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(54) Title: MEANS AND METHODS FOR TREATING LUNG HYPOPLASIA

(57) Abstract: The present invention relates to the field of lung disorders. In particular the invention relates to compositions of nanodiamond particles conjugated to VEGF for the treatment of conditions suffering from lung hypoplasia and lung hypertension such as for example congenital diaphragmatic hernia.



Means and methods for treating lung hypoplasiaField of the invention

The present invention relates to the field of lung disorders. In particular the invention relates to compositions of nanodiamond particles conjugated to VEGF for the treatment of conditions suffering from lung hypoplasia and lung hypertension such as for example congenital diaphragmatic hernia.

Introduction of the invention

Congenital diaphragmatic hernia (CDH) is a congenital malformation (birth defect) of the diaphragm and affects approximately 1 in 2000 newborns (300-400 new cases per year in the UK). CDH is a life-threatening pathology in infants, and a major cause of death due to two complications: pulmonary hypoplasia (decreased airway number) and pulmonary hypertension due to thickened blood vessels). In the more severe cases, mortality exceeds 80%. The lung pathology is caused mainly by intra-abdominal organs herniating into the chest and compressing the lungs thus preventing their growth. This condition is an ideal target for prenatal (in utero) treatment, as damage occurs before birth and cannot be treated postnatally. The only available treatment to date involves occlusion of the fetal trachea (at around 26-28 weeks gestational age), with encouraging initial results (the therapy is currently tested in a multicentre clinical trial; <http://www.totaltrial.eu/>). Although tracheal occlusion has been shown to be beneficial in this setting, it is likely that further therapies will be required in order to promote lung growth and improve outcomes. VEGF is described in the art as a pivotal growth factor for healthy lung development, and it has activity on both the airways (promotes pulmonary epithelial cell proliferation and maturation), as well as the pulmonary blood vessels (promotes angiogenesis, as well as blood vessel remodelling). Previously it was shown that the administration of recombinant VEGF is useful for the treatment of respiratory distress syndrome (see US7307060) due to an increased lung maturation and surfactant production. In our recent research we however observed that the acute administration of VEGF causes vascular leakage in the lungs and oedema formation. We therefore sought a more optimal administration of VEGF. In the present invention we identified an optimal treatment for CDH via the combination of the standard tracheal occlusion approach for CDH treatment and diamond nanoparticle-bound VEGF (nanodiamond-VEGF; ND-VEGF). In particular we show that the prenatal intra-pulmonary delivery of VEGF coupled to biocompatible nanoparticles induces dramatic lung growth and reverses defects in aveoli and arterioles when used in combination with tracheal occlusion in a pre-clinical model for CDH. Remarkably the observed effect is not present when unconjugated VEGF is administered in our model. Even more remarkable is that the ND-VEGF also correct the pulmonary hypertension which occurs in CDH. Further

advantages of delivering VEGF coupled to nanoparticles is that these particles enter the cell by endocytosis and are inert, not affecting normal cell function and not causing an immune response. We believe that one of the key aspects of the invention is that the ND-VEGF platform allows targeted and gradual release of VEGF in the lung, which mimics the spatially and temporally controlled release of VEGF during normal lung development.

Figures

Figure 1: effect of tracheal occlusion on fetal pulmonary uptake of nanodiamond particles following intra-tracheal in utero administration. The left panel shows the uptake of fluorescent (AF488-ND) nanodiamond particles of fetal pneumocytes in conditions without tracheal occlusion (TO). The right panel shows the enhanced uptake of the AF488-ND particles in combination with tracheal occlusion (TO).

Figure 2: localization of nanodiamonds following in utero administration to the fetal lungs with tracheal occlusion. The left panel shows the uptake of free AF488. The right panel depicts nanodiamond conjugated AF488.

Figure 3: chemical scheme depicting the conjugation of VEGF to nanodiamonds. The three steps are outlined in the materials and methods section.

Figure 4: measurement of the lung to body weight ratio (LBWR). The LBWR ratio is depicted as a %. It is apparent that the administration of nanodiamond-VEGF (ND-VEGF + TO) particles to the pre-clinical CDH model have a similar LBWR as the healthy animal. The beneficial effect is absent in the presence of SU5416 (a potent and selective inhibitor of VEGF Receptor 2 [KDR/FLK-1]).

Figure 5: measurement of the alveolar septal wall thickness. It is apparent that the administration of nanodiamond-VEGF (ND-VEGF + TO) particles to the pre-clinical model of CDH have a similar alveolar septal wall thickness as the healthy animal.

Figure 6: measurement of the alveolar internal diameter. It is striking that the administration of nanodiamond-VEGF (ND-VEGF + TO) particles correct the alveolar internal diameter in the pre-clinical CDH model to the same alveolar diameter as in the healthy animal.

Figure 7: measurement of pulmonary arteriole medial thickness. Administration of nanodiamond-VEGF (ND-VEGF + TO) particles restored the arteriole medial thickness to that observed in a healthy animal.

Detailed description of the invention

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings
5 described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is
10 specifically stated. Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated
15 herein.

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring
20 Harbor Press, Plainsview, New York (2012); and Ausubel et al., Current Protocols in Molecular Biology (Supplement 100), John Wiley & Sons, New York (2012), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

In the present invention we have shown that prenatal intra-pulmonary delivery of VEGF with
25 biocompatible nanoparticles induces dramatic lung growth and reverses structural alveolar and arterial abnormalities in an experimental animal model of congenital diaphragmatic hernia (CDH). While not limiting the invention to a particular mode of action of mechanism we believe that the lack of measurable effects of unconjugated VEGF suggests that gradual release - mimicking the spatial and temporal expression of VEGF in normal lung development - is a
30 requirement for bioactivity in this experimental model.

Accordingly in a first embodiment the invention provides nanodiamond particles which are chemically conjugated to VEGF. The term 'chemically conjugated' is equivalent to 'chemically coupled' and means that a chemical bond (id est a covalent) bound is present between the nanodiamond particles and the VEGF protein.

The term “nanodiamonds” is known to the skilled person and a review describing the properties and applications of nanodiamonds is available from Mochalin VN *et al* (2012) *Nature Nanotechnology* Vol 7, p. 11-23.

In a particular embodiment nanodiamond particles chemically conjugated to VEGF are used for treating respiratory distress syndrome in a mammal.

Respiratory distress syndrome (RDS) is a breathing disorder that affects newborns. RDS rarely occurs in full-term infants. The disorder is more common in premature infants born about 6 weeks or more before their due dates. RDS is more common in premature infants because their lungs are unable to make enough surfactant. Surfactant is a liquid that coats the inside of the lungs. It helps keep them open so that infants can breathe in air once they're born. Without enough surfactant, the lungs collapse and the infant has to work harder to breathe. In some cases, the baby is unable to inhale enough oxygen to support the body's organs. Without proper treatment, the lack of oxygen leads to irreversible organ and brain damage.

In yet another embodiment nanodiamond particles chemically conjugated to VEGF are used for treating lung hypoplasia.

In yet another embodiment nanodiamond particles chemically conjugated to VEGF are used for treating disorders wherein lung hypoplasia occurs.

Pulmonary hypoplasia or lung hypoplasia is incomplete development of the lungs, resulting in an abnormally low number or size of bronchopulmonary segments or alveoli. Lung hypoplasia most often occurs secondary to other fetal abnormalities that interfere with normal development of the lungs, such as congenital diaphragmatic hernia (CDH). Primary (idiopathic) pulmonary hypoplasia is rare and usually not associated with other maternal or fetal abnormalities. Incidence of pulmonary hypoplasia ranges from 9–11 per 10,000 live births and 14 per 10,000 births. Pulmonary hypoplasia is a common cause of neonatal death. It also is a common finding in stillbirths, although not regarded as a cause of these.

In yet another embodiment nanodiamond particles chemically conjugated to VEGF are used for treating lung hypertension.

Pulmonary hypertension or lung hypertension is an increase of blood pressure in the lung vasculature. The disorder is characterized by a narrowing of blood vessels connected to and with the lungs, with resultant thickening and stiffening of affected blood vessels (fibrosis), and increased blood pressure in the lungs and impairment of blood flow. Like pulmonary hypoplasia, pulmonary hypertension often develops in patients with CDH.

In yet another embodiment nanodiamond particles chemically conjugated to VEGF are used for treating lung hypertension and lung hypoplasia.

In yet another embodiment nanodiamond particles chemically conjugated to VEGF are used for treating congenital diaphragmatic hernia.

- 5 In yet another embodiment the invention provides nanodiamond particles conjugated to VEGF wherein the chemical linker between the nanodiamond particles and VEGF comprises an amide bond and a reducible disulfide bond.

In yet another embodiment the invention provides a method for producing nanodiamond particles chemically linked to VEGF comprising the following steps: i) reacting amino-
10 nanodiamond particles with sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate to generate ND-SPDP, ii) producing thiolated VEGF and iii) coupling the products obtained in steps i) and ii) to generate nanodiamond particles chemically linked to VEGF.

In a particular embodiment the nanodiamond conjugated VEGF particles of the invention release the VEGF part in a time period of between 2-14 days. In another particular
15 embodiment the nanodiamond conjugated VEGF particles of the invention release the VEGF part in a time period of between 4-14 days. In yet another particular embodiment the nanodiamond conjugated VEGF particles of the invention release the VEGF part in a time period of between 6-14 days. In a certain embodiment, the nanodiamond conjugated VEGF particles of the invention release the VEGF part in a time period of between 2-3 days.

20 Although the present invention exemplifies the use of VEGF-A chemically coupled to nanodiamond particles, the present invention is not limited to the VEGF-A isoform. The skilled person is aware that multiple isoforms of VEGF-A exist as a result from alternative splicing. For example in humans there also exists the VEGF121 isoform, the VEGF145 isoform, the VEGF189 and the VEGF206 isoform (the rodent orthologs of these proteins contain one fewer
25 amino acid). Thus it is clear that all these isoforms of VEGF are within the scope of the invention.

In a specific embodiment the nanodiamond particles conjugated to VEGF are formulated with a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably). The preferred administration of the nanodiamond particles conjugated to VEGF is intratracheal
30 administration via the fetal trachea. Preferably the intratracheal administration is followed by tracheal occlusion (TO).

Yet another way of administration of the nanodiamond particles conjugated to VEGF is intra-amniotic administration.

An amount effective to treat the disorders hereinbefore described depends on the usual factors such as the nature and severity of the disorders being treated and the weight of the mammal. However, a unit dose will normally contain 0.01 to 50 mg for example 0.01 to 10 mg, or 0.05 to 2 mg of the identified compound or a pharmaceutically acceptable salt thereof.

- 5 It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting
- 10 the application. The application is limited only by the claims.

Examples

1. Nanodiamond-VEGF particles improve lung growth in an experimental CDH model

The administration of prenatal nanodiamonds did not have noticeable adverse fetomaternal effects. Nanodiamonds coupled to Alexa Fluor (ND-FL) demonstrated efficient nanodiamond (ND) uptake by fetal pulmonary epithelium that was enhanced by tracheal occlusion (TO), see Figure 1. ND-FL were not found in any other fetal or maternal tissues, see Figure 2. Nanodiamond particles coupled to VEGF (ND-VEGF) in combination with TO was associated with improved lung growth (LBWR: $5.5 \pm 0.3\%$), which was significantly greater than that observed in VEGF+TO ($p < 0.01$), raw ND+TO ($p < 0.01$), vehicle+TO ($p < 0.01$) and sham ($p < 0.001$), but was found similar to healthy controls, see Figure 4. Moreover, ND-VEGF+TO resulted in thinner alveolar septa (mean transection length/airspace: 19.6 ± 1.8) see Figure 5, increased alveolar size (mean airspace chord length: 36.6 ± 2.2) see Figure 6, and decreased muscularisation of pulmonary arterioles (medial thickness: $20.3 \pm 0.9\%$), see Figure 7, compared to other treatment groups ($p < 0.01$ vs. all), but were similar to healthy controls. Although unconjugated VEGF+TO led to improvements in LBWR, airway and pulmonary vascular architecture, the effects did not exceed those seen in the vehicle+TO group. Co-administration of SU5416 (a VEGF Receptor-2 antagonist; Flk-1/KDR inhibitor) abrogated the beneficial effects of ND-VEGF, but did not affect outcomes in any other of the treatment groups.

We conclude that we have shown that prenatal intra-pulmonary delivery of VEGF with biocompatible nanoparticles induces dramatic lung growth and reverses structural arterial abnormalities via KDR/Flk-1 activation, when used in combination with TO in an experimental model of CDH. While not limiting the invention to a particular mode of action of mechanism we

tend to believe that the lack of measurable effects of unconjugated VEGF suggests that gradual release - mimicking the spatial and temporal expression of VEGF in normal lung development - is a requirement for bioactivity in this experimental model.

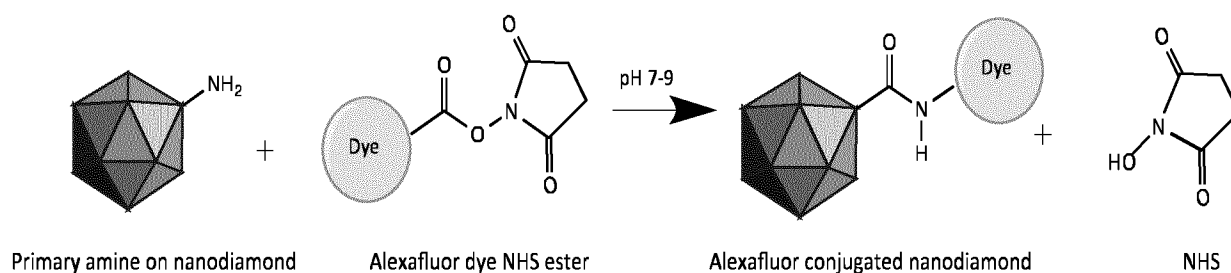
5 Materials and methods

1. General procedures

CDH was induced in fetuses of pregnant Wistar rats by administration of nitrofen at E9 (term=E22). Maternal laparotomy and hysterotomy were performed at E19, and nanodiamonds (ND; 2-8nm carbon nanoparticles; 75µg/mL in 50µL vehicle/saline) were administered into the fetal trachea followed by tracheal occlusion (TO). ND were either fluorescently-labelled (ND-FL), conjugated with recombinant VEGF164 (ND-VEGF; 2µg/mL VEGF164) or unlabelled/unconjugated (raw ND). Blinded assessment of lung-to-body weight ratio (LBWR), as well as airway and pulmonary vascular morphometric parameters was performed at E21.5 in CDH offspring. Comparisons were made between fetuses receiving ND-VEGF+TO, unconjugated VEGF+TO, raw ND+TO, vehicle+TO, and sham surgery. To examine the mechanism of action of VEGF in this setting, a potent and selective inhibitor of the VEGF-receptor-2 (KDR/Flk-1) was co-administered intra-tracheally to selected fetuses at the time of intervention (SU5416; 2mg/kg of fetal weight). Results are expressed as mean±SEM, and statistical analysis was performed using 1-way ANOVA with Bonferroni post-hoc tests.

2. Nanodiamond Alexa Fluor® (ND-AF)

To be able to track the localization of nanodiamonds in cells and in animals, nanodiamonds were labeled with Alexa Fluor® 488 or 647 carboxylic acid succinimidyl ester. The N-hydroxysuccinimide (NHS) ester is highly reactive towards primary amine groups and results in a stable amide bond (see scheme 1).



Scheme 1 depicts the NHS ester reaction chemistry between amine-terminated nanodiamond and Alexa Fluor® succinimidyl ester.

Alexa Fluor® 488 or 647 succinimidyl ester was reacted with ND-NH₂ particles at a weight ratio of 2:1, in a 50:50 volume mix of DMSO and 0.1 M sodium bicarbonate buffer (pH 8).

- 5 The reaction was maintained under stirring conditions for 3 hours, protected from light, and at room temperature. The solution was then washed 5 times with acetone and twice with ddH₂O via repeated centrifugation cycles (14.8K RPM, 99 minutes, 4°C). The resulting solution was subsequently transferred to a pre-weighed eppendorf tube and placed in a SAVANT DNA 120 speedvac concentrator (Thermo-Scientific, USA), to dry overnight.
- 10 Alexa Fluor® conjugated nanodiamond yield was calculated by subtracting the weight of the empty eppendorf tube from the weight of the eppendorf tube containing ND-AF particles.

3. Ultraviolet-visible spectroscopy (UV-Vis)

- 15 UV-Vis spectroscopy measures the optical absorbance of samples in the visible and near visible (near-infrared, near-UV) regions, and is a reflectance of electronic transitions from the ground to the excited states(19). UV-Vis measurements were performed to ascertain fluorophore conjugation to the ND surface and to quantify the amount of cargo functionalized.

4. Nanodiamond-fluorophore conjugates characterization

- 20 FITC conjugation was confirmed via a Jaz Oceanic Optics Spectrophotometer (USA), with the appearance of a 490 nm absorbance peak. Alexa Fluor® dye conjugation and the amount of dye conjugated to the ND surface was determined using a Varian Cary 300 Bio UV/Vis instrument and a Cary WinUV software package (Agilent, USA).

- 25 In essence, AF and ND-AF solutions were diluted 1 in 1000 in ddH₂O and their absorbance measured at 650 nm and at 494 nm for AF647 and 488 dyes, respectively. Dye concentration was determined using the Beer-Lambert equation: $A = \epsilon \cdot l \cdot c$, where A = absorbance (a.u.), ϵ = molar extinction coefficient ($M^{-1}cm^{-1}$), l = path length (cm), c = concentration (M); given a path length of 1 cm and molar extinction coefficients of 239,000 $M^{-1}cm^{-1}$ and 71000 $M^{-1}cm^{-1}$ for AF647 and 488, respectively. Concentrations were then multiplied by the dilution factor (i.e., 1000) and the degree of AF labeling on the ND surface was calculated using the following equation: (Concentration of dye on ND surface / Concentration of free dye) x 100%

5. Nanodiamond Vascular Endothelial Growth Factor (ND-VEGF) Coupling

ND-VEGF conjugation was a sequential process. ND-NH₂ particles were first reacted with the
 sulfhydryl-to-amine cross-linker sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-

propionamido)hexanoate (Sulfo-LC-SPDP), which contains an amine-reactive NHS ester group and a thiol-reactive pyridyldithiol group. The NHS ester arm of the linker reacts with amine terminated nanodiamond particles to form a stable amide bond, while the pyridyldithiol arm reacts with thiol-modified VEGF to yield reducible disulfide bonds(13-15) (see Figure 3).

5 (a) Nanodiamond SPDP (ND-SPDP) Functionalization:

Sulfo-LC-SPDP (Thermoscientific, USA) was reacted with ND-NH₂ particles overnight, at a molar excess of 377 in 0.1 M sodium bicarbonate buffer (pH 8).

10 The reaction was performed under stirring conditions, and at room temperature. The resulting solution was then washed thrice with ddH₂O via repeated centrifugation cycles (14.8K RPM, 99 minutes, 4°C), and subsequently transferred to a pre-weighed eppendorf tube and placed in a SAVANT DNA 120 speedvac concentrator (Thermo-Scientific, USA), to dry overnight.

ND-SPDP yield was calculated by subtracting the weight of the empty eppendorf tube from the weight of the eppendorf tube containing ND-SPDP particles.

(b) VEGF Thiolation:

15 2-Iminoethanol.HCL (Traut's reagent, Thermo-Scientific, USA) is a thiolation compound that reacts with primary amines to yield accessible sulfhydryl groups(27, 28).

20 Traut's reagent was reacted with lyophilized rat VEGF (in PBS) at a molar ratio of 4:1 in 0.1 M sodium bicarbonate buffer (pH 8), for 4 hours under stirring conditions, and at room temperature. The resultant mixture was then filtered through illustra NAP-10 columns (GE Healthcare Life Sciences, UK) to permit solvent exchange of residual PBS with sodium bicarbonate buffer.

(c) Bicinchoninic Acid (BCA) Protein Assay:

To quantify the yield of VEGF resulting from the thiolation reaction, a BCA assay (Thermo-Scientific, USA) was performed.

25 The BCA assay is a two-step reaction based on the reduction of cupric ions (Cu²⁺) to cuprous ions (Cu⁺) by protein, in alkaline medium (the biuret reaction). In the second step of the reaction, bicinchoninic acid reacts with the newly reduced cuprous ions (Cu⁺), resulting in a blue to purple color shift that can be detected calorimetrically(29, 30). A set of standards made of Bovine Serum Albumin (BSA) protein were used in this assay and ranged, in concentration,
30 from 31.25 µg/ml to 2000 µg/ml. In a 96-well plate, 20 µl of each standard and sample was added to 180 µl of BCA working reagent. The plate was then incubated at 37°C for 30 minutes and the absorbance measured at 550 nm, using a Titertek Multiscan MCC/340 plate reader

(Labsystems, Finland). Absorbance values from samples were compared to a generated standard curve to determine sample protein concentrations.

(d) Nanodiamond VEGF (ND-VEGF) Coupling:

ND-SPDP functionalized particles were reacted with thiolated VEGF at a molar ratio of 1:1.

- 5 The reaction was performed in 0.1 M sodium bicarbonate buffer (pH 8) overnight under stirring conditions, and at room temperature. The following day, the resulting product was washed thrice with ddH₂O, via sequential ultracentrifugation cycles (45000 RPM, 90 mins, 4°C), using a Beckman Ultracentrifuge with a T100.2 rotor (Beckman Coulter, USA), and resuspended in 1 ml of ddH₂O.

10 6. Enzyme-Linked Immunosorbent Assay (ELISA) for VEGF Quantification

An ELISA assay was performed, using a rat VEGF ELISA kit (Abcam, UK), to determine the quantity of VEGF conjugated to the nanodiamond surface.

- 15 The assay was used according to manufacturer protocols. Briefly, 100 µl of ND-VEGF samples of various dilutions and VEGF standards (supplied with the kit) were pipetted into a 96-well plate, pre-coated with rat VEGF antibody, in duplicate and incubated at 4°C overnight on a horizontal orbital microplate shaker at 500 RPM.

- The plate was washed 4 times with wash buffer, using an automated Denley Cellwash plate washer (Triad Scientific, USA) and 100 µl of biotinylated VEGF detection antibody was added to each well and incubated for 1 hour, with gentle shaking at room temperature. Following
20 which, the plate was washed 4 times and 100 µl of horseradish peroxidase streptavidin was added to each well for 45 minutes, in order to bind to the biotinylated detection antibody. The plate was subsequently washed 4 times and 100 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (chromogenic solution that develops proportionally to the amount of VEGF bound) was added to each well and incubated for 30 minutes, protected from light with gentle shaking.
25 50µl of stop solution was then added to quench the substrate reaction and the plate absorbance was read at 450 nm, using a Titertek Multiscan MCC/340 plate reader (Labsystems, Finland). Absorbance values from samples were compared to a generated standard curve to ascertain the concentration of VEGF, grafted onto NDs.

Claims

1. Nanodiamond particles chemically coupled to VEGF.
2. Nanodiamond particles of claim 1 for treating lung hypertension.
3. Nanodiamond particles of claim 1 for treating lung hypoplasia.
- 5 4. Nanodiamond particles of claim 1 for treating lung hypoplasia and lung hypertension.
5. Nanodiamond particles of claim 1 for treating congenital diaphragmatic hernia.
6. Nanodiamond particles linked to VEGF wherein the chemical linker comprises an amide bond and a reducible disulfide bond.
7. Nanodiamond particles of claim 6 for treating lung hypertension.
- 10 8. Nanodiamond particles of claim 6 for treating lung hypoplasia.
9. Nanodiamond particles of claim 6 for treating lung hypoplasia and lung hypertension.
10. Nanodiamond particles of claim 6 for treating congenital diaphragmatic hernia.
11. A method of producing nanodiamond particles chemically linked to VEGF comprising the following steps: i) reacting amino-nanodiamond particles with Sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate to generate ND-SPDP, ii) producing thiolated VEGF and iii) coupling the products obtained in steps i) and ii) to generate
15 nanodiamond particles chemically linked to VEGF.

Figure 1

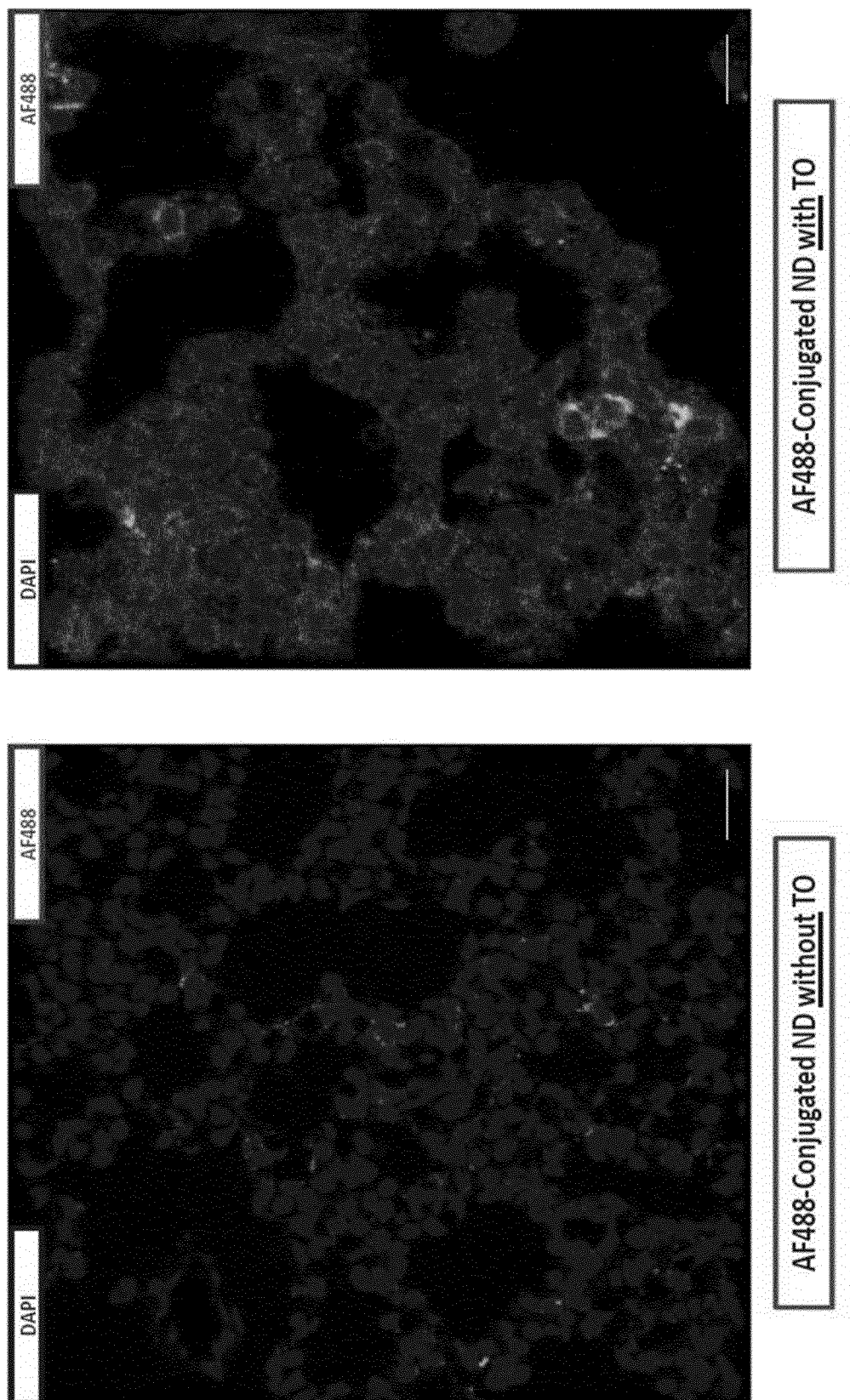


Figure 2

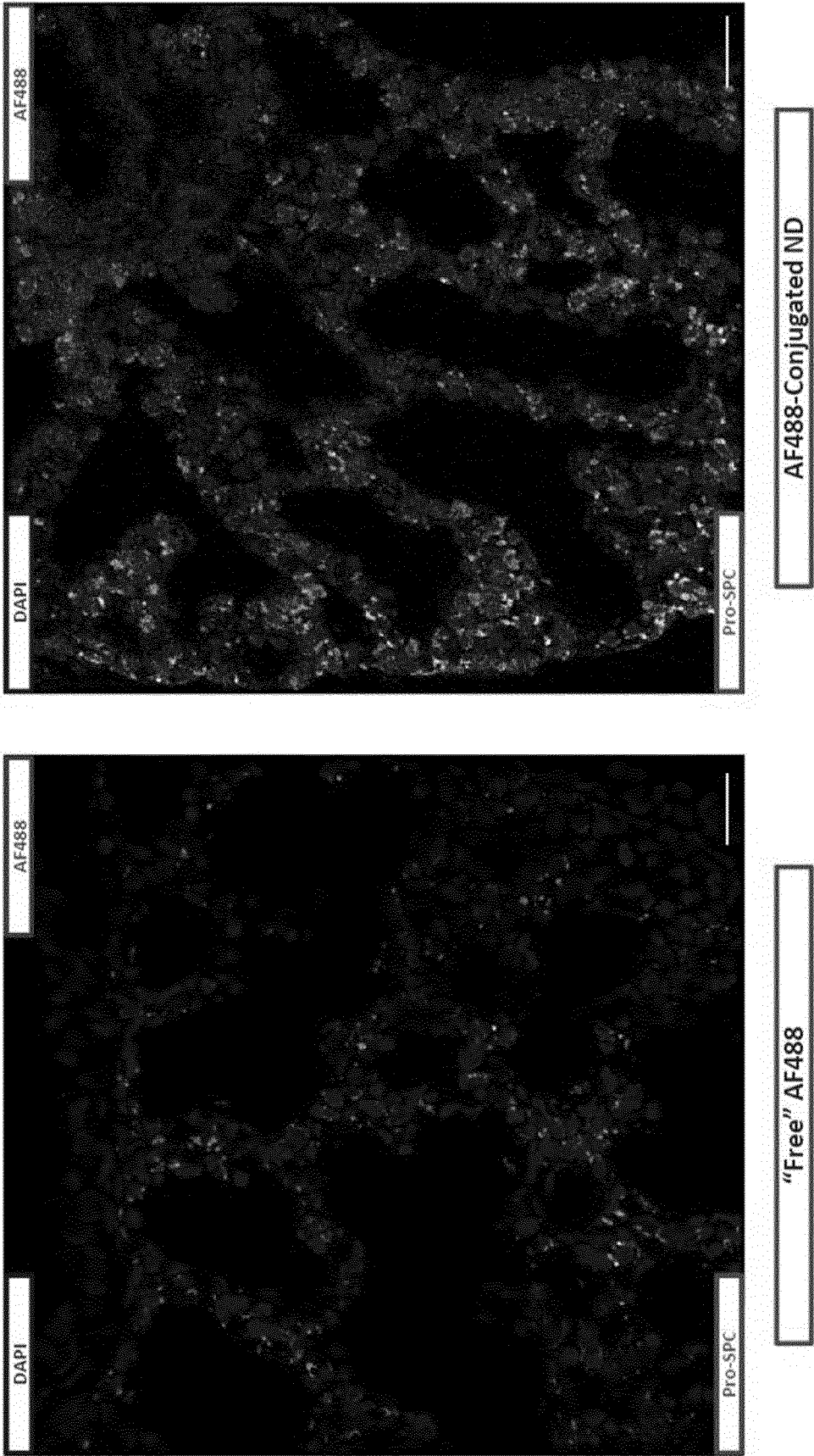


Figure 3

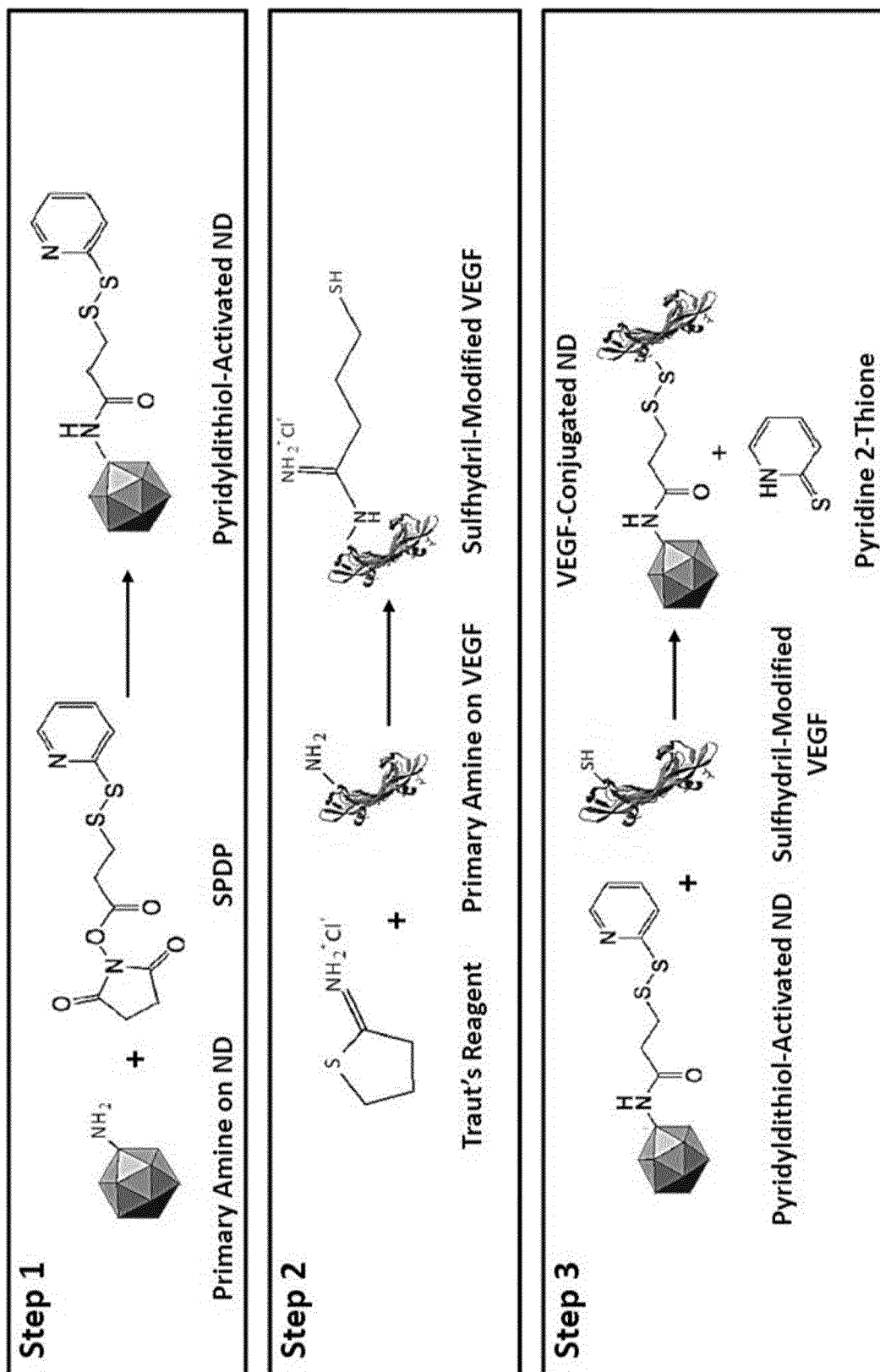


Figure 4

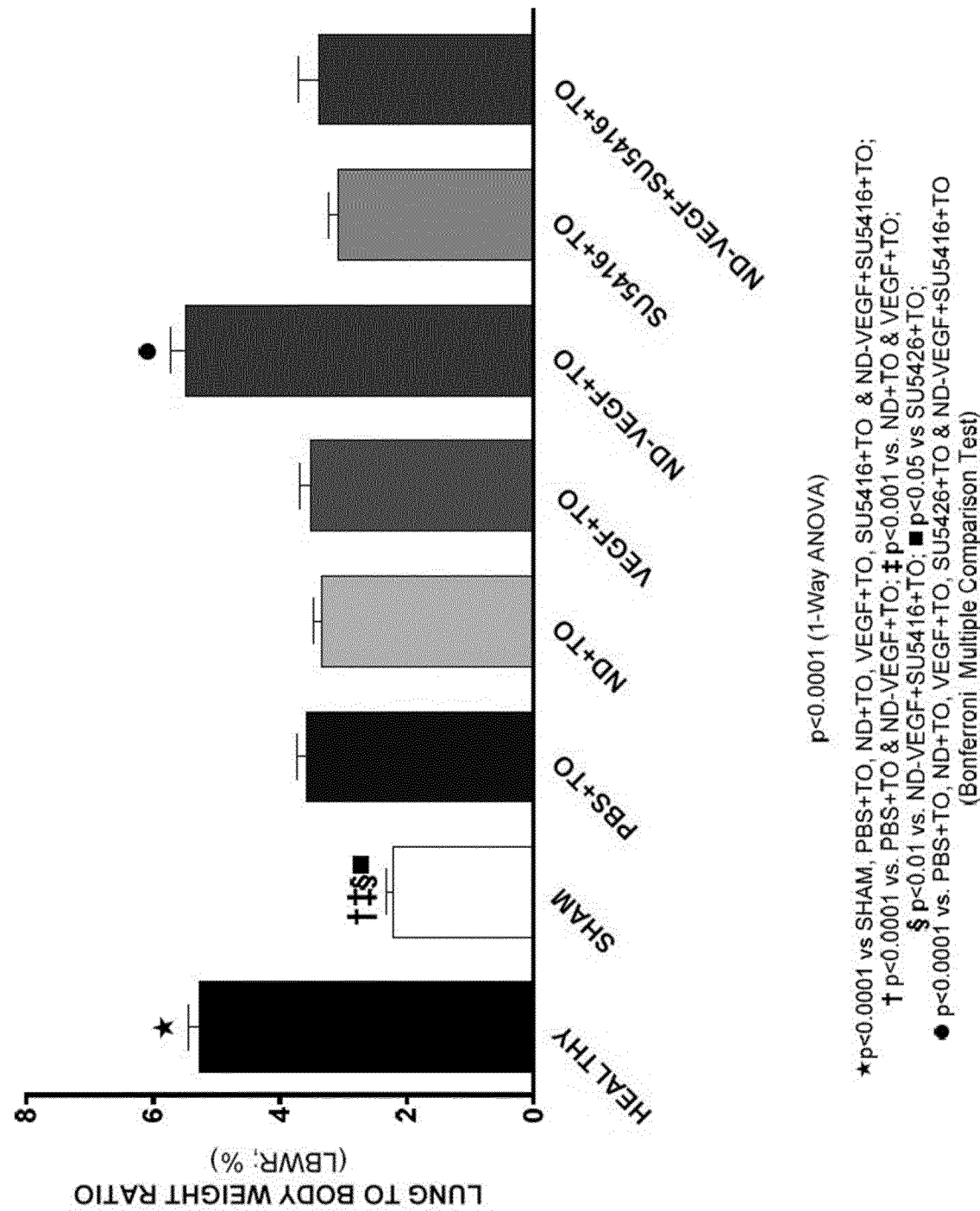


Figure 5:

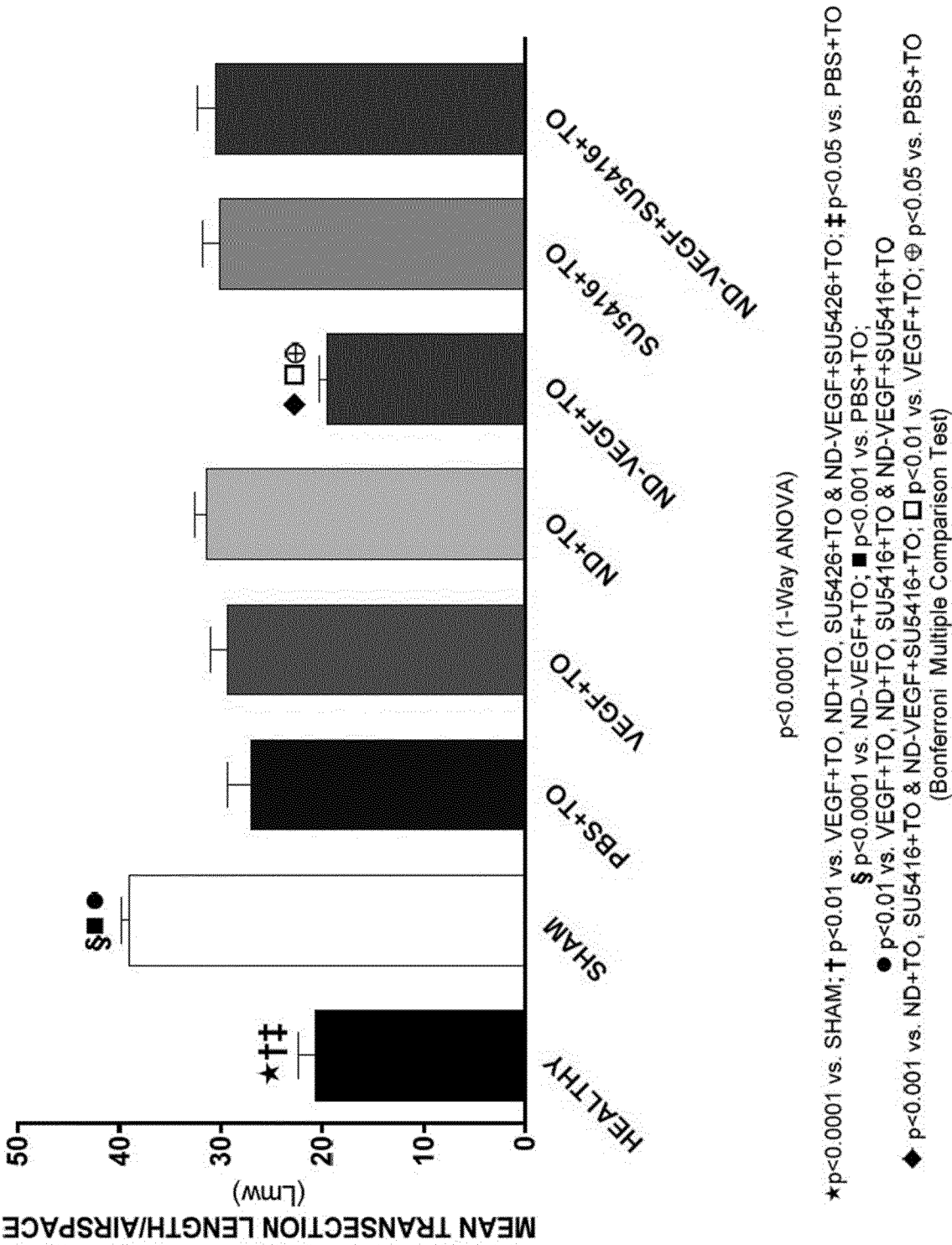


Figure 6

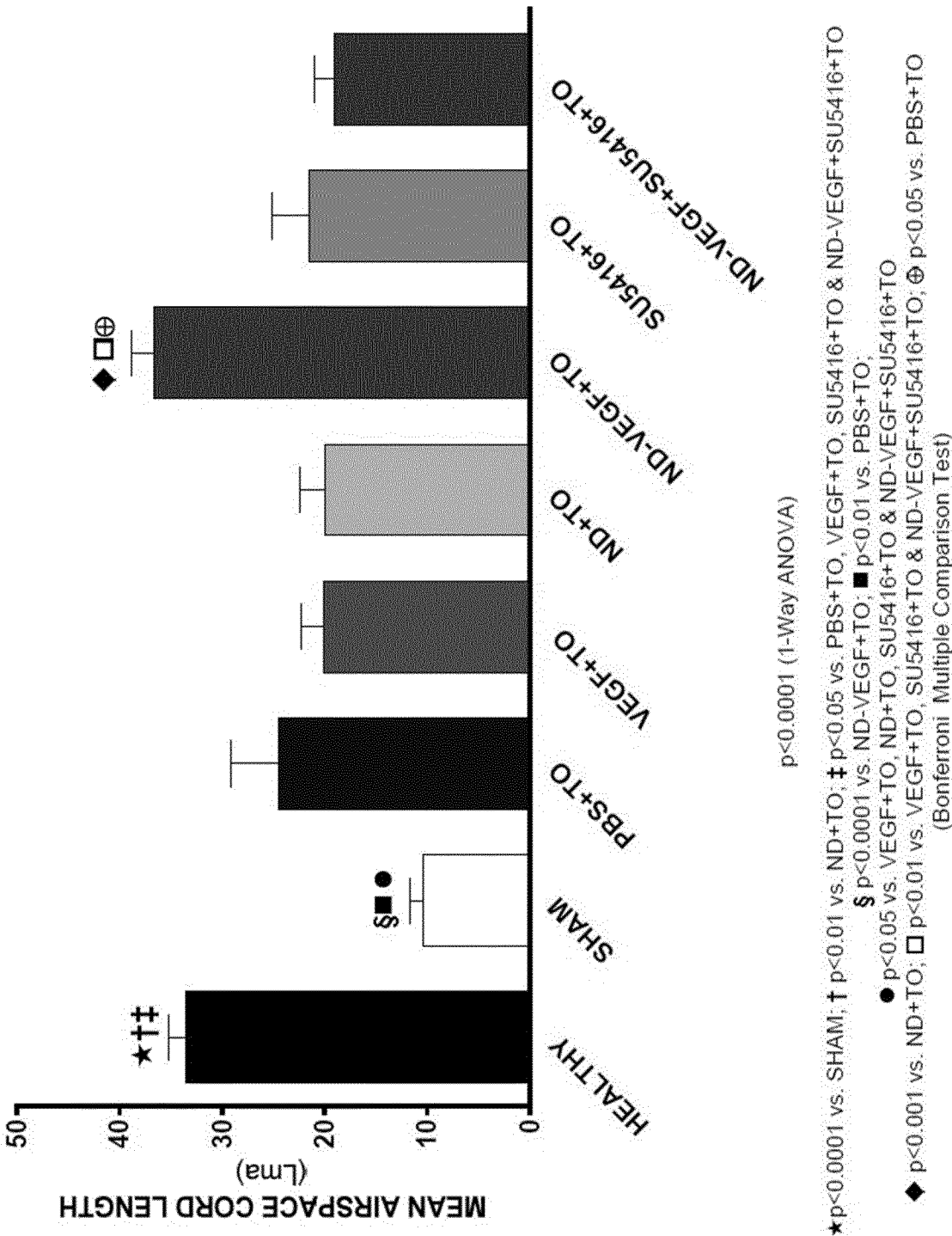
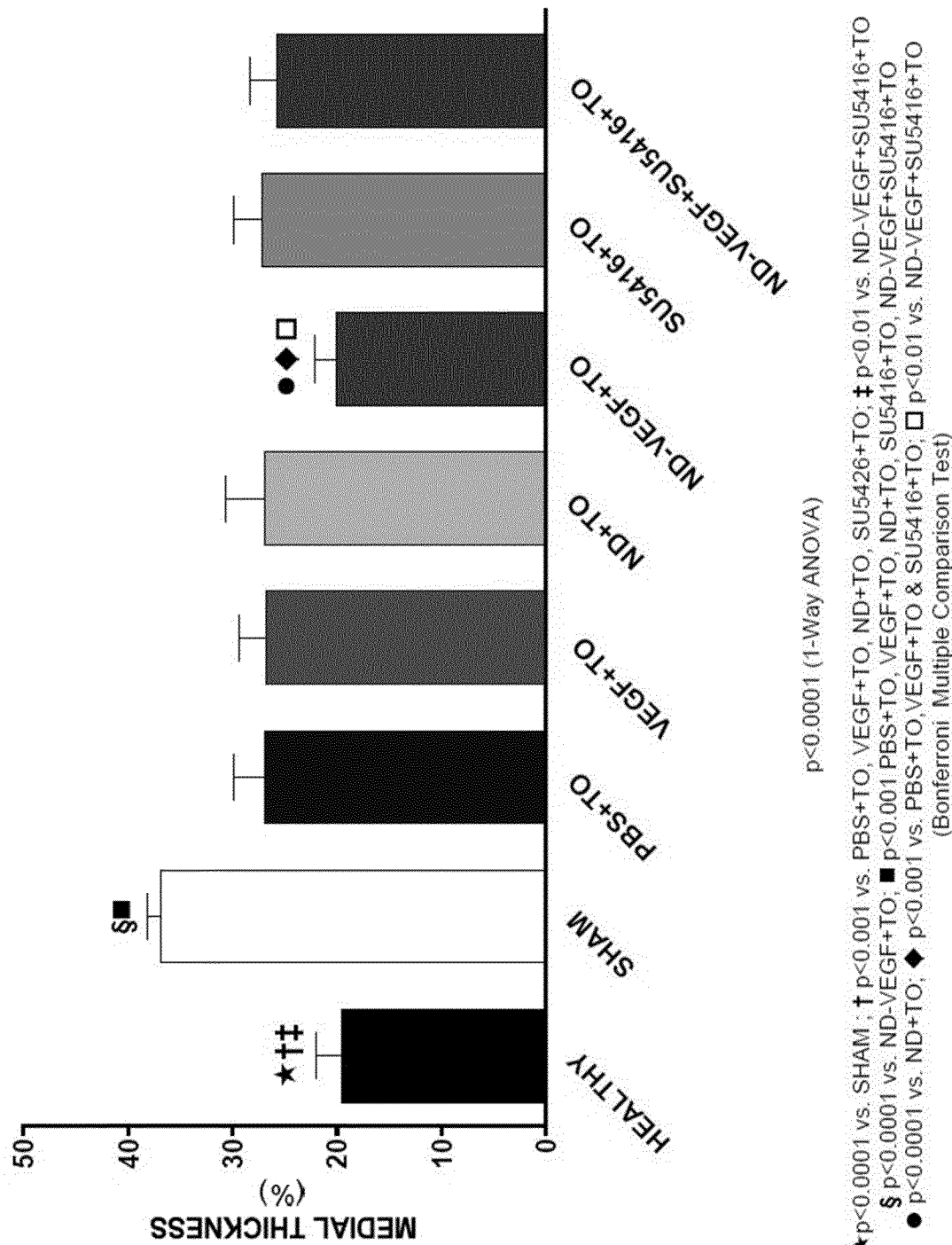


Figure 7



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/063214

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K47/48 A61K38/18 A61P11/00 B82Y5/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K B82Y

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KLARITSCH P ET AL: "439: Intra-amniotic VEGF administration in fetal rats with nitrofen induced lung hypoplasia - A placebo controlled study", AMERICAN JOURNAL OF OBSTETRICS & GYNECOLOGY, MOSBY, ST LOUIS, MO, US, vol. 199, no. 6, 1 December 2008 (2008-12-01), page S131, XP025985790, ISSN: 0002-9378, DOI: 10.1016/J.AJOG.2008.09.468 [retrieved on 2008-12-01] abstract ----- -/--	1-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/063214

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	DEBEER A ET AL: "Antenatal fetal VEGF therapy to promote pulmonary maturation in a preterm rabbit model", EARLY HUMAN DEVELOPMENT, SHANNON, IR, vol. 86, no. 2, 1 February 2010 (2010-02-01), pages 99-105, XP026939808, ISSN: 0378-3782, DOI: 10.1016/J.EARLHUMDEV.2010.01.025 [retrieved on 2010-02-23] abstract -----	1-11
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A	VADYM N. MOCHALIN ET AL: "The properties and applications of nanodiamonds", NATURE NANOTECHNOLOGY, vol. 7, no. 1, 18 December 2011 (2011-12-18), pages 11-23, XP55133599, ISSN: 1748-3387, DOI: 10.1038/nnano.2011.209 cited in the application page 20, section "Drug delivery" -----	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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