Protein synthesis 

LAM cells migration"— EMT

FIG. 14
src kinase inhibition as treatment for lymphangioleiomyomatosis and tuberous sclerosis

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technical field

[0002] The present disclosure generally concerns at least the fields of cell biology, molecular biology, and medicine.

background of the invention

[0003] Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutation in either the tuberous sclerosis complex 1 (TSC1) or TSC2 tumor suppressor genes (1). Lymphangioleiomyomatosis (LAM), pulmonary manifestation of TSC (2), is a progressive cystic lung disease affecting primarily women of childbearing age. LAM affects 30-40% of women with TSC (3,4) and is characterized by abnormal and potentially metastatic growth of atypical smooth muscle-like LAM cells within lungs and axial lymphatics. Clinical and genetic data suggest a link between the loss of TSC2 function and cell invasion and metastasis. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that positively regulates cell growth, proliferation, and survival (5). TSC2 is a negative regulator of the mTOR complex 1 (mTORC1) (6,7). Therefore, hyper-activation of mTORC1 and inhibition of autophagy are observed in TSC2/- LAM cells (8). However, many of the clinical and pathological features of LAM remain unexplained by our current understanding of the function of these genes. Activation of mTORC1 is sensitive to inhibition by rapamycin, which has been used in the treatment of LAM (9,10). Rapamycin treatment improved pulmonary functions and reduced the size of angiomyolipoma (AML) in TSC and LAM patients. Unfortunately, cessation of rapamycin therapy was followed by regrowth of tumors and the decline of pulmonary functions (9,10). Accordingly, alternative or combinational therapies are needed to treat LAM. Identification of novel therapeutic targets, other than mTOR, would allow such therapy.
BRIEF SUMMARY

[0004] Embodiments of the disclosure are directed to methods and/or compositions for use in medicine, such as at least treatment of a medical condition in which Src kinase is activated or at levels above normal. In particular embodiments, the medical condition is not one or more types of cancer. In particular embodiments, cells in the individual with the particular medical condition have activation of Src kinase and/or downregulation of E-cadherin. In certain embodiments, the individual has lymphangioleiomyomatosis or tuberous sclerosis complex. In some cases, an individual with tuberous sclerosis complex also has lymphangioleiomyomatosis.

[0005] In embodiments, an individual with lymphangioleiomyomatosis or tuberous sclerosis complex is treated with one or more inhibitors of Src kinase (which may alternatively be referred to as "Src"). The individual may have one or more symptoms of lymphangioleiomyomatosis or tuberous sclerosis complex. The individual may have been diagnosed with lymphangioleiomyomatosis or tuberous sclerosis complex. In some cases, the individual is a female that has one or more of the following symptoms: shortness of breath on exertion; cough; hemoptysis, as a result of vascular congestion; recurrent pneumothorax and/or chylous pleural effusion and chylous ascites, as a result of lymphatic obstruction.

[0006] Aspects of the disclosure encompass the use of Src inhibitors for treatment of a medical condition, such as lymphangioleiomyomatosis or tuberous sclerosis complex. An individual with tuberous sclerosis complex may or may not also have lymphangioleiomyomatosis.

[0007] Aspects of the disclosure encompass a pharmaceutical composition for the treatment of lymphangioleiomyomatosis and/or tuberous sclerosis complex, comprising one or more Src inhibitors.

[0008] Aspects of the disclosure encompass the use of a Src inhibitor in the manufacture of a medicament for the treatment of lymphangioleiomyomatosis and/or tuberous sclerosis complex.

[0009] Any suitable Src inhibitor may be employed for treatment of lymphangioleiomyomatosis or tuberous sclerosis complex. In some cases, more than one Src inhibitor is employed. In particular aspects, in addition to being treated with one or more Src inhibitors, the individual is also treated with one or more mTOR inhibitors.
[0010] Embodiments of the disclosure include the use of AZD0530 (saracatinib), which is Src tyrosine kinase inhibitor, as a novel therapy for subjects with lymphangioleiomyomatosis and tuberous sclerosis complex. Saracatinib is a potent orally bioavailable and well tolerated Src inhibitor.

[0011] The present disclosure shows that Src is activated in TSC2−/− cells and that increased Src activation contributes to down regulation of E-cadherin and raises the oncogenic abilities of these cells. Thus, Src inhibition represents a therapeutic strategy to up-regulate E-cadherin in TSC2−/− cells and reduce their oncogenic and metastatic potential. The increased Src activity in TSC2−/− cells, in particular aspects, is caused by inhibition of autophagy associated with hyper-activation of mTOR. In certain embodiments, autophagy is one mechanism to eliminate excessive activated Src kinase in TSC2−/− cells. In particular embodiments, the use of Src inhibitors, either alone or in combination with mTOR inhibitors, leads to a reduction in tumor growth and prevents dissemination of TSC2−/− cells.

[0012] In embodiments, there is a method of treating lymphangioleiomyomatosis or tuberous sclerosis complex in an individual, comprising the step of providing to the individual a therapeutically effective amount of at least one sarcoma (Src) kinase inhibitor. In specific embodiments, the Src kinase inhibitor is selected from the group consisting of Saracatinib, Dasatinib, Ponatinib, PPl, PP2, PP3, Bosutinib, KX2-391, PPI, NVP-BHG712, A 419259 trihydrochloride, AZM 475271, Damnacanthal, Herbimycin A, KB SRC 4, Lavendustin A, Lyn peptide inhibitor, MNS, 1-Naphthyl PPI, PD 166285 dihydrochloride, PD 180970, Piceatannol, pp60 c-src (521-533) (phosphorylated), Src II, BMS-354825, KX2, 391, XL-228, TG100435/TG100855, DCC2036, a combination thereof, and functional derivatives thereof.

[0013] In certain cases, a method for treating lymphangioleiomyomatosis or tuberous sclerosis complex in an individual further comprises the step of providing to the individual a therapeutically effective amount of at least one mammalian target of rapamycin (mTOR) inhibitor. The mTOR inhibitor may be selected from the group consisting of rapamycin, epigallocatechin gallate (EGCG), caffeine, curcumin, resveratrol, temsirolimus, everolimus, Ridaforolimus, BEZ235, AZD8055, KU-0063794, PP242, INK 128, GDC-0349, PI-103, WYE-354, GSK1059615, SAR245409, PP-121, OSI-027, PKI-587, GSK2126458, WAY-600, WYE-125132, WYE-687, GDC-0980, CH5132799, PF-04691502, NVP-BGT226, AZD2014, Torin 1, and Torin 2.
[0014] In certain cases, a method for treating lymphangioleiomyomatosis or tuberous sclerosis complex in an individual further comprises the step of providing to the individual a therapeutically effective amount of an additional therapy for the individual with lymphangioleiomyomatosis or tuberous sclerosis complex. The additional therapy for lymphangioleiomyomatosis may comprise anti-estrogen therapy, inhibition of interleukin-2, lung transplantation, statin, anti-vascular endothelial growth factor-D (VEGF-D) therapy, anti-Collagen I therapy, fulvestrant; inhibitors of the dual-specificity kinases MEK-1 and MEK-2; rho kinase inhibitors; the NCI domain of the alpha3 chain of type IV collagen; biguanide, a glitazone, a sulphonylurea, a sulfonamide, AMPK activator, mTOR inhibitor, an alpha-adrenergic antagonist, human interferon-gamma, an agent that increases endogenous interferon-gamma levels, or a combination thereof, for example. The additional therapy for tuberous sclerosis complex may comprise surgical resections therapy, for example.

[0015] In certain cases, a method for treating lymphangioleiomyomatosis or tuberous sclerosis complex in an individual further comprises the step of examining the individual for at least one symptom of lymphangioleiomyomatosis or tuberous sclerosis complex.

[0016] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.
BRIEF DESCRIPTION OF THE DRAWINGS

[0017] For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawing, in which:

[0018] Fig. 1. Sections of normal and LAM lungs were stained with LAM cell marker HMB45 antibody (A), or lysed and analyzed by immunoblot using antibodies against S6 and phospho-S6 (B), p62 (C), Src kinase and phospho-Src (Y416) kinase (D), or STAT3 and phospho-STAT3 (Y705) (E). Blotting with β-actin antibody was used as a loading control. Scale bar, 100 µm.

[0019] Figure 2. Activation of Src and STAT3 in TSC2-/− cells (EEF8). (A-B) Cell lysates of EEF4 and EEF8 were subjected to immunoblot using antibodies against Src kinase and phospho-Y416-Src (A) or STAT3 and phospho-Y705-STAT3 (B). (C) EEF4 and EEF8 cells were fixed and immunolabeled by phospho-STAT3 antibodies followed by IgG conjugated to Alexa Fluor 594 (red). Cells were stained with 4’,6-diamidino-2-phenylindol dihydrochloride (DAPI) to visualize nuclei (blue). Graph indicates percentage of pStat3 (nuclear localization) positive cells (n=3). (D) EEF4 and EEF8 cells were treated with either DMSO or Src kinase inhibitors PP2 (25 µM), or SU6656 (Su: 10 µM) for 4 hr prior to lysis of cells. Cell lysates were subjected to immunoblot analysis with indicated antibodies. (E) HeLa cells were transfected with control nontarget (NT) siRNA or TSC2-specific siRNA or for 72 hr. Cells were lysed and immunoblot was done. Graphs indicate quantification of phospho-Src and phospho-Stat3, normalized to total Src or STAT3 expression, respectively, n=3. (F) EEF4 cells were transfected for 24 hr with Src kinase plasmid or LacZ control plasmid. Cell lysates were analyzed, compared to EEF8, by immunoblotting. Blotting with β-actin antibody was used as a control. Data are mean ± SD, *P<0.05, **P<0.001. Scale bar, 10 µm.

[0020] Figure 3. Src kinase mediated phosphorylation of focal adhesive kinase (FAK) and activation of MAPK pathway in in TSC2-/− cells (EEF8). (A-E) EEF4 and EEF8 cells were analyzed by Real time PCR (A) or by immunoblot (B-D). Graphs indicate the quantification of the immunoblots, n=3-6. (F) EEF4 cells were transfected for 24 hr with a plasmid encoding Src kinase or LacZ as a control and analyzed by immunoblot. Graph indicates quantification of the immunoblots. Phospho-Erk(Thr202/Tyr204) was normalized to total Erk expression, n=3. (G) EEF8 cells were transfected with Src specific siRNA or
control (NT) siRNA for 72 hr and analyzed by immunoblot. Graph indicates the quantification of the immunoblots, n=4. (H) EEF8 cells were treated for 4 hr with either DMSO as a vehicle or with Src kinase inhibitors PP2 (25 µM), SU6656 (10 µM), dasatinib (0.5 µM) or Saracatinib (1 µM) and then analyzed by immunoblot. Blotting with actin antibody was used as a loading control. Data represent mean ± SD, n >3, **P<0.001.

[0021] Figure 4. TSC2 deficiency or overexpression of Src promotes EMT. EEF4 and EEF8 cells or human lungs (E) were analyzed by immunoblots, immunofluorescence microscopy or real time PCR (graphs in C and F) to evaluate E-cadherin (A-B), Snail (C-E), or MMP9 (F). In B and D, cells were stained with DAPI to visualize nuclei (blue). In (G), EEF4 cells were transfected for 16 hr with Src plasmid or LacZ control vector and analyzed by immunoblot. β-actin antibody was used as a loading control. Graphs in G indicate the quantification of the immunoblots. Data represent mean ± SD, n ≥ 3. **P<0.001. Scale bar, 10 µm.

[0022] Figure 5. Src inhibition reduces EMT markers in TSC2-/- cells. EEF8 cells were treated with vehicle only (DMSO), dasatinib (Dasa; 0.5 µM), saracatinib (Sara; 1 µM) or rapamycin (Rapa; 1μg/ml) for 24 hr. (A) Snail expression was analyzed by real time PCR (upper panel) or by immunoblot (lower panel), n=4. (B-C) Cell lysates were subjected to immunoblot analysis using antibodies against Src, phospho(Y416)-Src and Snail (B), or MMP9 (C). Graphs indicate the quantification of the immunoblots, n=3. (D) Cell lysates were analyzed by zymogram of Gelatin. Graph indicates the quantification of the zymogram, n=6. (E) EEF8 cells were transfected for 72 hr with Src-specific siRNA or non-target (NT) siRNA and analyzed by immunoblot. Graph indicates the quantification of the immunoblots, n=4. (F) Cell lysates of (E) were analyzed for MMP9 mRNA using real time PCR (n=4). Data are mean +SD, *p < 0.05.

[0023] Figure 6. Src inhibition attenuates migration activity and invasiveness of TSC2-/-cells. (A) EEF8 cells were grown to confluence in 100 mm tissue culture plate and scratched with sterile pipet tip; DMSO, rapamycin (1μg/ml), dasatinib (0.5 µM) or saracatinib (1µM) was added to cultures. Scratched areas were imaged at the start (0 h) and 18 hr later to assess the degree of wound healing. Representative images are shown. (B) Quantification of cell motility was done by measuring the wound areas in 6 random fields for each treatment condition. (C) The invasive properties of EEF4 and EEF8 cells were studied using Matrigel inserts. Serum-deprived cells (5x104 cells) were loaded in the upper
compartment of the chambers. DMSO, rapamycin, dasatinib or saracatinib was added for 18 hr. Cells on the surface of the Matrigel were visualized by staining with 1% Toluidine blue. Representative images are shown. (D) The invading cells were counted in 6 random fields. Data are expressed as mean ± SD, n ≥ 3. **P<0.001. Scale bar, 100 μm.

[0024] Figure 7. Src inhibition reduces lung colonization of TSC2/- cells in vivo. EEF8-Luciferase cells were treated for 18 hr with DMSO, rapamycin (Rapa; 1 μg/ml), saracatinib (Sara; 1μM) or by both rapamycin and saracatinib. Cells were then injected intravenously into female CB17 SCID mice and after 6 hr lung colonization was measured using bioluminescence. Representative images are shown (A). Total photon flux/second present in the chest region after injection of EEF8 cells is expressed as a percentage of DMSO treated EEF8 (B). Lungs were dissected 24 hr post cell injection and bioluminescence was imaged in Petri dish (C). Total photon flux/second present in the dissected lungs after injection of EEF8 cells is expressed as a percentage of DMSO treated EEF8 (D). Data are mean ± SD, n ≥ 3. *P<0.05, **P<0.001 compared to DMSO treated cells.

[0025] Figure 8. mTOR activation in TSC2-deficient cells. EEF4 and EEF8 cells were analyzed by immunoblot using antibodies against Tuberin (A), mTOR and phospho- mTOR (B), phospho-p70S6 kinase, ribosomal phospho-S6 and S6 proteins (C), or p62 and LC3 (D). β-actin antibody was used as a loading control. p62 mRNA level was measured using real time PCR (E). Data are expressed as mean ± SD, n ≥ 3.

[0026] Figure 9. Knockdown of TSC2 in Hela cells up-regulates mTOR activity. Hela cells were transfected for 72 hr with 50 nM TSC2-specific siRNAs or control (NT) siRNA. Cells were lysed and immunoblot analysis was done to evaluate tuberin, S6 and phospho-S6 ribosomal protein. Blotting with β-actin antibody was used for loading control.

[0027] Figure 10. Autophagy inhibition results in Src kinase activation. (A-B) EEF4 cells were transfected for 72 hr with ATG7-specific siRNA or control (NT) siRNA. Untreated EEF4 cells were used as a control (ctrl). Immunoblot analysis was done on cell lysates. Graph indicates the quantification of the immunoblots. Phospho-Src was normalized to total Src expression, n=3. (C) ATG7+/+ or ATG7-/- mouse embryonic fibroblasts were lysed and immunoblot was performed. (D) ATG5+/+ or ATG57- mouse embryonic fibroblasts were lysed and immunoblot was performed. (E) EEF4 cells were treated with chloroquine (CQ;
50 µM) for 48 hr and immunoblot analysis was done. (F) EEF8 cells were starved for 4 hr and then lysates were analyzed by immunoblot. Graph indicates the quantification of the immunoblots, n=4. Blotting with β-actin antibody was used as a control.

[0028] Figure 11. Src kinase inhibitors decrease proliferation of TSC2-/- cells. (A) Equal numbers of EEF4 and EEF8 cells were cultured for 24 hr and cell proliferation was evaluated using XTT dye. (B) EEF8 cells were incubated, for 24 h, with DMSO, rapamycin (1 µg/ml), dasatinib (0.5 µM) or saracatinib (1 µM) and cell proliferation was estimated. Data are expressed as mean ± SD, n ≥ 3, *P<0.05, **P<0.001.

[0029] Figure 12. Effects of Src inhibition on migration activity and invasiveness of EEF4 cells. (A) EEF4 cells were grown to confluence in 100 mm tissue culture plates and scratched with sterile pipet tip; DMSO, rapamycin (lug/ml), dasatinib (0.5 uM) or saracatinib (luM) was added. Scratched areas were imaged at the start (0 hr) and 18 hr later to assess the degree of wound healing. Representative images are shown. (B) Quantification of cell motility was done by measuring the wound areas in 6 random fields for each treatment condition. (C) The invasive properties of EEF4 cells were studied using Matrigel inserts. Serum-deprived cells (5x10^4 cells) were loaded in the upper compartment of the chambers. DMSO (as a vehicle), rapamycin (µg/ml), dasatinib (0.5 µM) or saracatinib (1 µM) was added for 18 hr. Cells on the surface of the Matrigel were visualized by staining with 1% Toluidine blue. Representative images are shown. (D) The invading cells were counted in 6 random fields. Data are mean ± SD, n ≥ 3, **P<0.001.

[0030] Figure 13. Effect of Src inhibition on lung colonization of EEF4 cells in vivo. (A) EEF4-Luciferase cells were treated for 18 hr with DMSO or saracatinib (Sara; 1µM). Cells were then injected intravenously into female CB17 SCID mice and after 6 hr lung colonization was measured using bioluminescence. Representative images are shown. (B) Total photon flux/second present in the chest region after injection of EEF4 cells is expressed as a percentage of DMSO treated EEF4 (n=3). (C) Lungs were dissected 24 hr post cell injection and bioluminescence was imaged in Petri dish. (D) Total photon flux/second present in the dissected lungs after injection of EEF8 cells is shown as a graph (n=3). Data are mean ± SD. *P<0.05, compared to DMSO treated cells.

[0031] Figure 14. Proposed model of Src role in LAM. The absence of TSC2 gene results in mTOR activation in LAM cells. Activation of mTOR increases protein synthesis and
proliferation and inhibits autophagy. Autophagy inhibition causes accumulation of active Src kinase that induces the inhibition of E-cadherin and upregulates SNAIL MMP9. These events promote EMT in LAM cells and thus enhance their migration and oncogenic properties.

DETAILED DESCRIPTION OF THE INVENTION


I. [0033] General Embodiments

[0034] Embodiments of the disclosure encompass methods and/or compositions for treatment and/or prevention of a medical condition associated with activation of Src kinase, including at least lymphangioleiomyomatosis and/or tuberous sclerosis complex. In some cases, the individual also receives inhibitors of mTOR. In particular embodiments, methods and/or compositions are provided to an individual to delay or inhibit the onset of at least one symptom of lymphangioleiomyomatosis and/or tuberous sclerosis complex, including to prevent development of LAM or additional manifestations of TSC, such as in a subject with TSC, for example.

II. [0035] Lymphangioleiomyomatosis (LAM)

[0036] An individual with LAM or that is susceptible to LAM may be treated with one or more methods of the disclosure. An individual may be treated with methods of the disclosure prior to or following diagnosis or confirmed diagnosis of LAM. The individual may be subjected to steps for diagnosing LAM, in some aspects.

[0037] Symptoms of LAM may include one or more of the following: shortness of breath on exertion; cough; hemoptysis, as a result of vascular congestion; recurrent pneumothorax; chylous pleural effusion and chylous ascites, as a result of lymphatic obstruction.

[0038] Diagnosis of LAM may occur in one of a variety of ways. A medical provider may utilize a CT scan and/or lung biopsy, for example. In patients with typical cystic changes on high resolution CT scanning serum levels of greater than 800 pg/ml are considered to be diagnostic for LAM. For lung biopsy, one may employ video-assisted thoracoscopic biopsy or transbronchial biopsy. In some cases, the diagnosis of LAM can be made with confidence on clinical grounds (without biopsy) in patients with typical cystic changes on high resolution CT scanning of the lung and findings of tuberous sclerosis, angiomyolipoma or chylothorax.
Exemplary methods for diagnosis of LAM are described in U.S. Patent No. 7,811,776; U.S. Patent No. 8,058,018; or U.S. Patent No. 8,278,060, all of which are incorporated by reference herein in their entirety.

In some cases, the individual receiving the therapy of the disclosure has received or is also receiving or will receive an additional LAM therapy. Therapies for LAM that may be utilized in conjunction with the methods of the present invention include fulvestrant; inhibitors of the dual-specificity kinases MEK-1 and MEK-2; rho kinase inhibitors; the NCI domain of the alpha3 chain of type IV collagen; biguanide, a glitazone, a sulphonylurea, a sulfonamide, AMPK activator, mTOR inhibitor, an alpha-adrenergic antagonist and/or human interferon-gamma or an agent that increases endogenous interferon-gamma levels. In some cases, an individual will receive, has received, or is also receiving one or more of statins, anti-vascular endothelial growth factor-D (VEGF-D) therapy, and anti-Collage I therapy.

Therapies for LAM that may be utilized in conjunction with the methods of the present invention also include at least those treatments for LAM described in U.S. Patent No. 8,530,457; U.S. Patent No. 8,299,096; U.S. Patent No. 8,227,419; U.S. Patent No. 8,058,227; U.S. Patent No. 7,816,335; U.S. Patent No. 7,229,614; U.S. Patent No. 6,310,088; U.S. Patent No. 6,187,924, U.S. Patent Application Publication No. 20120258937; or U.S. Patent Application Publication No. 2013004436, all of which are incorporated by reference herein in their entirety.

III. Tuberous Sclerosis Complex

In aspects of the disclosure, an individual with tuberous sclerosis complex or that is susceptible to tuberous sclerosis complex is treated with one or more methods of the disclosure. The individual may or may not have lymphangioleiomyomatosis associated with the tuberous sclerosis complex. The individual is provided with an effective amount of one or more Src inhibitors and may also be provided with an effective amount of one or more mTOR inhibitors.

In some aspects of the disclosure, an individual is diagnosed with tuberous sclerosis complex as part of some methods of the disclosure. The diagnosis may occur by any suitable method or methods, including one or more of the following: taking a personal and family history; examination of particular body parts (such as the skin under a Wood's lamp
(hypomelanotic macules), the fingers and toes (ungual fibroma), the face (angiofibromas) and the mouth (dental pits and gingival fibromas); cranial imaging with non-enhanced CT; cranial imaging with MRI (looking for cortical tubers and subependymal nodules); renal ultrasound (looking for angiomyolipoma or cysts); fundoscopy (looking for retinal nodular hamartomas or achromic patch); and, if an infant, an echocardiogram (looking for rhabdomyoma). The number of particular features that are identified provide criteria of whether or not a diagnosis is definite.

[0045] In embodiments, the individual with TSC has Lymphangioleiomyomatosis in the lungs. The individual may alternatively or additionally have other TSC symptoms, such as Facial angiofibromas or forehead plaque, Nontraumatic unglu or periungual fibroma, Hypomelanotic macules, Shagreen patch (connective tissue nevus), Cortical tuber, and or other symptoms (see Roach E, Sparagana S (2004). "Diagnosis of tuberous sclerosis complex". Journal of Child Neurology 19 (9): 643-9).

[0046] In certain aspects, an individual with TSC is provided one or more therapeutic methods in addition to the methods of the disclosure. In some cases, the additional therapeutic method(s) is a treatment for one or more symptoms, such as the following: rapamycin; everolimus (to treat subependymal giant cell astrocytomas and angiomyolipoma kidney tumors); antiepileptic drugs (for example, vigabatrin); and/or surgery, including dermabrasion and laser treatment.

IV. [0047] Src Kinase Inhibitors

[0048] Embodiments of the disclosure include providing an effective amount of one or more Src kinase inhibitors (which may be referred to as Src inhibitors) to an individual in need thereof. The individual may be known to have, or may be suspected of having, or may be susceptible to having lymphangioleiomyomatosis or tuberous sclerosis complex. In cases wherein more than one Src inhibitor is provided to an individual, they may be delivered at the same time or at different times. When delivered at the same time, they may or may not be comprised in the same formulation, and they may or may not be delivered by the same delivery route.

[0049] Particular Src inhibitors that may be employed in the invention include the following: Saracatinib, Dasatinib, Ponatinib, PP1, PP2, PP3, Bosutinib, KX2-391, PP1, NVP-BHG712, A 419259 trihydrochloride, AZM 475271, Damnacanthal, Herbimycin A, KB SRC 4,
Lavendustin A, Lyn peptide inhibitor, MNS, 1-Naphthyl PP1, PD 166285 dihydrochloride, PD 180970, Piceatannol, pp60 c-src (521-533) (phosphorylated), Src II, BMS-354825, KX2, 391, XL-228, TG100435/TG100855, DCC2036, or a combination thereof. However, in certain aspects a compound is employed that acts more specifically as a Src inhibitor, to reduce any potential side effects.

[0050] In particular aspects, one or more compounds as described in U.S. Patent No. 7,049,438 is employed in one or more methods of the invention.

[0051] In some cases, derivatives of known Src inhibitors that also have Src inhibition activity may be employed. The skilled artisan recognizes that one exemplary means to determine if a particular compound has Src inhibition activity is by testing Src phosphorylation on residue Tyrosine 416, for example. Additional tests include testing Src kinase activity in vitro using a measurable substrate. One can also measure the phosphorylation of down-stream cellular targets of Src such as focal adhesion kinase (FAK), or PAX (Baselga J, Cervantes A, Martinelli E, Chirivella I, Hoekman K, Hurwitz HI, et al. Phase I safety, pharmacokinetics, and inhibition of SRC activity study of saracatinib in patients with solid tumors. Clin Cancer Res, 16:4876-83, 2010).

V. [0052] mTOR Inhibitors

[0053] In some aspects of the invention, in addition to Src inhibitor methods, an individual is provided with an effective amount of one or more mTOR inhibitors. The one or more mTOR inhibitors may be provided to the individual before, during, and/or after the individual is given an effective amount of one or more Src inhibitors.

[0054] Particular mTOR inhibitors that may be employed in the invention include at least the following: rapamycin, epigallocatechin gallate (EGCG), caffeine, curcumin, resveratrol, temsirolimus, everolimus, Ridaforolimus, BEZ235, AZD8055, KU-0063794, PP242, INK 128, GDC-0349, PI-103, WYE-354, GSK1059615, SAR245409, PP-121, OSI-027, PFK-587, GSK2126458, WAY-600, WYE-125132, WYE-687, GDC-0980, CH5132799, PF-04691502, NVP-BGT226, AZD2014, Torin 1, Torin 2.

VI. [0055] Pharmaceutical Preparations

[0056] Pharmaceutical compositions of the present invention comprise an effective amount of one or more Src inhibitors (and optionally, mTOR inhibitors) dissolved or dispersed
in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of an pharmaceutical composition that contains at least one Src inhibitor active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington: The Science and Practice of Pharmacy, 21st Ed. Lippincott Williams and Wilkins, 2005, incorporated herein by reference. Moreover, for animal (e.g., human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards, such as required by FDA Office of Biological Standards.

[0057] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the pharmaceutical compositions is contemplated.

[0058] The Src inhibitor may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, transdermally, intrathecally, intraarterially, intraperitoneally, intranasally, intravaginally, intrarectally, topically, intramuscularly, subcutaneously, mucosally, orally, topically, locally, inhalation (e.g., aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in creams, in lipid compositions (e.g., liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

[0059] The Src inhibitor may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, e.g., those
formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as formulated for parenteral administrations such as injectable solutions, or aerosols for delivery to the lungs, or formulated for alimentary administrations such as drug release capsules and the like.

[0060] Further in accordance with the present invention, the composition of the present invention suitable for administration is provided in a pharmaceutically acceptable carrier with or without an inert diluent. The carrier should be assimilable and includes liquid, semi-solid, i.e., pastes, or solid carriers. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of a the composition contained therein, its use in administrable composition for use in practicing the methods of the present invention is appropriate. Examples of carriers or diluents include fats, oils, water, saline solutions, lipids, liposomes, resins, binders, fillers and the like, or combinations thereof. The composition may also comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (e.g., methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[0061] In accordance with the present invention, the composition is combined with the carrier in any convenient and practical manner, i.e., by solution, suspension, emulsification, admixture, encapsulation, absorption and the like. Such procedures are routine for those skilled in the art.

[0062] In a specific embodiment of the present invention, the composition is combined or mixed thoroughly with a semi-solid or solid carrier. The mixing can be carried out in any convenient manner such as grinding. Stabilizing agents can be also added in the mixing process in order to protect the composition from loss of therapeutic activity, i.e., denaturation in
the stomach. Examples of stabilizers for use in an the composition include buffers, amino acids such as glycine and lysine, carbohydrates such as dextrose, mannose, galactose, fructose, lactose, sucrose, maltose, sorbitol, mannitol, etc.

[0063] In further embodiments, the present invention may concern the use of a pharmaceutical lipid vehicle compositions that includes one or more Src inhibitors, one or more lipids, and an aqueous solvent. As used herein, the term "lipid" will be defined to include any of a broad range of substances that is characteristically insoluble in water and extractable with an organic solvent. This broad class of compounds are well known to those of skill in the art, and as the term "lipid" is used herein, it is not limited to any particular structure. Examples include compounds which contain long-chain aliphatic hydrocarbons and their derivatives. A lipid may be naturally occurring or synthetic (i.e., designed or produced by man). However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof. Of course, compounds other than those specifically described herein that are understood by one of skill in the art as lipids are also encompassed by the compositions and methods of the present invention.

[0064] One of ordinary skill in the art would be familiar with the range of techniques that can be employed for dispersing a composition in a lipid vehicle. For example, the Src inhibitor may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid, contained or complexed with a micelle or liposome, or otherwise associated with a lipid or lipid structure by any means known to those of ordinary skill in the art. The dispersion may or may not result in the formation of liposomes.

[0065] The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount may vary according to the response of the subject. The
practitioner responsible for administration will, in any event, determine the concentration of
active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[0066] In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0067] In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered, based on the numbers described above.

[0068] A. Alimentary Compositions and Formulations

[0069] In preferred embodiments of the present invention, the Src inhibitors are formulated to be administered via an alimentary route. Alimentary routes include all possible routes of administration in which the composition is in direct contact with the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered orally, buccally, rectally, or sublingually. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft- shell
gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

[0070] In certain embodiments, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz et al., 1997; Hwang et al., 1998; U.S. Pat. Nos. 5,641,515; 5,580,579 and 5,792, 451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, etc. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. When the dosage form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Gelatin capsules, tablets, or pills may be enterically coated. Enteric coatings prevent denaturation of the composition in the stomach or upper bowel where the pH is acidic. See, e.g., U.S. Pat. No. 5,629,001. Upon reaching the small intestines, the basic pH therein dissolves the coating and permits the composition to be released and absorbed by specialized cells, e.g., epithelial enterocytes and Peyer's patch M cells. A syrup of elixir may contain the active compound sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

[0071] For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally- administered formulation. For example, a mouthwash may be prepared incorporating the active ingredient in the required amount in an
appropriate solvent, such as a sodium borate solution (Dobell's Solution). Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

[0072] Additional formulations which are suitable for other modes of alimentary administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

[0073] B. Parenteral Compositions and Formulations

[0074] In further embodiments, one or more Src inhibitors may be administered via a parenteral route. As used herein, the term "parenteral" includes routes that bypass the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered for example, but not limited to intravenously, intradermally, intramuscularly, intraarterially, intrathecally, subcutaneous, or intraperitoneally U.S. Pat. Nos. 6,753,514, 6,613,308, 5,466,468, 5,543,158; 5,641,515; and 5,399,363 (each specifically incorporated herein by reference in its entirety).

[0075] Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy injectability exists. It must be stable under the conditions of manufacture and storage and must be preserved against
the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (i.e., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0076] For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in isotonic NaCl solution and either added hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologies standards.

[0077] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a
previously sterile-filtered solution thereof. A powdered composition is combined with a liquid carrier such as, e.g., water or a saline solution, with or without a stabilizing agent.

[0078]  C. Miscellaneous Pharmaceutical Compositions and Formulations

[0079]  In other preferred embodiments of the invention, the active compound Src inhibitor may be formulated for administration via various miscellaneous routes, for example, topical (i.e., transdermal) administration, mucosal administration (intranasal, vaginal, etc.) and/or inhalation.

[0080]  Pharmaceutical compositions for topical administration may include the active compound formulated for a medicated application such as an ointment, paste, cream or powder. Ointments include all oleaginous, adsorption, emulsion and water-solubly based compositions for topical application, while creams and lotions are those compositions that include an emulsion base only. Topically administered medications may contain a penetration enhancer to facilitate adsorption of the active ingredients through the skin. Suitable penetration enhancers include glycerin, alcohols, alkyl methyl sulfoxides, pyrrolidones and luarocapram. Possible bases for compositions for topical application include polyethylene glycol, lanolin, cold cream and petrolatum as well as any other suitable absorption, emulsion or water-soluble ointment base. Topical preparations may also include emulsifiers, gelling agents, and antimicrobial preservatives as necessary to preserve the active ingredient and provide for a homogenous mixture. Transdermal administration of the present invention may also comprise the use of a “patch”. For example, the patch may supply one or more active substances at a predetermined rate and in a continuous manner over a fixed period of time.

[0081]  In certain embodiments, the pharmaceutical compositions may be delivered by eye drops, intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering compositions directly to the lungs via nasal aerosol sprays has been described e.g., in U.S. Pat. Nos. 5,756,353 and 5,804,212 (each specifically incorporated herein by reference in its entirety). Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga et al., 1998) and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts. Likewise, transmucosal drug delivery in the form of a polytetrafluoroethylene support matrix is described in U.S. Pat. No. 5,780,045 (specifically incorporated herein by reference in its entirety).
The term aerosol refers to a colloidal system of finely divided solid of liquid particles dispersed in a liquefied or pressurized gas propellant. The typical aerosol of the present invention for inhalation will consist of a suspension of active ingredients in liquid propellant or a mixture of liquid propellant and a suitable solvent. Suitable propellants include hydrocarbons and hydrocarbon ethers. Suitable containers will vary according to the pressure requirements of the propellant. Administration of the aerosol will vary according to subject's age, weight and the severity and response of the symptoms.

EXAMPLES

The following examples are provided for further illustration of the present invention, and do not limit the invention. The examples provided herein are for illustrative purposes only, and are in no way intended to limit the scope of the present invention. While the invention has been described in detail, and with reference to specific embodiments thereof, it will be apparent to one with ordinary skill in the art that various changes and modifications can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. Experiments and exemplary procedures are described below which provide additional enabling support for the present invention.

EXAMPLE 1

SRC KINASE ACTIVATION PROMOTES EPITHELIAL-MESENCHYMAL TRANSITION IN LYMPHANGIOLEIOMYOMATOSIS

Lymphangioleiomyomatosis (LAM) is a progressive cystic lung disease associated with Tuberous Sclerosis Complex (TSC). LAM cells are found in blood, urine, and chylous fluids and lung of LAM patients. LAM cells in lungs are thought to represent metastasized secondary tumors. The behavior of LAM cells, with respect to their infiltrative growth pattern and metastatic potential indicate that they are undergoing epithelial-mesenchymal transition (EMT). E-cadherin and matrix metallopeptidase-9 (MMP9) are critical regulators of EMT. The regulation of these mediators in LAM was characterized herein.

Lung tissues of normals and of patients with LAM were obtained from National Disease Research Interchange. As a model of LAM cells, Eker rat Embryos Fibroblasts TSC2-/- mutant cells (EEF8) were used, and their wild-type counterparts TSC2+/+(EEF4) were
used. Protein expression was evaluated by Western analysis or immunofluorescence microscopy. mRNA level was evaluated by Real-Time PCR.

[0086] Increased Src activity in human LAM lungs and in EEF8 cells was identified. Furthermore, the expression and localization of E-cadherin and the transcriptional repressor SNAIL in EEF8 cells were notably different, compared to that of EEF4 cells. In EEF4, E-cadherin was localized predominantly at the plasma membrane but in EEF8 cells E-cadherin was considerably decreased and dispersed at the cytoplasm. In cells undergoing EMT, increased SNAIL level and its nucleus translocation are responsible for E-cadherin suppression. In EEF8 cells we found increased SNAIL mRNA and enhanced nuclear localization of SNAIL protein. Src inhibitors dasatinib and saracatinib reduced SNAIL and MMP9 in EEF8 cells. Dasatinib and saracatinib also attenuated the enhanced cell motility of EEF8 cells, as measured by wound-healing assay.

[0087] Src is activated in the human LAM lungs. Src activation leads to overexpression of SNAIL transcription factor that represses E-cadherin and increases MMP9 transcription. These effects alter cell adhesion properties and activates EMT program in the TSC2/- deficient cells. In particular embodiments, treatment of LAM patients includes Src inhibitors that can reduce the oncogenic and metastatic potential of LAM cells.

EXAMPLE 2

ENHANCED SRC ACTIVATION IN LAM LUNGS

[0088] Tissue samples were evaluated of lungs of normal subjects and of subjects with LAM. LAM lungs showed collections of LAM cells, which were identified by HMB45 antibodies (Fig. 1A) (12, 21). Phosphorylation of the ribosomal protein S6 was increased in LAM lung tissues compared to normal lungs (Fig. 1B). These data confirm that mTOR is activated in lung tissues of subjects with LAM, as expected to occur secondary to TSC2 deficiency. One of the consequences of mTOR activation is inhibition of autophagy, which was evident by the accumulation of the autophagy substrate p62 (19) in LAM lungs (Fig. 1C). Importantly, there was increased phosphorylation of Src on Tyr416 in LAM lung tissues compared to normal lungs (Fig. 1D). These findings were further confirmed by analyzing human LAM lungs by immunofluorescence. Moreover, there was strong correlation between expression of phospho-Src and HMB45 positive cells. However, some HMB45 negative cells
contained phospho-Src as well, consistent with the notion that not all LAM cells are HMB45 positive. Phosphorylation of Tyr416, in the activation loop of the kinase domain, upregulates Src kinase activity. These data indicate that Src is activated in lung tissues of subjects with LAM. To confirm that Src activation in LAM lungs had functional consequences, activation of signal transducer and activator of transcription 3 (STAT3), which is a downstream mediator of Src, was tested. LAM lungs had elevated phosphorylated STAT3 (Fig. IE), indicating that STAT3 is activated in LAM lungs, consistent with a prior report (20).

[0089] It was considered that the increased activities of Src and STAT3, observed in LAM lungs, were specific to LAM cells. Cells were isolated from LAM lung explants and LAM cells using antibodies against HMB45 were identified. Cells positive for HMB45 staining had increased phospho-(Y416)-Src and phospho-(Y705)-STAT3, whereas non-LAM cells did not exhibit such increase. These data confirm that LAM cells have increased Src and STAT3 activities.

EXAMPLE 3

SRC AND STAT3 ARE ACTIVATED IN TSC2-/- CELLS

[0090] Eker rat embryos fibroblasts (EEF) TSC2+/+ wild-type (EEF4) and TSC2-/- mutant cells (EEF8) were studied. These cells are well characterized as a cellular model for LAM and TSC (14,22). EEF8 (TSC2-/-) did not express Tuberin, had increased activity of mTOR and suppressed autophagy (Fig. 8). Activation of mTOR was evident by increased phosphorylation of mTOR and of its substrate p70S6 kinase. Inhibition of autophagy was shown by reduction of LC3-II and by increased level of autophagy substrate p62 protein. Accumulation of p62 in EEF8 cells was not caused by increase of its mRNA. The above data confirmed prior reports that TSC2-/- EEF8 cells have the molecular features of LAM cells. It was then considered if TSC2-/- cells have increased Src activity. Phosphorylation of Src was increased in the TSC2-/- cells (Fig. 2A). TSC2-/- cells had increased phosphorylated STAT3 (Fig. 2B). Increased STAT3 translocation to the nucleus was observed in TSC2-/- cells (Fig. 2C). Importantly, inhibition of Src, by PP2 or Su6656, reduced STAT3 phosphorylation (Fig. 2 D). It was then confirmed if the increase in Src and STAT3 activities was a direct result of TSC2 deficiency. To this end, small interfering RNA (siRNA)-mediated knockdown of TSC2 in Hela cells resulted in increased Src and STAT3 activities (Fig. 2E); essentially recapitulating the phenotype of TSC2-/- EEF8 cells. That phenotype was also confirmed by the finding of
increased phosphorylation of ribosomal protein S6 (Fig. 9), which indicated that the activation of mTOR was similar to that observed in EEF8 cells. Moreover, overexpression of Src kinase in wild-type EEF4 cells led to increased Src activity and increased STAT3 phosphorylation (Fig. 2F). These data indicate that TSC2-/- cells have increased Src activity, similar to that found in human LAM lungs. They also show that STAT3 activation in TSC2-/- cells is a downstream event of Src activation.

EXAMPLE 4

ENHANCED ACTIVATION OF SRC-KINASE SIGNALING PATHWAY IN TSC27- CELLS

The activation of STAT3 in TSC2-/- cells indicated that other Src downstream substrates might also be activated in these cells. One important Src partner is focal adhesion kinase (FAK). Levels of FAK were increased in EEF8 cells, likely secondary to increase of its mRNA (Fig. 3A-B). Moreover, overphosphorylation of FAK on Y397 and Y925 sites in EEF8 cells (Fig. 3C-D). FAK- Y397 autophosphorylation plays a role in FAK binding to Src kinase and forming of active FAK-Src complex (23). Recruitment of Src kinase results in phosphorylation of FAK-Y925 and triggers a Ras-dependant activation of MAP kinase pathway (24). To evaluate MAP kinase pathway activation in EEF8 cells, phosphorylation of Erk was examined. There was an increased level of phosphorylated Erk in EEF8 cells, compared to EEF4 cells (Fig. 3E). To determine the effect of Src kinase on the Erk phosphorylation Src kinase was overexpressed in EEF4 cells and there was markedly increased level of Erk phosphorylation (Fig. 3F). To confirm that the increased phosphorylation of Erk in EEF8 was caused by Src kinase, it was determined that Src-specific siRNA lead to a decrease of Erk phosphorylation in EEF8 cells (Fig. 3G). Moreover, Src kinase inhibition by any of four different inhibitors (PP2, SU6656, dasatinib, saracatinib) reduced Erk phosphorylation in EEF8 cells (Fig. 3H). Taken together, these data indicate the Src signaling pathway is activated in TSC27- cells.

EXAMPLE 5

AUTOPHAGY INHIBITION RESULTS IN SRC KINASE ACTIVATION

Loss TSC2 gene leads to mTOR activation and autophagy inhibition (25). Recently, a role for autophagy has been shown in degradation of active Src (17). It was considered that autophagy inhibition in EEF8 contributes to Src activation in these cells. To
characterize this, siRNA was used to knockdown autophagy related gene 7 (ATG7) in wild-type EEF4 cells. ATG7 knockdown resulted in inhibition of autophagy as shown by reduction of autophagy marker LC3 type II, and increased active phosphorylated Src (Fig. 10A-B). Furthermore, mouse embryonic fibroblasts (MEF) derived from ATG7-/ mice ATG57- mice had increased active Src (Fig. 10 C-D). Finally, treatment of wild-type EEF4 cells with autophagy-lysosome pathway inhibitor chloroquine resulted in increased active Src (Fig. 10E). Thus, autophagy inhibition caused by several independent methods led to accumulation of active Src kinase. Moreover, the phospho-Src levels decreased after induction of autophagy, by starvation, in TSC2-deficient EEF8 cells (Fig. 10F). These data indicated that autophagy was involved in modulation of Src kinase activity in the TSC2-/ cells.

EXAMPLE 6

TSC2 DEFICIENCY OR OVEREXPRESSION OF SRC PROMOTES EMT

[0093] To evaluate EMT in TSC2-/ cells, the level and cellular distribution of E-cadherin was examined. The expression and cellular localization of E-cadherin in EEF8 cells were notably altered (Fig. 4 A-B). In wild-type cells (EEF4), E-cadherin was readily detectable and localized predominantly at the plasma membrane, where it is known to play a critical role in adherens junction formation. In contrast, in TSC2-/ cells (EEF8), there was much lower expression of E-cadherin and it did not co-localize with plasma membrane. Instead, most of E-cadherin signals were found in punctate cytosolic structures. One possible explanation for the reduction in E-cadherin in TSC2-/ cells could be due to an increase of its transcriptional repressor Snail. There was marked increase in the expression of Snail mRNA and protein in EEF8 cells (Fig. 4C). Snail activity, measured by its nuclear translocation, was also more pronounced in EEF8 cells (Fig. 4D). Importantly, there was increased level of Snail in the samples of human LAM lungs (Fig. 4E). Further, the increase in Snail expression was limited to LAM cells identified by positive staining for HMB45. Matrix metalloproteinase 9 (MMP9), an important marker of EMT, was markedly increased in EEF8 cells (Fig. 4F). To confirm a role for the observed increased Src in TSC2-/ cells in promotion of EMT, wild-type EEF4 cells were transfected with Src and then evaluated several EMT markers. Src overexpression resulted in reduction of E-cadherin and increased levels of Snail and MMP9 (Fig. 4G), essentially recapitulating the phenotype observed in TSC2-/ cells. These dramatic changes in the expression and localization of E-cadherin could account for the decrease in cell adhesion,
increased motility, invasiveness and metastatic potential of TSC2-/− cells, in certain embodiments of the invention.

EXAMPLE 7

SRC INHIBITION REDUCES EMT MARKERS IN TSC2-/− CELLS

[0094] To validate Src as a potential therapeutic target in LAM, TSC2-/− cells (EEF8) were treated with exemplary Src inhibitors dasatinib or saracatinib (26, 27). Both inhibitors reduced levels of Snail, whereas rapamycin had no effect (Fig. 5 A-B). Src inhibition also reduced levels of MMP9, as determined by immunoblot and further confirmed by gelatin zymogram (Fig. 5 C-D). Overall, dasatinib and saracatinib appeared to have equivalent effects on Src activation (phosphorylation) and on EMT markers. In additional experiments, siRNA-mediated knockdown of Src resulted in decrease of expression of Snail and Mmp9 (Fig. 5E-F). These data are consistent with prior reports of increased immunoreactivity for MMPs in lung biopsy specimens from subjects with LAM and TSC2-deficient LAM-like cells (32,33,34) and suggest that inhibition or genetic knockdown of Src could reduce EMT in TSC2-/− cells.

EXAMPLE 8

SRC INHIBITION ATTENUATES MIGRATION ACTIVITY OF TSC2-/− CELLS

[0095] Using wound-healing assay, we found that inhibition of Src kinase by dasatinib or saracatinib led to considerable reduction of migration ability of TSC2-/− cells (Fig. 6A-B). In contrast, mTOR inhibitor rapamycin had no significant effect on cell migration. These data suggest that Src inhibition is likely to reduce migration and metastatic ability of TSC2-/− cells. It should be noted, however, that the migration assay results, in part, reflect reduction of cell proliferation by Src inhibitors. We found that EEF8 cell proliferation was increased compared to control cells and that Src inhibitors significantly decreased the proliferation of EEF8 cells (Fig. 11). Src inhibition also reduced migration activity of EEF4 cells (Fig. 12A-B).
EXAMPLE 9

SRC INHIBITION REDUCES INVASIVENESS OF TSC2-/- CELLS

[0096] The invasive properties of EEF4 and EEF8 cells were studied using Matrigel inserts. After incubation for 18 hr, the membranes were processed and the invading cells were counted in 6 random fields. Invasive cells were counted as the number of migrating cells per field. TSC2-/- cells (EEF8) were much more invasive than control cells (Fig. 6C-D). This behavior is consistent with the notion that TSC2-/- cells have increased invasive and migratory properties, likely secondary to EMT in these cells. The effect of Src inhibition on the invasive properties of EEF8 cells was evaluated. Src inhibition by dasatinib or saracatinib markedly reduced the invasiveness properties of TSC2-/- cells. In contrast, mTOR inhibitor rapamycin had no significant effect. Further, there was no effect of Src inhibitors on the invasiveness in the EEF4 cells, probably because of low invasiveness of these cells (Fig. S7C-D). These data indicate that Src inhibition is likely to reduce invasiveness of TSC2-/- cells and that effect is specific for such cells.

EXAMPLE 10

SRC INHIBITION REDUCES LUNG COLONIZATION OF TSC2-/- CELLS IN VIVO

[0097] The effect of Src inhibition was evaluated on the metastatic potential for TSC2-/- cells in vivo. Luciferase-expressing TSC2-/- (EEF8-Luc) cells were engineered to allow in vivo imaging following their injection into mice. EEF8-Luc cells were pre-treated with vehicle (DMSO), rapamycin (1 µg/ml), saracatinib (1 µM) or both rapamycin and saracatinib.

[0098] 1x10^6 cells were then intravenously injected into female CB17 SCID mice. Six hours following injection of cells, and 10 minutes prior to imaging, animals were injected intraperitoneally with 120 mg/kg, Luciferin. Bioluminescent signals were recorded using Xenogen in vivo imaging system (Fig. 7 A-B). Total photon flux at the chest region was analyzed. Saracatinib significantly reduced the number of EEF8-Luc cells that was detected in the lungs. Rapamycin treatment had no significant effect. At 24 hr time point after EEF8 cell injection, mice were sacrificed and their lungs were dissected and imaged in Petri dish (Fig. 7 C-D). Further, in vivo experiments were conducted with injection of luciferase-expressing EEF4 cells treated with DMSO or saracatinib (Fig. 13). There was reduction in the EEF4 cells lung colonization after 24 hr but not after 6 hr of the cell injection. Thus, the
consequences of Src inhibition were more pronounced in TSC7- cells compared to control cells. These results indicate that Src inhibition can reduce the metastatic potential for TSC2-/- cells.

EXAMPLE 11

SIGNIFICANCE OF CERTAIN EMBODIMENTS

[0099] As described herein: 1) Src is activated in LAM cells; 2) Src activation contributes to the pathogenesis of LAM by promoting EMT in TSC2-/- LAM cells; 3) Src inhibition can attenuate the oncogenic and metastatic potential of LAM cells. An exemplary model based on the findings is depicted in Figure 14. In LAM cells, the loss of TSC2 gene results in hyperactivation of mTOR by Rheb. Activation of mTOR increases protein synthesis and proliferation of LAM cells and inhibits autophagy. Autophagosomes are involved in the elimination of active Src kinase from cells. Autophagy inhibition causes accumulation of phospho-Src(Y416) kinase. Activation of Src pathway upregulates EMT genes including Snail and MMP9 and leads to suppression of E-cadherin.

[0100] Recent evidence suggests that LAM cells might be undergoing EMT. One of the critical steps driving EMT is the repression of E-cadherin, resulting in loss of cell-cell adhesion. E-cadherin is a critical regulator of epithelial junction formation. Dysfunction of the E-cadherin-mediated cell adhesion system plays an important role in tumor progression of the relatively benign tumor to invasive, metastatic carcinoma.

[0101] Recent studies have shown that, in cancer cells in which the Src pathway is hyperactive, autophagosomes promote degradation of the active tyrosine Src kinase (17). Autophagy is inhibited in LAM cells due to the mTOR activation (7,25). In aspects of this disclosure, the role of autophagy in accumulation of active Src kinase in TSC2-/- cells as well as in other models of autophagy-deficient cells is shown. The results indicate that the Src kinase activation promotes migration and invasion of TSC2-/- cells, likely secondary to upregulation of Snail transcription factor, which suppresses E-cadherin expression. Similar role of Src in promoting cell migration and invasion via activation of EMT marker MMP9 has been previously described in breast cancer (28). Increased immunoreactivity for MMPs in lung biopsy specimens from patients with LAM (32,33) and TSC2-deficient LAM-like cells (34) were also described. Such activation of MMP9 plays the
critical role for the proteolysis and remodeling of the extracellular matrix that allows cancer cells to invade into the surrounding stroma and promotes metastasis.

[0102] Src family kinase inhibitor PP2 was found to enhance E-cadherin-mediated cell adhesion system, which resulted in the suppression of metastasis of cancer cells (18). Dasatinib and saracatinib are the most clinically studied SRC inhibitors (26,27). Preclinical studies in solid tumor cell lines have shown that both dasatinib and saracatinib consistently inhibit cell proliferation. As shown herein, the selective inhibition of Src kinase restores cell adhesion and reduces metastatic tendencies in LAM, in at least some embodiments. To characterize Src inhibition as a therapeutic strategy in LAM, dasatinib and saracatinib were used, which are the most clinically developed Src inhibitors (26,27). Dasatinib and saracatinib significantly decrease migration and invasion ability of TSC2-/- cells.

[0103] LAM cells exhibit increased activation of the mTOR pathway (29). Recent clinical trials in patients with TSC or LAM using mTOR inhibitor rapamycin showed that there was a reduction in the size of angiomyolipomas and, in some cases, improvement in lung function (9,30). However, cessation of therapy led to the regrowth of tumors and diminished pulmonary functions (9,10,31). Herein, rapamycin had no effect on the migration and invasion activity of TSC2-/- cells. These data are of particular clinical relevance because circulating LAM cells were found in the blood and plural fluid of women with LAM and these cells might be the source for invasion of LAM cells into the lungs (11). Thus, failure of rapamycin to affect cell migration or invasion of TSC2-/- cells may explain the transient nature of rapamycin efficacy in LAM. In certain aspects of the disclosure, the selective inhibition of Src restores cell adhesion and reduces metastatic tendencies of TSC2-/2 cells in LAM. Saracatinib treatment notably decreased lung colonization of TSC2-/2 cells in vivo. Rapamycin either alone or in combination with saractinib did not provide benefit and seemed to have paradoxical enhancing effect on colonization.

[0104] Taken together, the findings highlight a role of Src kinase in the pathogenesis of LAM. The data demonstrate that activated Src kinase promotes EMT in TSC2-/- cells and increases their metastatic potential. Src kinase inhibitors dasatinib and saracatinib notably decrease migration and invasion ability of TSC2-/- cells. These results are valuable for understanding the nature of EMT in LAM cells and provide a novel therapeutic target to prevent LAM cell dissemination. The efficacy of dasatinib and saracatinib, used as a
single agent or in combination with mTOR inhibitors improves treatment outcomes in LAM, in particular embodiments. The use of multiple drug therapy has the advantage of reducing the dose of each drug and thus can minimize side effect. Overall, the disclosure establishes Src as a novel therapeutic target in LAM.

EXAMPLE 12

EXEMPLARY MATERIALS AND METHODS

[0105] Exemplary materials and methods are provided herein, although the skilled artisan recognizes that there may be suitable alternative material and/or methods.

[0106] Reagents and antibodies. The following antibodies were used for immunoblot analysis: pSrc(Tyr416), pStat3(Tyr705), Stat3, pErkl/2(Thr202/Tyr204), Erkl/2, S6, pS6(Ser235/236), pFAK(Tyr925), pFAK(Tyr397), mTOR, U0126 (all from Cell Signaling), tuberin, rabbit E-cadherin, MMP9, Snail (all from Santa Cruz), mouse E-cadherin (BD), Src (Millipore), pSrc(Tyr418) (LifeSpan Biosciences) and HMB45 (Enzo Life Sciences). Src kinase inhibitors PP2 and SU6656 were purchased from Calbiochem. Rapamycin, dasatinib and saracatinib were purchased from LCLaboratories.

[0107] Cell culture and tissue samples. Eker rat embryonic fibroblasts EEF4 (TSC2+/+) and EEF8 (TSC2/-) were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 mixture (1:1) containing 10% heat-inactivated FBS. Lung tissues of normals and of subjects with LAM were obtained from National Disease Research Interchange. Plasmids, siRNA and cell transfection. Specific TSC2 (J-003029-11 and J-003029-12), ATG7 (J-0095596-11 and J-0095596-12) and Src (J-080144-11 and J-080144-12) siRNAs were purchased from Dharmacon. Cationic lipid-mediated transient transfection of plasmids was done using Lipofectamine 2000 (Invitrogen).

[0108] Immunofluorescence and histochemistry. Cells were grown on glass coverslips, fixed in either cold pure methanol or 4% paraformaldehyde, permeabilized by 0.2% Triton X-100, and blocked in 10% normal goat serum. Primary antibody incubation was done at 4oC overnight in a humidified chamber followed by a 30-min incubation at room temperature with Alexa fluor 594-labeled secondary antibodies. Coverslips were mounted by SlowFade gold antifade reagent with DAPI. Tissues sections were
deparaffinized, incubated overnight with primary antibodies at 4°C in a humidified chamber and then washed and incubated with biotinylated secondary antibodies for 30 min at room temperature. Slides were developed using Vectastatin Elite ABC kit (Vector Labs) and counterstained with hematoxylin. Images were viewed using a Zeiss Axiovert microscope.

[0110] Wound healing assay. Cells were plated in a 10 cm plate and allowed to form a confluent monolayer that was then scratched with a sterile pipette tip (200 μL), washed with medium to remove floated and detached cells. Wound areas were photographed (magnification 100x) at the start and 18 hr after treatment to assess the degree of wound closure. Data are expressed in um2x1000.

[0111] Cell invasion assay. Cells were studied using Matrigel inserts (BD Biosciences). Serum-deprived cells (5x10⁴ cells) were loaded in the upper compartment of the chambers and the bottom wells were filled with chemo-attractant (complete media with 10% FBS). After incubation for 18 hr, the membranes were processed and the non-migrating cells were removed from the upper chamber with a cotton swab and the inserts were fixed with methanol and stained with 1% Toluidine blue. The invading cells were counted in 6 random fields under a microscope.

[0112] Real-Time PCR. RNAs were purified using Rneasy Mini Kit (Qiagen) and cDNA synthesis was performed using cDNA Reverse Transcription Kit (Applied Biosystems). mRNA expression was measured using a real-time detection system (Applied Biosystems StepOnePlusTM) in 96-well optical plates using PerfeCTaTMqPCR FastMixTM (Quanta Biosciences). 18S was used as an endogenous control. All analysis was performed in triplicate, and means were used for statistical calculations.

[0113] Animal in vivo imaging. All animal studies were performed in accordance with protocol approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Female CB17-SCID mice, 6-8 weeks of age, were purchased from Jackson Laboratory. EEF8 cells were transfected with pGL4.51[pc2/CMV/Neo] vector (Promega) using Lipofectamine 2000 (Invitrogen). For intravenous injections, 1x10⁶ EEF8-Luc cells were injected into the retro-orbital vein. Ten minutes prior to imaging, animals were injected with 79301434.1
Luciferin (Promega; 120mg/kg, i.p.). Bioluminouscent signals were recorded using Xenogen in vivo imaging system (IVIS; Xenogen). Total photon flux at the chest regions was analyzed.

[0115] Statistical analysis. All blots and data are representative of at least three independent experiments. The Student's t test was used, and p values of less than 0.05 were considered to be statistically significant.

REFERENCES

[0116] All patents and publications mentioned in this specification are indicative of the level of those skilled in the art to which the invention pertains. All patents and publications herein are incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference in their entirety.


[0148] 32. Hayashi T, Fleming MV, Stetler-Stevenson WG, Liotta LA, Moss J, Ferrans VJ, Travis WD. (1997) Immunohistochemical study of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in pulmonary lymphangioleiomyomatosis (LAM). Hum Pathol, 28(9), 1071-1078.


[0151] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.
CLMALS

What is claimed is:

1. A method of treating lymphangioleiomyomatosis or tuberous sclerosis complex in an individual, comprising the step of providing to the individual a therapeutically effective amount of at least one sarcoma (Src) kinase inhibitor.

2. The method of claim 1, wherein the Src kinase inhibitor is selected from the group consisting of Saracatinib, Dasatinib, Ponatinib, PP1, PP2, PP3, Bosutinib, KX2-391, PP1, NVP-BHG712, A 419259 trihydrochloride, AZM 475271, Damnacanthal, Herbimycin A, KB SRC 4, Lavendustin A, Lyn peptide inhibitor, MNS, 1-Naphthyl PP1, PD 166285 dihydrochloride, PD 180970, Piceatannol, pp60 c-src (521-533) (phosphorylated), Src II, BMS-354825, KX2, 391, XL-228, TG100435/TG100855, DCC2036, a combination thereof, and functional derivatives thereof.

3. The method of claim 1, further comprising the step of providing to the individual a therapeutically effective amount of at least one mammalian target of rapamycin (mTOR) inhibitor.

4. The method of claim 3, wherein the mTOR inhibitor is selected from the group consisting of rapamycin, epigallocatechin gallate (EGCG), caffeine, curcumin, resveratrol, temsirolimus, everolimus, Ridaforolimus, BEZ235, AZD8055, KU-0063794, PP242, INK 128, GDC-0349, PI-103, WYE-354, GSK1059615, SAR245409, PP-121, OSI-027, PKI-587, GSK2126458, WAY-600, WYE-125132, WYE-687, GDC-0980, CH5132799, PF-04691502, NVP-BGT226, AZD2014, Torin 1. and Torin 2.

5. The method of claim 1, further comprising the step of providing an additional therapy for the individual with lymphangioleiomyomatosis or tuberous sclerosis complex.
6. The method of claim 5, wherein the additional therapy for lymphangioleiomyomatosis comprises anti-estrogen therapy, inhibition of interleukin-2, lung transplantation, statin, anti-vascular endothelial growth factor-D (VEGF-D) therapy, anti-Collagen I therapy, fulvestrant; inhibitors of the dual-specificity kinases MEK-1 and MEK-2; rho kinase inhibitors; the NCI domain of the alpha3 chain of type IV collagen; biguanide, a glitazone, a sulphonylurea, a sulfonamide, AMPK activator, mTOR inhibitor, an alpha-adrenergic antagonist, human interferon-gamma, an agent that increases endogenous interferon-gamma levels, or a combination thereof.

7. The method of claim 5, wherein the additional therapy for tuberous sclerosis complex comprises surgical resections therapy.

8. The method of claim 1, further comprising the step of examining the individual for at least one symptom of lymphangioleiomyomatosis or tuberous sclerosis complex.
FIG. 1
FIG. 4
FIG. 6
FIG. 8
FIG. 12
### INTERNATIONAL SEARCH REPORT

**International application No.**
PCT/US1 3/68490

#### A. CLASSIFICATION OF SUBJECT MATTER

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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)

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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)


#### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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