the agent under study. Dose response relationship is plotted as a graph, called dose response curve. The first point along the curve where a response, i.e. total effects inclusive death and malformation, above zero is reached is referred as the threshold concentration, or no observed adverse effect concentration (NOAEC). Above the threshold concentration, undesirable adverse effects, i.e. death and malformation, will still appear and the effect will be stronger as the concentration increases. Therefore, in this tier, the NOAEC of the agent on teleost is determined. The preferred stage of embryos to be examined is 24 or 48 hpf.

[0008] In the third tier, it is determined whether the agent at the level of NOAEC, instead of gross abnormalities, will induce any adverse effect in organ level and cellular level. Therefore, safety of an agent will be evaluated by mean of cytotoxicity test, organ toxicity test and cardiotoxicity test. In addition, angiogenic modulating activity will be tested at NOAEC. In this tier, visualization of intersegmental vessels is by microangiography imaging technology in which fluorescent dye, e.g. fluorescent microbead or fluorescent dextrans, are injected into the blood circulation such that it will be diffused to all registered blood vessels by blood circulation. Alteration of intersegmental vessels will be as indicator for angiogenic modulating activity. An agent with any positive results in one of the test or negative results in angiogenic modulating activity will be discarded. Otherwise, it will be considered as safe with angiogenic modulating activity.

Detail Description of the Invention

[0009] The present invention provides a biological method of screening an agent for angiogenic modulating activity as defined in the appended claims.

[0010] A variety of agents, either synthetic or natural sources, can be screened using the present invention. Agents can be small molecular in pure form or crude extraction of herb or crude extraction of a combination of different herbs. Agents should be administrated in soluble form, with a variety of carrier solvents, e.g. ethanol, DMSO. The maximum concentrations of these solvents at which no malformation is induced in zebrafish are reported (Hallare et al., 2006). For example, the level of DMSO and ethanol used in zebrafish embryo assay should be below 1.5% and 1% respectively. The agent should first dissolve in the carrier solvent, if necessary. Then it is added to the embryo media contained 20 fertilized eggs or healthy embryos at desired concentration. The preferred starting time of addition is prior the onset of angiogenesis, i. e. 24 hpf. Any effect induced by the presence of administrated agent will be observed after certain interval period of time, e.g 24 hours or 48 hours or more. Egg collection, picking up fertilized eggs and identification of healthy embryos are well known to those of ordinary skill in the art (Westerfield, 1995).

[0011] In the first tier, fertilized egg at 4 hpf are bathed in embryo medium containing various concentrations of agent to be tested with 10-fold increment in each concentration. The total number of concentrations to be tested is seven. Therefore, the range of concentrations to be tested covers 7 orders of magnitude. After incubation for a certain time period (e.g. 96 hours), embryos are fixed and blood vessels are visualized by color staining which specifically stains endogenous alkaline phosphatase activity predominantly in vascular endothelial cells. Number of intersegmental vessels is counted in each individual embryo. Number of embryos exhibiting decrease or increase in the number of intersegmental vessels is counted in each concentration. Agent at any concentration inducing alteration of the number of intersegmental vessels will be stated as a potential lead and passed to tier 2. Simultaneously, numbers of dead embryos are also counted in each concentration. This data will serve as preliminary range of concentrations to be tested in the following second tier.

[0012] In the second tier, occurrence of death and malformation is counted upon exposure of teleost to different concentrations of the potential lead agent identified in tier 1. The concentration range to be tested is preliminarily determined in the range-finding experiment in the first tier. Concentrations to be tested are chosen in equal placement. Percentage of death and malformation in each concentration is determined and plotted in a graph to show the dose response relationship. A dose response curve is fitted to all the data points and the concentration corresponding to the highest concentration at which there are no statistically significant increase in the percentage of adverse effects between exposed group and control group is determined as NOAEC. This concentration level will then be used in the third tier. If for any agent no NOAEC can be determined, it will be discarded from the testing.

[0013] Aim of the third tier is to find out any side effect of the agent at the concentration level not causing any gross adverse effects, such as death and malformation in teleost. Therefore, tests included in the third tier are cellular toxicity test, organ-specific toxicity test and cardiotoxicity test. In cytotoxicity test, number of died cells (apoptotic cells) is quantified by flow cytometry. By flow cytometry, one can analyze, in addition to amount of dead cells, the amount of cells in different stages of cell cycle in a large number of sample size, like 300 teleosts in a single experiment. In organ-specific toxicity, morphology of organ is studied by fluorescent *in situ* hybridization with organ specific probe. The morphology of organ is quantified by 3D reconstruction of the optical sections from confocal images. Any change in the size and shape of the organ may reveal the toxic effects induced by the agent tested. Cardiotoxicity is to test whether the positive candidate is able to affect cardiac functions, such as cardiac output and cardiac rhythm. Cardiotoxicity is an important test because more and more drugs are showed to exhibit side effects on the heart, even in rare frequent, and needed to be withdrawn from market. Finally, the effect of angiogenic modulating activity of the positive candidate is evaluated at the NOEC. The positive candidate that does not induce any cytotoxic, organ-specific toxic and cardiotoxic effects and exhibits angiogeneic modulating activity at the NOEC is said to pass the third tier and, thus, this screening bioassay.

Examples

1. Material and Methods

a. Egg collection

[0014] Zebrafish mate and spawn with the light on. In order to collect eggs, it is necessary to protect the eggs from being eaten by the adults. The egg collection box used is made of acrylic with a metal mesh mounted on top. This is then to be covered with plastic seaweed so that the adults will mate where the plastic seaweed is, and the eggs that are spawned will fall into the acrylic box. The eggs are to be protected from adult fish consumption with a metal mash. The collection box should be removed after 30 min duration of the light period. The eggs are then to be collected by pouring fish water from the collection box through a fish net. The eggs should be rinsed under running tap water for 30 seconds, then transferred into a 90-mm Petri dish with 15 ml of embryo medium (19.3 mM NaCl, 0.23 mM KCl, 0.13 mM $MgSO_4$□$7H_2O$, 0.2 mM $Ca(NO_3)_2$, 1.67 mM Hepes (pH 7.2)). The dish should then be kept in an incubator at 28°C.

b. Administration of agents

[0015] Agents are dissolved in a desired concentration such that the highest testing concentration contains the concentration of carrier solvent (e.g. DMSO, ethanol) not more than the recommended tolerant concentration. Agents are to be added directly to the medium solution with 4 hpf of the fish embryos. Assays should be performed in Petri dishes (60 mm x 15 mm, Falcon, BD Bioscience, San Jose, California). Each Petri dish is to hold 20 embryos.

[0016] As a primary screen for compound effects in tier 1, each agent is tested at 8 different concentrations, with 1 order of magnitude different to determine which concentration will provide the most information. For dose response study in tier 2, range of concentrations to be tested cover from the lowest at which no adverse effect is observed to the highest concentration at which all embryos are killed.

[0017] For the cytotoxicity, cardiotoxicity and organ toxicity in tier 2 and mechanmistic study in tier 3, the only concentration to be tested is the NOAEC determined from dose response curve. For each concentration of the agnet, 6 ml of culture medium is to be added to twenty embryos cultured in a Petri dish. Fifteen replications per agent per concentration (i.e. 20 x 15 = 300 embryos for each concentration of an agent) are then tested. The effect could be observed at different time points, e.g. 28 hpf, 52 hpf or 76 hpf. Furthermore, the further experiments to examine toxicity and angiogenesis can be performed following agent treatment.

c. Toxicity test

[0018] To determine the toxicity of a compound for a biological system, an observable and well-defined endpoint must be identified. Mortality is one of these widely used endpoints. The index of lethal effects describing the potency of a compound is called LC50, which is defined as the dosage of a chemical causing death in at least 50% of the animals tested. In addition to lethality, adverse effects such as malformation in morphology induced by a compound can be used as an observable endpoint. These adverse outcomes along with death are grouped as total adverse effects. The potency of a compound to induce adverse effects is defined as EC50, which is the concentration of a compound causing at least 50% of the animals tested to exhibit abnormal appearances as well as death.

[0019] Mortality and total adverse effect were determined to be observable endpoint in the present study. After administering a compound to the fish embryos, the embryos are to be maintained at 28°C until 28 hpf and 52 hpf, respectively. Twenty-four and forty-eight hours after adding the compound to the medium in which the fish embryos are cultured, the embryos are then visually inspected for mortality, movements and gross morphological defects under a dissecting microscope (Zeiss, Jena, Germany). The number of dead embryos of the 20 embryos tested in each experiment is then to be scored. Data collected from the 15 replicates are to be tested for the null hypothesis that no differences existed between mortality over all concentrations. Analyses of variance are then done, along with the post-hoc Tukey's honestly significant difference test to determine the differences in mortality between treatment concentrations and the untreated control. Likewise, analyses of variance and the post-hoc Tukey honest significant difference test are both used to determine the differences in total adverse effects between treatment concentrations and the untreated control. The software Statistica for Windows (StatSoft, Tulsa, OK, USA) is to be used for the calculations.

d. Endogenous alkaline phosphatase staining

[0020] Embryos older than 72 hpf can be used for endogenous alkaline phosphatase staining. Specifically, embryos are fixed in 4% paraformaldehyde (PFA) in PBS and 1% Triton x-100. Embryos are fixed for overnight at 4°C and then washed four times in PBS/0.1% Tween-20 (PBT) for 5 minutes. For staining, the embryos are equilibrated three times in a TMNT buffer (0.1M Tris-HCl pH 9.5; 50 mM MgCl; 0.1M NaCl; 0.1% Tween 20) for 10 minutes at room temperature. After the embryos equilibrated, they are stained in substrate solution (4.5 μl of 75 mg/ml nitro blue tetrazolium (NBT) and 3.5 μL of 50 mg/ml X-phosphate for 1 ml of TMNT solution). After staining for 12 minutes, all the blood vessels in the fish embryo are labeled. The staining reaction is stopped by washing three times in PBT and embryos are fixed in 4% paraformaldehyde in PBS at room temperature for 30 minutes. The embryos are then washed briefly in PBT twice and 10. minutes 4 times. After washing, embryos should be immersed in 70% glycerol in PBS before examination and image capturing on a CCD camera.

[0021] To examine pro-/anti-angiogenic activity induced by a compound, the number of intersegmental vessels is required to be counted under a stereomicroscope or a compound microscope. Stained embryos are positioned laterally in glycerol. The region of examination is from the first intersegmental vessels to the one just above the anus. In general, there should be 12-13 pairs. The advantage is that there is a constant number of intersegmental vessels in 3-day embryos which makes the comparison between the control and treatment groups easier. Another advantage of using the zebrafish for this type of assay is that the subintestinal vessels, which are located over the yolk, are both sensitive to factors which affect vessel formation and are easily assayed by this method. The subintestinal vessels are normally present on the dorsolateral surface of the yolk of zebrafish embryos by 48 hours of development. They form a distinct basket shape that extends 50-100μm from the ventral edge of the somite over the yolk. By assaying the subintestinal vessels at 72 hours of development (24 hours after the subintestinal vessels normally appear), the normal variation in the timing of the vessel formation is avoided.

e. Microangiography

[0022] Procedures of microangiography are adopted from Weinstein et al. (Nat Genet, 1995), with modifications. Embryos are to be mounted laterally in 0.3% agarose as described below. Fluoresceinated and carboxylated latex microbeads (0.02-μm, cat# F-8787, Molecular Probes, USA) are then injected into the sinus venosus. The bead suspension should be diluted 1:1 with 2% BSA, sonicated at maximum power for 5 minutes, then centrifuged at maximum speed for 5 minutes. The supernatant is then to be transferred to a new tube for injection. Glass microneedles are then prepared from 1 mm OD capillaries (World Precision Instruments, cat # TW100-4 or TW100F-4 for glass without or with an internal filament) by using a Narishige PC-10 micropipette puller. To make these microneedles, this micropipette puller is required to pull the glass capillary vertically using the gravitational force of its own weight. A double pull is set in which the first pull is at a temperature of 76°C and the second pull is at a temperature of 64°C. Microneedles are then to be broken open by a Narishige MF-900 micro-forge to give an opening of approximately 5 μm in width. The tip of microneedle needs to be broken by pushing against a small drop of glass melted on to the platinum wire of the micro-forge, monitored at 10X object of the micro-forge. No pipette holding is needed in this protocol since the embryos are mounted in agarose. The bead suspension is then loaded into a microneedle at the wider end with a micropipette loader. A glass microneedle is then inserted obliquely into the sinus venosus. Many (20+) small boluses of bead suspension should be injected over the course of up to a minute. Confocal images are acquired after letting the embryo recover for 3-5 minutes. The instrument setting is like the one described below. After injection, fluorescent beads will circulate around the whole vascular network, lighting up each blood vessel with active blood flow from the heart. Confocal images (Carl Zeiss confocal microscope equipped with LSM version 5) are then acquired for detailed reconstruction of the 3D structure of the zebrafish embryo vascular system.

[0023] Embryo prepared for microangiography should be immobilized by agarose. Agarose solution (0.3%) is prepared and kept at around 45°C on a heat block. Embryos are to be transferred to a clean slide by using a Pasteur pipette with a broad opening. To avoid yolk sac damaging, a small drop of solution should be added to the embryo before addition of pre-heat 0.3% agarose to the embryo. With a 27G needle, the embryo is arranged to the appropriated position just before the agarose hardens (it usually requires a minute or two).

[0024] To start with, a laser lamp with the appropriate wave length (e.g., 488 nm laser for FITC/fluorescein and acridine orange) should be switched on first. The pinhole size is then adjusted to a value, so that the airy value is 1.00. The "Find" button in the side menu bar is used to adjust detector gain, offset and amplifier gain automatically. Detector gain and amplifier gain, if necessary, are to be adjusted to enhance intensity and for the detector offset to suppress background noise. The position of starting optical section ("slice") is defined and marked by selecting "Mark first". Similarly, the position of ending optical section is defined and marked by selecting "Mark last". The interval and number of slice was defined by setting "X:Y:Z: = 1:1:1" so that the resolution of the Z-axis is set as X and Y resolution. Series stack confocal images are then captured, saved, and recorded in CD-R for backup. Confocal images can be exported as TIFF (16-bit raw image) by using the standalone version of LSM image browser (Carl Zeiss) for analysis in other image analysis software, e.g. MetaMorph (Universal Imaging, USA).

f. whole mounted *in situ* hybridization

[0025] In addition to performing visual screens, specific molecular changes in teleost tissues can be detected by *in situ* hybridization of RNA or antibody staining of specific proteins. A digoxigenin-labeling kit from Roche can be used to label the antisense RNA probes. Antisense probes are synthesized by linearizing plasmids pBlueScript (10μg) with appropriate restriction enzymes in a 50 μl cocktail. Agarose gel electrophoresis is to be used as a monitor to make sure the plasmid is totally digested, and an equal volume of phenol/chloroform (Gibco BRL, Life Technology, USA) is used to stop the reaction. The mixture is then centrifuged on a bench top microcentrifuge under room temperature for 3 minutes at full speed (13,000 rpm). The aqueous phase should be transferred to a new tube followed by the addition of 1/10 volumes of 3M sodium acetate (pH 8.0) (Sigma, USA) and 2 volumes of ethanol (Sigma, USA), centrifuged for 30 minutes at room temperature. The pellet is then washed with 70% ethanol and left to air dry on bench, resuspended in an appropriate amount of RNAase-free (DEPC; Sigma, USA) water to a concentration of 0.5 μg/μl.

[0026] Linearized plasmids are then transcribed with T3 RNA polymerase or T7 RNA polymerase in a transcription cocktail, incubated for 2 hours at 37°C for the preparation of non-radioactive digoxigenin transcripts. The reaction is to be stopped by adding 1μl of EDTA (0.5M, pH8.0). The addition of 2.5μl of LiCl (4M) and 75μl of cold ethanol is done to precipitate the RNA probe by centrifuging at full speed for 30 minutes at 4°C. The pellet is then washed with 70% ethanol, left for air dry on bench and resuspended in RNAse-free (DEPC) water.

[0027] Whole mount *in situ* hybridization can be carried out as described by Westerfield (1994) with modifications (Cheng et al. 2000): Embryos at 24 hpf are to be dechorionated by using a pair of forceps and fixed in PBS (phosphate-buffered saline) with 4% paraformaldehyde (PFA) and 1% Triton X-100 at 4°C overnight. Briefly, antisense RNA is then synthesized by linearising the plasmid and transcribing with T7 polymerase and Digoxigenin-11-UTP (Roche, Basel, Switzerland). The embryos should be transferred to methanol and stored at -20°C to increase permeability. They are then washed and lightly digested with 10μg/ml proteinase K in PBT (PBS with 0.1% Tween 20) before incubating with the antisense probes in *situ* hybridization solution (50% formamide, 5 x SSC, 50μg/ml Heparin, 500μg/ml tRNA, 9mM citric acid, pH 6.0, and 0.1% Tween 20) at 65-70°C overnight. Following hybridization, probes are removed with high-stringency washes. In brief, embryos are washed twice in 50% Formamide: 50% (2 x SSC/0.1%). Tween-20), each for 30 minute. Followed by 2x SSC/0.1% Tween-20 at 65°C for 15 minutes, 2 x SSC and 0.2 x SSC each twice for 30 min, at 65°C. Embryos were subsequently incubated with pre-absorbed sheep anti-digoxigenin-alkaline phosphatase Fab fragments (Roche, Basel, Switzerland) on a nutator at 4°C overnight. After washing 6 times in PBT, 5-bromo-4-chloroindolyl phosphate was added as substrate and nitro blue tetrazolium as coupler (Roche, Basel, Switzerland) for color staining.

g. Apoptosis

[0028] Embryos treated with agents (15 replicates) are pooled into a single 90-mm Petri dish coated with 0.3% agarose and washed three times in an embryo culture medium. Dechorionation is done under a stereomicroscope by using a pair of forceps to tear off the chorion. The embryos are then transferred to a 15-ml culture tube filled with 8-ml trypsin solution (0.5 mg/ml trypsin in a solution of 0.14M of NaCl, 0.05M of KCl, 0.005M of glucose, 0.007M of $NaHCO_3$ and 0.7mM of EDTA). Then the embryos are to be triturated through a narrow bore Pasteur pipette until they are dissociated with continuous checking under a dissection microscope. The cell suspension is then centrifuged at 1000X g for 7 minutes at 4°C. The supernatant is discarded and cells are resuspended in 5ml PBS to wash away the trypsin. They are then centrifuged again at 1000X g for 7 minutes at 4°C, followed by resuspension in 5ml of PBS and centrifuged at 1000X g for 7 minutes at 4°C. The aqueous solution is discarded, and the pellet is then resuspended completely in 200 μl of PBS. Following this, 2 ml of 70% ethanol is added and the mixture is incubated at -20°C overnight. The sample is then centrifuged at 1000X g for 7 minutes at 4°C and pellet is added with 100 μl of propidium iodide (400 μg/ml) and 100 μl of RNase (1 mg/ml), incubated at 28.5°C for 30 minutes before flow cytometry analysis.

h. Cardiotoxicity

[0029] Embryo prepared for microangiography should be immobilized by agarose. Agarose solution (0.3%) is prepared and kept at around 45°C on a heat block. Embryos are to be transferred to a clean slide by using a Pasteur pipette with a broad opening. To avoid yolk sac damaging, a small drop of solution should be added to the embryo before addition of pre-heat 0.3% agarose to the embryo. With a 27G needle, the embryo is arranged to their lateral position just before the agarose hardens. After agarose hardening, circulation at the tail region is examined under dissection microscope with CCD camera connected either to a personal computer equipped with video grabbing device or to digital video camera. In the case of digital video camera, data stored in mini-DV tape is transferred back to personal computer via i-Link connection and saved in AVI format for video image analysis. After video image analysis, data for the power spectrum of blood cell movement is obtained. Two parameters are derived from the power spectrum. The first parameter representing the heart rate is the basic frequency component which is the value of frequency with biggest power value. The second parameter is the ratio of the power value of the basic frequency component to the total power value of the whole spectrum. This ratio is correlated with the heart beat rhythmicity.

2. Results

a. Tier 1

[0030] Totally, 1431 single compounds, 107 herbs singularly and 13 formulas had been screened in tier 1. These agents were chosen from books of Traditional Chinese Medicine. Out of 1431 compounds, there were 11 showing anti-angiogenic modulating activity at least in one of the tested concentration. The phenomenon of anti-angiogenesis was the absence of at least one intersegmental vessel. Out of 107 herbs, there were 11 showing anti-angiogenic modulating activity at least in one of the tested concentration. Out of 13 formulas tested, there were 4 showing anti-angiogenic modulating activity at least in one of the tested concentration while there were 3 showing pro-angiogenic modulating activity at least in one of the tested concentration. Same agents purchased from different supplier also exhibited similar response.

b. Tier 2

[0031] Twenty nine agents passed the first tier and were tested in the second tier. General toxicity tests have been carried out. The NOAECs for each agent were then been determined. Among them, there were 6 agents (4 herbs and 2 formulas) that no NOAEC could be determined and thus they were not further tested in the third tier.

c. Tier 3

[0032] Toxicity test at the NOAEC was carried out. All 11 compounds at their NOAECs induced ectopic apoptosis in teleost embryos and were failed in the cytotoxicity test. Therefore, they were failed in the third tier and no further toxicity test was carried out. Five out of 7 herbs induced ectopic apoptosis at their NOAECs while the remaining 2 passed the cytotoxicity test. Among these 2, only one exhibited antiangiogenic activity and passed organ-specific toxicity tests and cardiotoxicity at its NOAEC. Furthermore, 3 out of 5 formulas did not induce ectopic apoptosis at their NOAEC and proceeded to further tests while the remaining 2 failed in the cytotoxicity test. Among the 3 passing the cytotoxicity test, one failed to induce any change in vasculature at its NOAEC. But there was 1 exhibited anti-angiogenic activity at its NOAEC while there was 1 exhibited pro-angiogenic activity. These 2 formulas had passed the organ-specific toxicity tests and cardiotoxicity test. Therefore, there were 3 agents, including 1 herb and 2 formulas, passed all 3 tiers tests.

References

[0033]

Cross, L. M., Cook, M. A., Lin, S., Chen, J. N. and Rubinstein, A. L. (2003). Rapid Analysis of Angiogenesis Drugs in a Live Fluorescent Zebrafish Assay. Arterioscler. Thromb. Vasc. Biol. 23, 911-912.

Epstein, F. H. and Epstein, J. A. (2005). A Perspective on the Value of Aquatic Models in Biomedical Research. Exp. Biol. Med. (Maywood) 230, 1-7.

Goldsmith, P. (2004). Zebrafish as a Pharmacological Tool: The how, Why and when. Curr. Opin. Pharmacol. 4,504-512.

Hallare, A., Nagel, K., Kohler, H. R. and Triebskorn, R. (2006). Comparative Embryotoxicity and Proteotoxicity of Three Carrier Solvents to Zebrafish (Danio Rerio) Embryos. Ecotoxicol. Environ. Saf. 63, 378-388.

Hasan, J., Shnyder, S. D., Bibby, M., Double, J. A., Bicknel, R. and Jayson, G. C. (2004). Quantitative Angiogenesis Assays in Vivo--a Review. Angiogenesis 7, 1-16.

Kidd, K. R. and Weinstein, B. M. (2003). Fishing for Novel Angiogenic Therapies. Br: J. Pharmacol. 140, 585-594.

Lawson, N. D. and Weinstein, B. M. (2002). In Vivo Imaging of Embryonic Vascular Development using Transgenic Zebrafish. Dev. Biol. 248, 307-318.

Liekens, S., De Clercq, E. and Neyts, J. (2001). Angiogenesis: Regulators and Clinical Applications. Biochem. Pharmacol. 61, 253-270.

Parng, C., Seng, W. L., Semino, C. and McGrath, P. (2002). Zebrafish: A Preclinical Model for Drug Screening. Assay Drug Dev. Technol. 1, 41-48.

Risau, W. (1997). Mechanisms of Angiogenesis. Nature 386, 671-674.

Serbedzija, G. N., Flynn, E. and Willett, C. E. (1999). Zebrafish Angiogenesis: A New Model for Drug Screening. Angiogenesis 3, 353-359.

Staton, C. A., Stribbling, S. M., Tazzyman, S., Hughes, R., Brown, N. J. and Lewis, C. E. (2004). Current Methods for Assaying Angiogenesis in Vitro and in Vivo. Int: J. Exp. Pathol. 85, 233-248.

Taraboletti, G. and Giavazzi, R. (2004). Modelling Approaches for Angiogenesis. Eur. J. Cancer 40, 881-889.

Westerfield, M. (1995). The Zebrafish Book. A Guide for the Laboratory use of Zebrafish. Eugene: Univ. of Oregon Press.

Claims

1. A biological method of screening an agent for angiogenic modulating activity, comprising a hierarchical 3 -tier biological screening assay system:

   a. a first tier wherein teleost embryos are used to screen an agent having angiogenic modulating activities;
   b. a second tier wherein "no observed adverse effect concentration (NOAEC) of the agent on teleost embryos is determined from a dose-response relationship describing the change in the effects of gross abnormalities including death and malformation caused by differing levels of concentrations of the agent;
   c. a third tier wherein it is determined whether the agent at the NOAEC will induce any adverse effect at the organ level and cellular level of the teleost embryos as well as having angiogenic modulating activities.

2. The method of claim 1, wherein the teleost embryo is a zebrafish or medaka embryo.

3. The method of claim 2, wherein the zebrafish or medaka embryos are treated by agent at concentrations covering 7 orders of magnitude in the first tier.

4. The method of claim 2, wherein the earliest time and latest time to add agent to medium is 4 hour post fertilization and 20 hour post fertilization respectively.

5. The method of claim 2, wherein the first tier comprises a step to visualize and analysis of the pattern of vascular system in zebrafish or medaka.

6. The method of claim 5, wherein the zebrafish or medaka are examined between 3 to 5 days post fertilization, and the pattern of vascular system is visualized by staining endothelial cells with color substrates or fluorescent substrates in fixed zebrafish or medaka, or by injecting fluorescent substrates into the circulation system in live zebrafish or medaka.

7. The method of claim 5, wherein the analysis tactic is by counting the number of intersegmental blood vessels, either totally from head to tail or in particular region.

8. The method of claim 2, wherein the second tier comprises a step to determine NOAEC of the agent on zebrafish or medaka at different concentrations for at least 1-day exposure time.

9. The method of claim 2, wherein the third tier comprises a step to measure the size and the shape of the target organs.

10. The method of claim 9, wherein the organ is visualized by organ-specific substrates.

11. The method of claim 10, wherein the organ-specific substrates are fluorescence substrates.

12. The method of claim 2, wherein occurrence of dead cells are determined in at least 100 zebrafish or medaka per concentration in the third tier.

13. The method of claim 2, wherein the third tier comprises a step to determine cardiotoxicity of agents.

14. The method of claim 13, wherein the zebrafish or medaka are at least 48 hour post fertilization.

15. The method of claim 13, wherein the parameters to be measured are heart rate and heart beat rhythmicity.

Patentansprüche

1. Biologisches Verfahren zum Screening eines Agens nach angiogen-modulierender Aktivität, umfassend ein hierarchisches, 3-stufiges biologisches Screening-Testsystem:

   a. eine erste Stufe, worin Teleostei-Embryos zum Screenen eines Agens mit angiogenmodulierenden Aktivitäten verwendet werden;
   b. eine zweite Stufe, worin die "Konzentration mit keiner beobachteten nachteiligen Wirkung (NOAEC)" des Agens auf Teleostei-Embryos aus einem Dosis-Wirkungs-Verhältnis bestimmt wird, welches die Änderung in den Wirkungen von groben Anomalitäten, einschließlich Tod und Missbildung, beschreibt, die durch unterschiedliche Konzentrationen des Agens verursacht werden;
   c. eine dritte Stufe, worin bestimmt wird, ob das Agens bei der NOAEC irgendeine nachteilige

Wirkung auf Organ- und Zellebene der Teleostei-Embryos hervorruft sowie angiogen-modulierende Aktivitäten aufweist.

2. Verfahren nach Anspruch 1, wobei der Teleostei-Embryo ein Zebrafisch- oder Medaka-Embryo ist.

3. Verfahren nach Anspruch 2, wobei die Zebrafisch- oder Medaka-Embryos mit dem Agens in Konzentrationen, welche 7 Größenordnungen umfassen, in der ersten Stufe behandelt werden.

4. Verfahren nach Anspruch 2, wobei der früheste und der späteste Zeitpunkt zum Zugeben des Agens zum Medium 4 Stunden nach der Befruchtung bzw. 20 Stunden nach der Befruchtung ist.

5. Verfahren nach Anspruch 2, wobei die erste Stufe einen Schritt zur Visualisierung und Analyse der Struktur des Gefäßsystems beim Zebrafisch oder Medaka umfasst.

6. Verfahren nach Anspruch 5, wobei der Zebrafisch oder Medaka zwischen 3 bis 5 Tage nach der Befruchtung untersucht werden und die Struktur des Gefäßsystems durch Färbung der Endothelzellen mit Farbsubstraten oder fluoreszierenden Substraten in fixiertem Zebrafisch oder Medaka oder durch Injizieren von fluoreszierenden Substraten in das Blutkreislaufsystem im lebenden Zebrafisch oder Medaka visualisiert wird.

7. Verfahren nach Anspruch 5, wobei die Analysevorgehensweise darin besteht, die Anzahl von intersegmentalen Blutgefäßen, entweder insgesamt von Kopf bis Schwanzflosse oder in einem bestimmten Bereich, zu zählen.

8. Verfahren nach Anspruch 2, wobei die zweite Stufe einen Schritt zur Bestimmung der NOAEC des Agens bei Zebrafisch oder Medaka bei verschiedenen Konzentrationen während einer zumindest eintägigen Expositionszeit umfasst.

9. Verfahren nach Anspruch 2, wobei die dritte Stufe einen Schritt zur Messung der Größe und der Form der Ziel-Organe umfasst.

10. Verfahren nach Anspruch 9, wobei das Organ mittels organspezifischer Substrate visualisiert wird.

11. Verfahren nach Anspruch 10, wobei die organspezifischen Substrate Fluoreszenzsubstrate sind.

12. Verfahren nach Anspruch 2, wobei das Auftreten von toten Zellen in zumindest 100 Zebrafischen oder Medaka pro Konzentration in der dritten Stufe bestimmt wird.

13. Verfahren nach Anspruch 2, wobei die dritte Stufe einen Schritt zur Bestimmung der Kardiotoxizität der Agenzien umfasst.

14. Verfahren nach Anspruch 13, wobei der Zebrafisch oder Medaka mindestens 48 Stunden nach der Befruchtung ist.

15. Verfahren nach Anspruch 13, wobei die zu messenden Parameter die Herzfrequenz und die Herzschlagrhythmik sind.

Revendications

1. Procédé biologique de sélection d'un agent pour une activité modulant l'angiogenèse, comprenant un système de test de sélection biologique à 3 niveaux hiérarchiques :

a. un premier niveau où des embryons téléostéens sont utilisés pour sélectionner un agent ayant des activités modulant l'angiogenèse ;
b. un second niveau où la « concentration sans effet néfaste observé (CSENO) » de l'agent sur des embryons téléostéens est déterminée d'après une relation dose-réponse décrivant le changement dans les effets d'anomalies prononcées incluant la mort et les malformations causées par différents niveaux de concentrations de l'agent ;
c. un troisième niveau où on détermine si l'agent à la CSENO induira un quelconque effet néfaste au niveau des organes et au niveau cellulaire des embryons téléostéens et a des activités modulant l'angiogenèse.

2. Procédé selon la revendication 1 où l'embryon téléostéen est un embryon de poisson zèbre ou de medaka.

3. Procédé selon la revendication 2 où les embryons de poisson zèbre ou de medaka sont traités par un agent à des concentrations couvrant 7 ordres de grandeur dans le premier niveau.

4. Procédé selon la revendication 2 où le moment le plus précoce et le moment le plus tardif pour ajouter un agent au milieu sont 4 heures après la fécondation et 20 heures après la fécondation respectivement.

5. Procédé selon la revendication 2 où le premier niveau comprend une étape de visualisation et d'analyse de la configuration du système vasculaire dans le poisson zèbre ou le medaka.

6. Procédé selon la revendication 5 où le poisson zèbre ou le medaka est examiné entre 3 et 5 jours après la fécondation, et la configuration du système vasculaire est visualisée par coloration de cellules endothéliales avec des substrats colorés ou des substrats fluorescents dans un poisson zèbre ou un medaka fixé, ou par injection de substrats fluorescents dans le système circulatoire dans un poisson zèbre ou un medaka vivant.

7. Procédé selon la revendication 5 où la tactique d'analyse est par comptage du nombre de vaisseaux sanguins intersegmentaires, totalement de la tête à la queue ou dans une région particulière.

8. Procédé selon la revendication 2 où le second niveau comprend une étape pour déterminer la CSENO de l'agent sur un poisson zèbre ou un medaka à différentes concentrations pendant une durée d'exposition d'au moins 1 jour.

9. Procédé selon la revendication 2 où le troisième niveau comprend une étape pour mesurer la taille et la forme des organes cibles.

10. Procédé selon la revendication 9 où l'organe est visualisé par des substrats spécifiques d'organe.

11. Procédé selon la revendication 10 où les substrats spécifiques d'organe sont des substrats fluorescents.

12. Procédé selon la revendication 2 où la survenue de cellules mortes est déterminée dans au moins 100 poissons zèbres ou medakas par concentration dans le troisième niveau.

13. Procédé selon la revendication 2 où le troisième niveau comprend une étape pour déterminer la cardiotoxicité d'agents.

14. Procédé selon la revendication 13 où les poissons zèbres ou medakas sont à au moins 48 heures après la fécondation.

15. Procédé selon la revendication 13 où les paramètres à mesurer sont la fréquence cardiaque et la rythmicité des battements cardiaques.

REFERENCES CITED IN THE DESCRIPTION

Patent documents cited in the description

- WO 9942606 A **[0004]**

Non-patent literature cited in the description

- **Cross, L. M. ; Cook, M. A. ; Lin, S. ; Chen, J. N. ; Rubinstein, A. L.** Rapid Analysis of Angiogenesis Drugs in a Live Fluorescent Zebrafish Assay. *Arterioscler. Thromb. Vasc. Biol.,* 2003, vol. 23, 911-912 **[0033]**
- **Epstein, F. H. ; Epstein, J. A.** A Perspective on the Value of Aquatic Models in Biomedical Research. *Exp. Biol. Med. (Maywood),* 2005, vol. 230, 1-7 **[0033]**
- **Goldsmith, P.** Zebrafish as a Pharmacological Tool: The how, Why and when. *Curr. Opin. Pharmacol.,* 2004, vol. 4, 504-512 **[0033]**
- **Hallare, A. ; Nagel, K. ; Kohler, H. R. ; Triebskorn, R.** Comparative Embryotoxicity and Proteotoxicity of Three Carrier Solvents to Zebrafish (Danio Rerio) Embryos. *Ecotoxicol. Environ. Saf.,* 2006, vol. 63, 378-388 **[0033]**
- **Hasan, J. ; Shnyder, S. D. ; Bibby, M. ; Double, J. A. ; Bicknel, R. ; Jayson, G. C.** Quantitative Angiogenesis Assays in Vivo--a Review. *Angiogenesis,* vol. 7, 1-16 **[0033]**
- **Kidd, K. R. ; Weinstein, B. M.** Fishing for Novel Angiogenic Therapies. *Br: J. Pharmacol.,* vol. 140, 585-594 **[0033]**
- **Lawson, N. D. ; Weinstein, B. M.** In Vivo Imaging of Embryonic Vascular Development using Transgenic Zebrafish. *Dev. Biol.,* 2002, vol. 248, 307-318 **[0033]**
- **Liekens, S. ; De Clercq, E. ; Neyts, J.** Angiogenesis: Regulators and Clinical Applications. *Biochem. Pharmacol.,* 2001, vol. 61, 253-270 **[0033]**
- **Parng, C. ; Seng, W. L. ; Semino, C. ; McGrath, P.** Zebrafish: A Preclinical Model for Drug Screening. *Assay Drug Dev. Technol.,* 2002, vol. 1, 41-48 **[0033]**
- **Risau, W.** Mechanisms of Angiogenesis. *Nature,* 1997, vol. 386, 671-674 **[0033]**
- **Serbedzija, G. N. ; Flynn, E. ; Willett, C. E.** Zebrafish Angiogenesis: A New Model for Drug Screening. *Angiogenesis,* 1997, vol. 3, 353-359 **[0033]**
- **Staton, C. A. ; Stribbling, S. M. ; Tazzyman, S. ; Hughes, R. ; Brown, N. J. ; Lewis, C. E.** Current Methods for Assaying Angiogenesis in Vitro and in Vivo. *Int: J. Exp. Pathol.,* 2004, vol. 85, 233-248 **[0033]**
- **Taraboletti, G. ; Giavazzi, R.** Modelling Approaches for Angiogenesis. *Eur. J. Cancer,* 2004, vol. 40, 881-889 **[0033]**
- **Westerfield, M.** The Zebrafish Book. A Guide for the Laboratory use of Zebrafish. Eugene: Univ. of Oregon Press, 1995 **[0033]**

专利名称(译)	使用硬骨鱼胚胎筛选具有血管生成调节活性的药剂的方法		
公开(公告)号	EP2027283A4	公开(公告)日	2010-03-03
申请号	EP2007701966	申请日	2007-01-08
[标]申请(专利权)人(译)	香港城市大学		
申请(专利权)人(译)	香港城市大学		
当前申请(专利权)人(译)	香港城市大学		
[标]发明人	CHENG SHUK HAN CITY UNIV OF HONG KONG CHAN POKWOK CITY UNIV OF HONG KONG		
发明人	CHENG, SHUK HAN, CITY UNIVERSITY OF HONG KONG CHAN, POKWOK, CITY UNIVERSITY OF HONG KONG		
IPC分类号	C12Q1/00 G01N31/00 G01N33/48 G01N33/53 G01N33/50		
CPC分类号	G01N33/5014 G01N33/5088 G01N2333/4603 G01N2333/515		
代理机构(译)	COMOGLIO，ELENA		
其他公开文献	EP2027283B1 EP2027283A1		
外部链接	Espacenet		

摘要(译)

本发明涉及使用透明硬骨鱼胚胎作为模型筛选具有血管生成调节活性的配方中的化合物，草药提取物或草药组合提取物的3层系统的方法。