Abstract: This invention relates to the use of Rorl-antagonizing compounds to inhibit tumor growth and to treat cancer (e.g., lung cancer). The invention also provides methods of using Rorl to screen for compounds that inhibit tumor growth.

Title: METHODS AND COMPOSITIONS FOR TREATING CANCER
METHODS AND COMPOSITIONS FOR TREATING CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application Number 60/813,499 filed 13 June 2006. The full disclosure of this application is incorporated herein by reference in its entirety and for all purposes.

FIELD OF THE INVENTION

[0002] The present invention generally relates to methods for identifying agents that inhibit growth of tumor cells and to therapeutic methods for treating cancer.

BACKGROUND OF THE INVENTION

[0003] Lung cancer is a highly aggressive cancer that represents the leading cause of cancer deaths within the United States and throughout the world. There are two primary types of lung cancers: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Small cell lung cancer accounts for approximately 25% of lung cancer and spreads aggressively, and non-small cell lung cancer represents the majority (about 75%) of lung cancer. Non-small cell lung cancer can be histologically subclassified into squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. Each tumor expresses specific differentiation features or surface phenotype determinants, all of which distinguish these cells from normal cells. The development of monoclonal antibody diagnostic techniques has greatly enhanced the production of reagents capable of differentiating normal cells from cancer cells and differentiating types of cancer cells from other cancer cells.

[0004] Treatments of lung cancers still mainly depend on surgery, chemotherapy, and radiotherapy. This is because the molecular mechanisms underlying the pathogenesis of lung cancers remain largely unclear. Surgical resection is the mainstay of therapy for non-small cell carcinoma. For small cell lung cancer, which is biologically aggressive
and leads rapidly to death, intensive combined chemotherapy has demonstrated some efficacies. Active chemotherapeutic agents include cisplatin, etoposide, cyclophosphamide, vinblastine, vincristine, doxorubicin, and carboplatin. Unfortunately, the high initial response rates to first-line chemotherapy do not appear to translate into a survival benefit (Kohno and Kitahara, 2001, Gan To Kagaku Ryoho 28(4):448-53). Moreover, there are many undesirable side effects associated with chemotherapy.

There is an urgent need in the art for better understanding of the molecular mechanisms underlying lung cancer, as well as for better means for treating lung cancer that are more effective and less toxic. The present invention addresses this and other needs.

SUMMARY OF THE INVENTION

In one aspect, the invention provides methods for inhibiting growth and proliferation of tumor cells, the method comprising contacting the cell with an agent which down-regulates Ror1 cellular level or inhibits Ror1 kinase activity. Some of the methods are directed to inhibiting growth of lung cancer cells. Some of the methods are directed to inhibiting growth of tumor cells in a subject having or at risk of developing lung cancer. The agents that can be employed in the methods include nucleic acid agents such as, e.g., short interfering RNA (siRNA), microRNA (miRNA), short hairpin RNA (shRNA), anti-sense nucleic acid, and a complementary DNA (cDNA). The agents can also be antagonist antibodies that specifically bind to Ror1 and inhibit Ror1 kinase activity.

In a related aspect, the invention provides methods for treating lung cancer in a subject. The methods entail administering to the subject a pharmaceutical composition that contains an effective amount of an agent which down-regulates Ror1 cellular level or inhibits Ror1 kinase activity.

In another aspect, the invention provides methods for identifying an agent for treating lung cancer. These methods involve first screening test compounds to identify one or more modulating compounds which down-regulates Ror1 cellular level or
inhibits RorI kinase activity; and then testing the identified modulating compounds for ability to inhibit lung cancer growth.

[0009] A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figures 1A-1C show that RorI mediates canonical Wnt-signaling in the lung cancer cell lines A549 and H460 cells.

[0011] Figure 2 shows that knockout of RorI inhibits growth of lung cancer cell lines A549 and H460.

[0012] Figures 3A-3B show that knockout of RorI mediates apoptosis in lung cancer cell line H460.

DETAILED DESCRIPTION

I. Overview

[0013] This invention is predicated in part on the discoveries by the present inventors that orphan tyrosine kinase receptor RorI is a therapeutic target in lung cancer. Receptor tyrosine kinases (RTKs) play crucial roles in developmental morphogenesis by regulating cellular proliferation, differentiation, migration, and death. As detailed in the Examples below, the present inventors discovered that RorI is expressed in lung cancer cell lines, that RorI mediates canonical Wnt-signal transduction in cancer cell lines, and that targeting RorI with siRNAs inhibits growth of the cancer cells and induces cell death.

[0014] In accordance with these discoveries, the invention provides methods for inhibiting growth of tumor cells, e.g., lung cancer cells. Also provided in the invention are therapeutic applications of RorI inhibitors in the treatment of subjects that suffer from cancers (e.g., lung cancer). These methods employing compounds (e.g., siRNAs, antibodies or small molecule organic compounds) which down-regulate cellular level or
enzymatic activities of Ror1. The invention also provides methods for identifying therapeutic agents for inhibiting tumor growth and for treating cancers, e.g., lung cancers. [0015] The following sections provide further guidance for practicing the methods of the invention, and for making and using the compositions of the invention.

II. Definitions

[0016] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: Oxford Dictionary of Biochemistry and Molecular Biology, Smith et al. (eds.), Oxford University Press (revised ed., 2000); Dictionary of Microbiology and Molecular Biology, Singleton et al. (Eds.), John Wiley & Sons (3rd ed., 2002); and A Dictionary of Biology (Oxford Paperback Reference), Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). In addition, the following definitions are provided to assist the reader in the practice of the invention.

[0017] The term "agent" or "test agent" includes any substance, molecule, element, compound, entity, or a combination thereof. It includes, but is not limited to, e.g., protein, polypeptide, small organic molecule, polysaccharide, polynucleotide, and the like. It can be a natural product, a synthetic compound, or a chemical compound, or a combination of two or more substances. Unless otherwise specified, the terms "agent", "substance", and "compound" can be used interchangeably.

[0018] The term "analog" is used herein to refer to a molecule that structurally resembles a reference molecule but which has been modified in a targeted and controlled manner, by replacing a specific substituent of the reference molecule with an alternate substituent. Compared to the reference molecule, an analog would be expected, by one skilled in the art, to exhibit the same, similar, or improved utility. Synthesis and screening of analogs, to identify variants of known compounds having improved traits (such as higher binding affinity for a target molecule) is an approach that is well known in pharmaceutical chemistry.

[0019] As used herein, "contacting" has its normal meaning and refers to combining two or more molecules (e.g., a test agent and a polypeptide) or combining
molecules and cells (e.g., a test agent and a cell). Contacting can occur in vitro, e.g., combining two or more agents or combining a test agent and a cell or a cell lysate in a test tube or other container. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

A "heterologous sequence" or a "heterologous nucleic acid," as used herein, is one that originates from a source foreign to the particular host cell, or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that, although being endogenous to the particular host cell, has been modified. Modification of the heterologous sequence can occur, e.g., by treating the DNA with a restriction enzyme to generate a DNA fragment that is capable of being operably linked to the promoter. Techniques such as site-directed mutagenesis are also useful for modifying a heterologous nucleic acid.

The term "homologous" when referring to proteins and/or protein sequences indicates that they are derived, naturally or artificially, from a common ancestral protein or protein sequence. Similarly, nucleic acids and/or nucleic acid sequences are homologous when they are derived, naturally or artificially, from a common ancestral nucleic acid or nucleic acid sequence. Homology is generally inferred from sequence similarity between two or more nucleic acids or proteins (or sequences thereof). The precise percentage of similarity between sequences that is useful in establishing homology varies with the nucleic acid and protein at issue, but as little as 25% sequence similarity is routinely used to establish homology. Higher levels of sequence similarity, e.g., 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% or more can also be used to establish homology.

A "host cell" refers to a prokaryotic or eukaryotic cell into which a heterologous polynucleotide can be introduced. The polynucleotide can be introduced into the cell by any means, e.g., electroporation, calcium phosphate precipitation, microinjection, transformation, viral infection, and/or the like.

The term "sequence identity" in the context of two nucleic acid sequences or amino acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence over a specified comparison window. A
"comparison window" refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are aligned optimally. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482; by the alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443; by the search for similarity method of Pearson and Lipman (1988) Proc. Nat. Acad. Sci U.S.A. 85:2444; by computerized implementations of these algorithms (including, but not limited to CLUSTAL in the PC/Gene program by Intelligentics, Mountain View, CA; and GAP, BESTFIT, BLAST, FASTA, or TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., U.S.A.). The CLUSTAL program is well described by Higgins and Sharp (1988) Gene 73:237-244; Higgins and Sharp (1989) CABIOS 5:151-153; Corpet et al. (1988) Nucleic Acids Res. 16:10881-10890; Huang et al (1992) Computer Applications in the Biosciences 8:155-165; and Pearson et al. (1994) Methods in Molecular Biology 24:307-331. Alignment is also often performed by inspection and manual alignment. In one class of embodiments, the polypeptides herein are at least 70%, generally at least 75%, optionally at least 80%, 85%, 90%, 95% or 99% or more identical to a reference polypeptide, e.g., a Rorl molecule described herein, e.g., as measured by BLASTP (or CLUSTAL, or any other available alignment software) using default parameters. Similarly, nucleic acids can also be described with reference to a starting nucleic acid, e.g., they can be 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more identical to a reference nucleic acid, e.g., as measured by BLASTN (or CLUSTAL, or any other available alignment software) using default parameters.

[0024] A "substantially identical" nucleic acid or amino acid sequence refers to a nucleic acid or amino acid sequence which comprises a sequence that has at least 90% sequence identity to a reference sequence using the programs described above (preferably BLAST) using standard parameters. The sequence identity is preferably at least 95%, more preferably at least 98%, and most preferably at least 99%. For example, the BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an
expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989)). Percentage of sequence identity is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Preferably, the substantial identity exists over a region of the sequences that is at least about 50 residues in length, more preferably over a region of at least about 100 residues, and most preferably the sequences are substantially identical over at least about 150 residues. In a most preferred embodiment, the sequences are substantially identical over the entire length of the coding regions.

[0025] The term "modulate" with respect to a biological activity of a reference protein (e.g., a Rorl molecule disclosed herein) or its fragment refers to a change in the expression level or other biological activities of the protein. For example, modulation may cause an increase or a decrease in expression level of the reference protein, enzymatic modification (e.g., phosphorylation) of the protein, binding characteristics (e.g., binding to another molecule), or any other biological (e.g., enzymatic), functional, or immunological properties of the reference protein. The change in activity can arise from, for example, an increase or decrease in expression of one or more genes that encode the reference protein, the stability of an mRNA that encodes the protein, translation efficiency, or from a change in other biological activities of the reference protein. The change can also be due to the activity of another molecule that modulates the reference protein (e.g., a kinase which phosphorylates the reference protein).

[0026] Modulation of a reference protein can be up-regulation (i.e., activation or stimulation) or down-regulation (i.e. inhibition or suppression). The mode of action of a
modulator of the reference protein can be direct, e.g., through binding to the protein or to
genomes encoding the protein, or indirect, e.g., through binding to and/or modifying (e.g.,
enzymatically) another molecule which otherwise modulates the reference protein.

The term "subject" includes mammals, especially humans. It also
encompasses other non-human animals such as cows, horses, sheep, pigs, cats, dogs,
mice, rats, rabbits, guinea pigs, monkeys.

A "variant" of a reference molecule refers to a molecule substantially
similar in structure and biological activity to either the entire reference molecule, or to a
fragment thereof. Thus, provided that two molecules possess a similar activity, they are
considered variants as that term is used herein even if the composition or secondary,
tertiary, or quaternary structure of one of the molecules is not identical to that found in the
other, or if the sequence of amino acid residues is not identical.

The term "inhibiting" or "inhibition," in the context of tumor growth or
tumor cell growth, refers to delayed appearance of primary or secondary tumors, slowed
development of primary or secondary tumors, decreased occurrence of primary or
secondary tumors, slowed or decreased severity of secondary effects of disease, or
arrested tumor growth and regression of tumors. The term "prevent" or "prevention"
refers to a complete inhibition of development of primary or secondary tumors or any
secondary effects of disease.

The term "treat" or "treatment" refers to arrested tumor growth, and to
partial or complete regression of tumors. The term "treating" includes the administration
of compounds or agents to prevent or delay the onset of the symptoms, complications, or
biochemical indicia of a disease (e.g., lung cancer), alleviating the symptoms or arresting
or inhibiting further development of the disease, condition, or disorder. Treatment may
be prophylactic (to prevent or delay the onset of the disease, or to prevent the
manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or
alleviation of symptoms after the manifestation of the disease.

The term "inhibition," in the context of enzyme inhibition, relates to
reversible enzyme inhibition such as competitive, uncompetitive, and noncompetitive
inhibition. This can be experimentally distinguished by the effects of the inhibitor on the
reaction kinetics of the enzyme, which may be analyzed in terms of the basic Michaelis-
Menten rate equation. Competitive inhibition occurs when the inhibitor can combine with the free enzyme in such a way that it competes with the normal substrate for binding at the active site. A competitive inhibitor reacts reversibly with the enzyme to form an enzyme-inhibitor complex \([EI]\), analogous to the enzyme-substrate complex.

Lung cancer includes both small cell and non-small cell lung cell lung cancer. Specific disease conditions include benign and metastatic carcinomas, adenocarcinomas, epithelial cell tumors, neoplasia, dysplasia, and hyperplasia of lung and lung associated tissues such as bronchial epithelium, pleural tissues, alveolar tissues. Lung cancer may be characterized by a high nuclear/cytoplasmic ratio, hyperchromasia, coarsely granular chromatin, absence of nucleoli, isolated cells and cellular and nuclear pleomorphism.

Mimetic refers to a molecule which, in shape and effect, mimics the shape and therefore the activity of another molecule or complex of molecules upon which it is designed.

**III. Inhibiting Tumor Growth with Rorl -Antagonizing Compounds**

The invention provides methods and compositions to treat cancer in a subject by inhibiting growth of tumor cells. The methods are also useful to prevent tumorigenesis in a subject. Subjects that are suitable for treatment with the methods of the invention are those who are suffering from various types of cancer or those who are at risk or have a predisposition of developing a cancer. A great number of diseases and conditions are amenable to treatment with methods and compositions of the present invention. Examples include tumors originated from lung, skin, breast, brain, gastrointestinal, genitourinary tract (e.g., kidney, bladder and urethra, prostate, testis), blood, the nervous system, bone and liver. They encompass solid tumors and metastatic tumors.

Typically, the methods involve administering to the subject in need of treatment a pharmaceutical composition that contains a Rorl -antagonizing agent of the present invention. The Rorl -antagonizing agent can be used alone or in conjunction with other known anti-cancer agents to provide synergistic effects in the subject. The Rorl-antagonizing agent down-regulates cellular level or inhibits a biological activity (e.g.,
kinase activity) of Ror1. These agents include compounds that can be identified in accordance with the screening methods described below, e.g., small molecule compounds or antibodies (e.g., antagonist antibodies). They also include compounds which specifically inhibit expression or down-regulate cellular level of Ror1.

[0036] In some preferred embodiments, the therapeutic applications of the invention are directed to inhibiting growth of lung cancer. Examples of lung cancer suitable for treatment include bronchogenic carcinoma, alveolar carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesothelioma. In some methods, Ror1-antagonizing agents used are nucleic acid agents such as short interfering RNA (siRNA), microRNA (miRNA), short hairpin RNA (shRNA), anti-sense nucleic acid, or complementary DNA (cDNA). Using these nucleic acid agents to specifically silence expression of a target gene has been well known and routinely practiced in the art. Such nucleic acid agents that specifically target Ror1 can be prepared using methods well known in the art. By way of example, siRNAs targeting a Ror1 gene can be utilized to down-regulate Ror1 expression level, as demonstrated in the Examples below.

Interference with the function and expression of endogenous genes by double-stranded RNA has been shown in various organisms such as C. elegans as described, e.g., in Fire et al., Nature 391:806-811, 1998; drosophila as described, e.g., in Kennerdell et al., Cell 95:1017-1026, 1998; and mouse embryos as described, e.g., in Wianni et al., Nat. Cell Biol. 2:70-75, 2000. Such double-stranded RNA can be synthesized by in vitro transcription of single-stranded RNA read from both directions of a template and in vitro annealing of sense and antisense RNA strands. Double-stranded RNA can also be synthesized from a cDNA vector construct in which a Ror1 gene is cloned in opposing orientations separated by an inverted repeat. Following cell transfection, the RNA is transcribed and the complementary strands reanneal. Double-stranded RNA targeting a Ror1 gene can be introduced into a cell (e.g., a lung tumor cell) by transfection of an appropriate construct. By way of example, sequences of the antisense and sense strands of a specific siRNA which down-regulates Ror1 expression are disclosed herein (SEQ ID NOS: 1 and 7). Other siRNAs targeting Ror1 as well as antisense oligonucleotides have been described in the art, e.g., Paganoni et al., J Cell Sci. 118:433-46, 2005.
The therapeutic applications of the invention can also employ Rorl-antagonizing agents that inhibit a biological activity of Rorl. These include compounds that can be identified in accordance with the below described screen methods. Suitable Rorl-antagonizing agents also include antagonist antibodies which specifically bind to an Rorl polypeptide and antagonize its kinase activity. Monoclonal antibody-based reagents are among those most highly preferred in this regard. Anti-Rorl antagonist antibodies can be generated using methods well known and routinely practiced in the art, e.g., *Monoclonal Antibodies—Production, Engineering And Clinical Applications*, Ritter et al., Eds., Cambridge University Press, Cambridge, UK, 1995; and *Harlow and Lane, Antibodies, A Laboratory Manual*, Cold Spring Harbor Press, 3rd ed., 2000. Radiolabeled monoclonal antibodies for cancer therapy, in particular, are well known and are described in, for instance, *Cancer Therapy With Radiolabeled Antibodies*, D. M. Goldenberg, Ed., CRC Press, Boca Raton, Fla., 1995.

Compounds which down-regulate Rorl expression or its kinase activity can be used in conjunction with other therapies. For example, subjects receiving surgery and radiation therapies can also be administered with a pharmaceutical composition of the present invention. In addition, chemotherapy, hormonal therapy and cryotherapy may also be combined with the therapeutic applications of the present invention to treat subjects suffering from cancers. The Rorl-antagonizing agents can also be used in a subject to prevent tumor growth or treat cancer together with the administration of other therapeutic compounds for the treatment or prevention of these disorders. When an Rorl-antagonizing agent is administered together with another anti-cancer agent, the two can be administered in either order or simultaneously. These therapeutic compounds may be chemotherapeutic agents, ablation or other therapeutic hormones, antineoplastic agents, monoclonal antibodies useful against cancers and angiogenesis inhibitors.

There are many anti-cancer drugs known in the art, e.g., as described in, e.g., *Cancer Therapeutics: Experimental and Clinical Agents*, Teicher (Ed.), Humana Press (1st ed., 1997); and *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, Hardman et al. (Eds.), McGraw-Hill Professional (10th ed., 2001). Examples of suitable anti-cancer drugs include 5-fluorouracil, vinblastine sulfate, estramustine phosphate, suramin and strontium-89. Examples of suitable
chemotherapeutic agents include Asparaginase, Bleomycin Sulfate, Cisplatin, Cytarabine, Fludarabine Phosphate, Mitomycin and Streptozocin. Hormones which may be used in combination with the present invention diethylstilbestrol (DES), leuprolide, flutamide, cyproterone acetate, ketoconazole and amino glutethimide.

IV. Screening for Novel Agents for Inhibiting Tumor Growth

[0040] Identification of the important role that Rorl plays in tumor cell growth provides novel methods to screen for drugs for inhibiting tumor growth and for treating cancers (e.g., lung cancer). Employing a Rorl molecule as a target, various biochemical and molecular biology techniques or assays well known in the art can be employed to practice the screening methods of the present invention. Such techniques are described in, e.g., Handbook of Drug Screening, Seethala et al. (eds.), Marcel Dekker (1st ed., 2001); High Throughput Screening: Methods and Protocols (Methods in Molecular Biology, 190), Janzen (ed.), Humana Press (1st ed., 2002); Current Protocols in Immunology, Coligan et al. (Ed.), John Wiley & Sons Inc (2002); Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press (3rd ed., 2001); and Brent et al., Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (ringbou ed., 2003).

[0041] To screen for Rorl antagonizing modulators, either an intact Rorl molecule or a fragment (e.g., a fragment containing its kinase domain) may be employed. Human Rorl as well as homologs from various other species can be used in the practice of the present invention. Rorl molecules from a number of species have all been well characterized. Polynucleotide and amino acid sequences encoding the Rorl molecules are all known in the art. For example, human Rorl has been characterized and its polynucleotide and amino acid sequences disclosed in, e.g., Masiakowski et al., J. Biol. Chem. 267: 26181-26190, 1992; Reddy et al., Oncogene 13:1555-1559, 1996; and Accession Nos. NM_005003 and NP_005003. Similarly, mouse Rorl has been disclosed in the art, e.g., Oishi et al., Genes Cells 4:41-56, 1999; Al-Shawi et al., Dev. Genes Evol. 211:161-171, 2001; Matsuda et al., Mech. Dev. 105:153-156, 2001; and Accession Nos. NM_013845 and NP_038873. Rorl from other species such as chimpanzee (Accession
Nos. XM_513458 and XP_513458) or fowl (Accession Nos. NM_204509 and NP_989840) are also known.

Any of these Rorl molecules can be employed to screen test compounds for Rorl modulators that inhibit tumor cell growth. Further, polynucleotide sequences or polypeptide sequences that are substantially identical to the sequence of any of the Rorl sequences disclosed herein can also be employed in the screening methods of the invention. Moreover, analogs or functional derivatives of Rorl can also be used in the screening. The fragments or analogs that can be employed in these assays usually retain one or more of the biological activities of Rorl (e.g., its kinase activity). Fusion proteins containing such fragments or analogs can also be used for the screening of test compounds. Functional derivatives of a Rorl molecule usually have amino acid deletions and/or insertions and/or substitutions while maintaining one or more of the bioactivities and therefore can also be used in practicing the screening methods of the present invention. A functional derivative of a given Rorl molecule can be prepared from a Rorl molecule by proteolytic cleavage followed by conventional purification procedures known to those skilled in the art. Alternatively, the functional derivative can be produced by recombinant DNA technology by expressing only fragments of a Rorl molecule that retain one or more of their bioactivities.

Test compounds or candidate agents that can be screened with methods of the present invention include polypeptides, beta-turn mimetics, polysaccharides, phospholipids, hormones, prostaglandins, steroids, aromatic compounds, heterocyclic compounds, benzodiazepines, oligomeric N-substituted glycines, oligocarbamates, polypeptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Some test agents are synthetic molecules, and others natural molecules. In some applications, the test agents are nucleic acids. Nucleic acid test agents can be naturally occurring nucleic acids, random nucleic acids, or "biased" random nucleic acids. In some embodiments, the screening methods are directed to screening inhibitory polynucleotides for agents that specifically down-regulate expression or cellular level of a Rorl molecule. Such inhibitory polynucleotides include, e.g., short interfering RNAs (siRNAs), microRNAs (miRNAs), short RNAs (shRNAs), anti-sense nucleic acids, and complementary DNAs (cDNAs).
Test agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. Combinatorial libraries can be produced for many types of compound that can be synthesized in a step-by-step fashion. Large combinatorial libraries of compounds can be constructed by the encoded synthetic libraries (ESL) method described in WO 95/12608, WO 93/06121, WO 94/08051, WO 95/35503 and WO 95/30642. Peptide libraries can also be generated by phage display methods (see, e.g., Devlin, WO 91/18980). Libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts can be obtained from commercial sources or collected in the field. Known pharmacological agents can be subject to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs. Combinatorial libraries of peptides or other compounds can be fully randomized, with no sequence preferences or constants at any position. Alternatively, the library can be biased, i.e., some positions within the sequence are either held constant, or are selected from a limited number of possibilities. 

Test agents also include antibodies that specifically bind to a Rorl molecule described herein. Typically, anti-Rorl antibodies are screened for ability to antagonize (i.e., suppress) a biochemical activity (e.g., kinase) of Rorl. The antibodies can be monoclonal or polyclonal. Such antibodies can be generated using methods well known in the art. For example, the production of non-human monoclonal antibodies, e.g., murine or rat, can be accomplished by, for example, immunizing the animal with a Rorl molecule or its antigenic fragment (see, e.g., Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Press, 3rd ed., 2000). Such an immunogen can be obtained from a natural source, by peptides synthesis or by recombinant expression. In addition, humanized forms of mouse antibodies can be generated by linking the CDR regions of non-human antibodies to human constant regions by recombinant DNA techniques. See Queen et al., Proc. Natl. Acad. Sci. USA 86, 10029-10033 (1989) and WO 90/07861. Human antibodies can be produced using phage-display methods. See, e.g., Dower et al., WO 91/17271; McCafferty et al., WO 92/01047.

In some other embodiments, test agents are small molecule organic compounds, e.g., chemical compounds with a molecular weight of not more than about 500 or 1,000. Preferably, high throughput assays are adapted and used to screen for such

[0047] In general, test compounds noted above are first screened to identify modulating compounds which down-regulate expression or cellular level of Rorl, or compounds which inhibit other biological activities of Rorl (e.g., its kinase activity). Once compounds which down-regulate Rorl cellular level or its enzymatic activities are identified, they are typically subject to further screening for ability to inhibit growth of tumor cells (e.g., lung tumor cells). This second screening step is typically performed in the presence of Rorl, e.g., using a tumor cell line which endogenously expresses Rorl.

[0048] In some methods, test agents are screened for ability to down-regulate expression or cellular level of Rorl. Preferably, cell-based assays are used to identify agents that inhibit Rorl expression. For example, each test compound (e.g., a siRNA) can be contacted with a cell (e.g., a lung tumor cell) and expression of Rorl is then measured. A potential Rorl-inhibitor is identified if the level of Rorl expression in the cell is substantially lower relative to the level of Rorl expression in cells that have not been treated with the compound. The level of Rorl expression can be detected by, for example, measuring the level of mRNA transcripts corresponding to or proteins encoded by a Rorl gene. In some embodiments, endogenous levels of a Rorl molecule can be directly monitored in cells normally expressing Rorl (e.g., a tumor cell). In some embodiments, expression or cellular level of a Rorl molecule can be examined in an expression system using cloned cDNA or genomic sequence encoding Rorl. Standard detection techniques well known in the art for detecting RNA, DNA, proteins and peptides can readily be applied to detect expression levels of Rorl. Such techniques may include detection with nucleotide probes or may comprise detection of the protein by, for example, antibodies or their equivalent. Types of probe include cDNA, riboprobes, synthetic oligonucleotides and genomic probes. Such techniques are described in, e.g., Sambrook et al., supra; and Brent et al., supra.
In some other methods, down-regulation of Ror1 expression is monitored by measuring expression of a reporter gene under the control of a transcription regulatory element of a Ror1 gene. In these methods, modulation of expression of a Ror1 gene is examined in a cell-based system by transient or stable transfection of an expression vector into cultured cell lines. Assay vectors bearing transcription regulatory sequences (e.g., promoter) of a Ror1 gene operably linked to reporter genes can be transfected into any mammalian host cell line for assays of promoter activity. As noted above, Ror1 genes have been characterized in the art, and their transcription regulatory sequences have all been delineated. See, e.g., Masiakowski et al., J. Biol. Chem. 267:26181-90, 1992; Reddy et al., Oncogene 13:1555-1559, 1996; and Katoh et al., Int J Mol Med. 15:533-8, 2005. Constructs containing a transcription regulatory element of a Ror1 gene that is operably linked to a reporter gene can be prepared using only routinely practiced techniques and methods of molecular biology (see, e.g., Sambrook et al. and Brent et al., supra).

General methods of cell culture, transfection, and reporter gene assay have been described in the art, e.g., Brent, supra; and Transfection Guide, Promega Corporation, Madison, WI (1998). Any readily transflectable mammalian cell line may be used to assay a Ror1 promoter function or to express a Ror1 gene, e.g., CHO, COS, HCT1 16, HEK 293, MCF-7, and HepG2 cell lines. When inserted into the appropriate host cell, the transcription regulatory elements in the expression vector induces transcription of the reporter gene by host RNA polymerases. Reporter genes typically encode polypeptides with an easily assayable enzymatic activity that is naturally absent from the host cell. Typical reporter polypeptides for eukaryotic promoters include, e.g., chloramphenicol acetyltransferase (CAT), firefly or Renilla luciferase, beta-galactosidase, beta-glucuronidase, alkaline phosphatase, and green fluorescent protein (GFP).

In some methods, test agents are screened for ability to inhibit a biological activity of Ror1, e.g., its kinase activity. In general, the methods involve adding a test agent to a sample containing a Ror1 molecule in the presence or absence of other molecules or reagents which are necessary to test the biological activity of Ror1, and determining an alteration in the biological activity of Ror1. For example, test compounds can be screened for ability to modulate the kinase activity of Ror1 in a kinase assay. The
substrate to be used in the screening can be a molecule known to be enzymatically modified by Rorl or a molecule that can be easily identified from candidate substrates for a given class of enzymes. For example, many kinase substrates are available in the art. See, e.g., www.emdbiosciences.com; and www.proteinkinase.de. In addition, a suitable substrate of a kinase can be screened for in high throughput format. For example, substrates of a kinase can be identified using the Kinase-Glo® luminescent kinase assay (Promega) or other kinase substrate screening kits (e.g., developed by Cell Signaling Technology, Beverly, Massachusetts). Rorl kinase activity can also be examined by employing and modifying many specific assays for receptor tyrosine kinases (RTKs) that are known in the art. For example, compounds modulating (e.g., down-regulate) Rorl kinase activity can be identified with the immune complex kinase described in Masiakowski et al., J Biol Chem. 267:26181-90, 1992. Examples of other kinase assays that can be used in the practice of the screen methods of the invention include, e.g., the high-throughput cell-based enzyme-linked immunosorbent assay as described in Baumann et al., J Biochem Biophys Methods. 60:69-79, 2004 or the scintillation proximity assay described in Bembenek et al., Assay Drug Dev Technol. 1:555-63, 2003.

Once modulating compounds which down-regulate Rorl expression level or its kinase activity have been identified, they are typically further tested for ability to modulate (e.g., inhibit) growth of a tumor cell (e.g., a cultured lung cancer cell). This further testing step is often needed to confirm that their modulatory effect on Rorl would indeed lead to inhibition of tumor cell growth. Typically, this screening step is performed in the presence of Rorl on which the modulating agent acts. Preferably, this screening step is performed in vivo using cells that endogenously express Rorl. As a control, effect of the modulating agents on the growth of a normal non-tumor cell can also be examined.

Many cell lines can be used in this screening step. These include the tumor cell lines described in the Examples herein, e.g., A549 or H460 lung cancer cells lines. Examples of other types of cancer cell lines include SKOV-3 (ovarian), ES-2 (ovarian), MDA-MB-231 (breast), A2058 (melanoma) and DU145 (prostate) cell lines. Many other tumor or non-tumor cell lines can also be used to screen for compounds that specifically inhibit tumor growth. These include tumor cell lines such as lung cancer cell lines NCI-H322M and NCI-H460; human glioblastoma cell line U373 (ATCC);
melanoma cell line SK-MEL-2; ovarian cancer cell line OVCAR-4; leukemia lines HL60 and RPMI-8226; colon lines COLO 205 and HCC-2998; brain tumor lines SF-539 and SNB-75; and breast cancer lines MCF7 and HS 578T (Monks et al., Anticancer Drug Des 12: 533-541, 1997; and Boyd and Paull, Drug Dev Res 34: 91-109, 1995). Non-tumor cell lines include, e.g., human embryonic kidney cell line (HEK293); human umbilical vein endothelial cell line (HUVEC); epithelial cell line MCF-IOA (Soule et al., Cancer Res. 50: 6075-6086, 1990); colon cell line (CCL-1840) and ovarian cell line (NOV-31 (Hirasawa et al., Cancer Research 62, 1696-1701, March 15, 2002).

[0054] In addition, ATCC provides many tumor/normal cell line pairs that are used to elucidate the underlying causes of cancers. They can also be employed to screen Rorl -antagonizing agents to identify selective anti-tumor agents. These tumor/normal cell line pairs include non-small cell lung cancer cell line (ATCC No. CCL-256) and normal peripheral blood cell line ATCC No. CCL-256.1; adenocarcinoma cell line ATCC No. CRL-5868 and normal peripheral blood cell line ATCC No. CRL-5957; malignant melanoma cell line ATCC No. CRL-1974 and normal cell line ATCC No. CRL-1980; basal cell carcinoma cell line ATCC No. CRL-7762 and normal skin cell line ATCC No. CRL-7761; colorectal adenocarcinoma cell line ATCC No. CCL-228 and normal lymph node cell line ATCC No. CCL-227; and giant cell sarcoma cell line ATCC No. CRL-7554 and normal bond cell line ATCC No. CRL-7553. Any of these cell line pairs can be used to screen the modulating agents for compounds that selectively inhibit migratory activity of tumor cells.

[0055] There are many available methods for measuring growth of a tumor cell or a non-tumorous control cell. For example, growth of the cells can be examined using the BrightGlo assay (Promega) as demonstrated in the Examples below. This assay quantifies the number of cells which harbor a luciferase reporter by monitoring luminescence from the cells with, e.g., an Analyst GT reader (Molecular Devices). Similar assays for the measurement of cell growth and viability have been described in the art, e.g., the luciferase viability assay (ELVA) reported by Coombe et al., J Immunol Methods. 215:145-50, 1998. In addition, many other assays routinely practiced in the art can also be employed to examine cell growth in the practice of the screening methods of the present invention. Examples of assays well known in the art for measuring cell

[0056] In addition to these in vitro methods, ability of the Rorl -antagonizing compounds to inhibit tumor cell growth can also be examined in an animal model. Various animal models of cancer are known in the art which can be employed in these studies. For example, mouse has been one of the most informative and productive experimental system for studying carcinogenesis (Sills et al., Toxicol Letters 120:187-198, 2001), cancer therapy (Malkinson, Lung Cancer 32:265-279, 2001; and Hoffman, Invest New Drugs 17:343-359, 1999), and cancer chemoprevention (Yun, Annals NY Acad Sci. 889:157-192, 1999). To transplant human cancer to an animal and to prevent its destruction by rejection, the immune system of the animal are compromised. For example, nude mice that are athymic congenitally have been used as recipients of a variety of human tumors. The severe combined immunodeficient (SCID) mice were also developed in which the acquired immune system is completely disabled by a genetic mutation. Human lung cancer was first used to demonstrate the successful engraftment of a human cancer in the SCID mouse model (Reddy, Cancer Res. 47:2456-2460, 1987). Subsequently, the SCID mouse model have been shown to allow disseminated metastatic growths for a number of human tumors, particularly hematologic disorders and malignant melanoma (Mueller, Cancer Metastasis Rev. 10:193-200, 1991; and Bankert et al., Trends Immunol. 22:386-393, 2001). Further, the advent of transgenic technology allows the mouse genome to become the primary mammalian genetic model for the study of cancer (Resor et al., Human Molec Genet. 10:669-675, 2001). Any of these animal models can be used to study the effect of the Rorl -antagonizing compounds on the growth of various types of human tumors (e.g., human lung tumor).

V. Pharmaceutical Compositions and Administration

[0057] The Rorl -antagonizing compounds of the present invention can be administered alone under sterile conditions to a subject in need of treatment. More
preferably, they are administered as an active ingredient of a pharmaceutical composition. Pharmaceutical compositions of the present invention typically comprise an effective amount of at least one Rorl- antagonizing agent described herein together with one or more acceptable carriers thereof. The compositions can also contain a second therapeutic agent noted above, e.g., a chemotherapeutic agent or other anti-cancer agent. Pharmaceutically carriers enhance or stabilize the composition, or to facilitate preparation of the composition. Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., nucleic acid, protein, or other type of compounds), as well as by the particular method used to administer the composition. They should also be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the subject. They may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral, sublingual, rectal, nasal, or parenteral. For example, an antitumor compound can be complexed with carrier proteins such as ovalbumin or serum albumin prior to their administration in order to enhance stability or pharmacological properties.

[0058] There are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., Remington: The Science and Practice of Pharmacy, Mack Publishing Co., 20th ed., 2000). Without limitation, they include syrup, water, isotonic saline solution, 5% dextrose in water or buffered sodium or ammonium acetate solution, oils, glycerin, alcohols, flavoring agents, preservatives, coloring agents starches, sugars, diluents, granulating agents, lubricants, and binders, among others. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax.

[0059] The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100% by weight. Therapeutic formulations are prepared by any methods well known in the art of pharmacy. See, e.g., Gilman et al., eds., Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; Remington: The Science and Practice of Pharmacy, Mack Publishing Co., 20th ed., 2000; Avis et al., eds.,
[0060] The therapeutic formulations can be delivered by any effective means that can be used for treatment. Depending on the specific antitumor agent to be administered, the suitable means include oral, rectal, vaginal, nasal, pulmonary administration, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) infusion into the bloodstream. For parenteral administration, antitumor agents of the present invention may be formulated in a variety of ways. Aqueous solutions of the modulators may be encapsulated in polymeric beads, liposomes, nanoparticles or other injectable depot formulations known to those of skill in the art. Additionally, the compounds of the present invention may also be administered encapsulated in liposomes. The compositions, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomal suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surfactants such a diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature.

[0061] The therapeutic formulations can conveniently be presented in unit dosage form and administered in a suitable therapeutic dose. A suitable therapeutic dose can be determined by any of the well known methods such as clinical studies on mammalian species to determine maximum tolerable dose and on normal human subjects to determine safe dosage. Except under certain circumstances when higher dosages may be required, the preferred dosage of an antitumor agent of the present invention usually lies within the range of from about 0.001 to about 1000 mg, more usually from about 0.01 to about 500 mg per day. The preferred dosage and mode of administration of an antitumor agent can vary for different subjects, depending upon factors that can be individually reviewed by the treating physician, such as the condition or conditions to be treated, the choice of composition to be administered, including the particular antitumor agent, the age, weight, and response of the individual subject, the severity of the subject's symptoms, and the
chosen route of administration. As a general rule, the quantity of an antitumor agent administered is the smallest dosage which effectively and reliably prevents or minimizes the conditions of the subjects. Therefore, the above dosage ranges are intended to provide general guidance and support for the teachings herein, but are not intended to limit the scope of the invention.

EXAMPLES

[0062] The following examples are provided to illustrate, but not to limit the present invention.

Example 1. Rorl mediates canonical Wnt-signaling in lung cancer cells

[0063] The Wnt signaling pathway regulates numerous processes in animal development and has increasingly been implicated in tissue homeostasis in adult organisms. The aberrant activation of Wnt signaling has also been associated with many types of cancer. However, the players of the Wnt-signaling pathways are still not jet completely identified. In addition, suitable therapeutic targets in the Wnt-pathway and effective therapeutic compounds are not jet identified. Wnt signaling has clearly emerged as a critical pathway in lung carcinogenesis; however, it is proposed that in lung cancer, a different mechanism exists than in e.g. colorectal cancer for activation of the Wnt-mediated signal. The role of each mechanism and their relationship to one another remain unclear.

[0064] The lung cancer cell lines A549 (ATCC) and H460 (ATCC) were seeded in 96-well plates in the density of 15,000 cells per well in 100 μl DMEM (Invitrogen) with 50% FBS (Invitrogen) (for A549) or RPMI (Invitrogen) with 50% FBS (for H460) 24 hours prior to transfection with siRNAs. The cells were respectively transfected with 50 nM of dicer substrate siRNA (IDT) against Rorl, LRP6 or β-catenin. Sequences of the antisense and sense strands of the siRNA targeting Rorl are, respectively, 5’-GUGGAAGAAACCACCUCCUUGCCGUUU-3’ (SEQ ID NO: 1) and 5’-ACGGCAAGGAGGUGGUUUCUCCAC-3’ (SEQ ID NO: 2). As a non-specific control, a non-coding siRNA was also used. For each well 0.2 μl of Lipofectamin
(Invitrogen) were incubated in 10 µl of OPTIMEM (Invitrogen) for 5 min. A solution of dicer-substrate siRNA (20 µM stock solution, final concentration 50 nM) in 10 µl OPTIMEM was mixed with the Lipofectamin-OPTIMEM solution and incubated for 20 min. 20 µl this Lipofectamin-siRNA solution were transferred into each well. To test the siRNA knockout of Rorl and LRP6, in parallel to this transfection, 150,000 cells of A549 and H460 cells were seeded in 12-well plates in 1 ml of medium and transfected 24 h later with the same 50 nM dicer-substrate siRNA. For each 12-well 2 µl of Lipofectamine were mixed with 100 µl OPTIMEM and mixed with a solution of 100 µl OPTIMEM with 50 nM dicer-substrate siRNA. 200 µl of this mixture were transferred into each well. 18 h after the transfection the medium of the cells on both the 96-well plates and on the 12-well plates was replaced with fresh medium. The cells in the 96-well plates were transfected with the TOPFLASH reporter construct to measure the impact of the Rorl knockout on the activation of the canonical Wnt-signaling. For each well 10 µl of OPTIMEM (Invitrogen) were mixed with 0.3 µl of FUGENE (Roche) according to the protocol of Roche. After 5 min 0.1 µg of the TOPFLASH DNA were mixed with this solution and incubated for 15 min. 10 µl of this mixture were transferred into each well. 24 h later the medium on the cells was exchanged to Wnt3A-conditioned medium or medium only. Another 24 h later luciferase activity was detected with BrightGlo assay (Promega). In parallel a CellTiterGo (Promega) assay was performed on identically treated cells to detect the cell number and to control the effect of the siRNAs on the cell number. The luminescence was detected with Analyst GT (Molecular Devices). The BrightGlo values were normalized on the CellTiterGlo values. The percentage of cell number was normalized on the non-coding siRNA. All experiments were performed in triplicates.

In parallel to the BrightGlo assay, effects of siRNA (Rol-siRNA or LRP-siRNA) treatment on Rorl expression level in the cells were examined by fluorescence activated cell sorting (FACS) analysis. Specifically, cells in the 12-well plates were harvested with 500 µl Trypsin (Invitrogen) and washed with 2 ml of PBS. 500,000 cells were incubated with a Rorl- or a LRP6-antibody (R&D) 1:100 in 100 µl of PBS for 1 h. The cells were then washed with 4 ml of PBS and incubated 1:200 for 1 h with an APC-conjugated anti-goat antibody (Jackson Laboratories). The cells were washed once with 4
ml of PBS and resuspended in 300 µl of PBS and analyzed for APC-fluorescence (LSRII, BD).

The results from these studies are shown in Figure 1. As shown in the figure, knockout of Rorl with siRNAs decreases the activity of the Topflash promoter down to 5% in H460 cell lines. From the FACS analysis, it was found that siRNA knockout of Rorl resulted in a Rorl expression level that was reduced by about 95% (data not shown). This is consistent with the reduction in the Topflash promoter activity. Thus, these results indicate that Rorl is a regulator in the canonical Wnt-pathway. Similarly, it was observed that in A549 cells the Topflash promoter activity is decreased by 50%. The reason for the smaller effect compared to H460 cells is that A549 cells express higher levels of Rorl. Specifically, results from the FACS analysis showed that siRNA knockout resulted in a Rorl level that is reduced by only 50% (data not shown). In conclusion, our data demonstrate that Rorl mediates canonical Wnt-signaling in the lung cancer cell lines A549 and H460.

Example 2. Knockout of Rorl inhibits growth of lung cancer cells

The lung cancer cell lines A549-luc and H460-luc were seeded in 96-well plates in the density of 5,000 cells per well in 100 µl DMEM (Invitrogen) with 50% FBS (Invitrogen) (for A549) or RPMI (Invitrogen) with 50% FBS (for H460) 24 hours prior to transfection with siRNAs. The cells were transfected with 50 nM of dicer substrate siRNA (IDT - Integrated DNA Technology) against Rorl, LRP6, β-catenin. As unspecific control a non-coding siRNA was used (IDT). As a control for growth inhibition siRNA against polo-kinase was used. For each well 0.2 µl of Lipofectamin (Invitrogen) were incubated in 10 µl of OPTIMEM (Invitrogen) for 5 min. A solution of dicer-substrate siRNA (20 nM stock solution, final concentration 50 nM) in 10 µl OPTIMEM was mixed with the Lipofectamin-OPTIMEM solution and incubated for 20 min. 20 µl this Lipofectamin-siRNA solution were transferred into each well. In order to test siRNA knockout of Rorl and LRP6, 50,000 cells of A549 and H460 cells were also seeded in 12-well plates in 1 ml of medium and transfected 24 h later with the same 50 nM dicer-substrate siRNA. For each 12-well 2 µl of Lipofectamin were mixed with 100 µl OPTIMEM and mixed with a solution of 100 µl OPTIMEM with 50 nM dicer-substrate
siRNA. 200 µl of this mixture were transferred into each 12 well. 18 h after the transfection the medium of the cells on both the 96 well plates and on the 12 well plates was replaced with fresh medium. 72 h after the transfection the amount of cells was detected with BrightGlo assay (Promega). The luminescence was detected with Analyst GT (Molecular Devices). The percentage of cell number was normalized to cells treated with the non-coding siRNA. All experiments were done in triplicats.

As shown in Figure 2, results from these studies showed that knockout of Rorl with siRNA decreases the cell number of H460-luc cells to 12% of the control. As demonstrated in the FACS analysis, Rorl siRNA treatment reduced Rorl levels by 95% in H460 cells. This indicates that the growth of H460 cells is dependent on the expression level of Rorl. In A549 cells the growth is reduced to 50% of the controls. This is also consistent with the reduced level (about 50%) of Rorl in Rorl-siRNA treated A549 cells relative to control cells. In conclusion, the efficiency of the knockout correlates with the reduction in growth. These results demonstrate that the growth of both lung cancer cell lines, A549 and H460, is dependent on the expression of Rorl, and that targeting Rorl inhibits the growth of these lung cancer cell lines.

Example 3. Knockout of Rorl mediates apoptosis in lung cancer cells

The lung cancer cell lines H460-luc was seeded in 12-well plates in the density of 150,000 cells per well in 1 ml RPMI (Invitrogen) with 50% FBS 24 hours prior to transfection with siRNAs. The cells were transfected with 50 nM of dicer substrate siRNA (IDT) against Rorl, LRP6, b-catenin. As a non-specific control, a non-coding siRNA was used (IDT). As a control for growth inhibition, siRNA against polo-kinase was used. For each well 2 µl of Lipofectamin (Invitrogen) were incubated in 100 µl of OPTIMEM (Invitrogen) for 5 min. A solution of dicer-substrate siRNA (20 µM stock solution, final concentration 50 nM) in 100 µl OPRIMEM was mixed with the Lipofectamin-OPTIMEM solution and incubated for 20 min. 200 µl this Lipofectamin-siRNA solution were transferred into each well. 18 h after the transfection the medium of the cells on both the 96 well plates and on the 12 well plates was replaced with fresh medium. 72 h after the transfection the amount of apoptotic cells was detected with AnnexinV-PE according to the manufactures protocol (BD Biosciences).
This experiment was performed two times independently. The average of the data with standard deviations is shown. As shown in Figure 3, results from these experiments clearly show that the knockout of Rorl with siRNAs induces apoptosis in H460 cells.

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It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

All publications, patents, patent applications, polynucleotide and polypeptide sequence accession numbers and other documents cited herein are hereby incorporated by reference in their entirety and for all purposes to the same extent as if each of these documents were individually so denoted.
We Claim:

1. A method for inhibiting growth of a tumor cell, the method comprising contacting the cell with an agent which down-regulates Rorl cellular level or inhibits Rorl kinase activity.

2. The method of claim 8, wherein the tumor cell is a lung tumor cell.

3. The method of claim 8, wherein the tumor cell is present in a subject having or at risk of developing lung cancer.

4. The method of claim 8, wherein the agent is selected from the group consisting of an short interfering RNA (siRNA), a microRNA (miRNA), a short hairpin RNA (shRNA), an anti-sense nucleic acid, and a complementary DNA (cDNA).

5. The method of claim 8, wherein the agent is an antibody that specifically binds to Rorl.

6. The method of claim 11, wherein the antibody inhibits the kinase activity of Rorl.

7. The method of claim 11, wherein the antibody is a monoclonal antibody.

8. A method for treating lung cancer in a subject, the method comprising administering to the subject a pharmaceutical composition comprising an effective amount of an agent which down-regulates Rorl cellular level or inhibits Rorl kinase activity.

9. The method of claim 8, wherein the agent down-regulates expression of a gene encoding Rorl.

10. The method of claim 9, wherein the agent is selected from the group consisting of an short interfering RNA (siRNA), a microRNA (miRNA), a short hairpin RNA (shRNA), an anti-sense nucleic acid, and a complementary DNA (cDNA).

11. The method of claim 8, wherein the agent is an antibody that specifically binds to Rorl.

12. The method of claim 11, wherein the antibody inhibits the kinase activity of Rorl.
13. The method of claim 11, wherein the antibody is a monoclonal antibody.

14. A method for identifying an agent for treating lung cancer, the method comprising (a) screening test compounds to identify one or more modulating compounds which down-regulates Rorl cellular level or inhibits Rorl kinase activity; and (b) testing the identified modulating compounds for ability to inhibit lung cancer growth.

15. The method of claim 14, wherein the test compounds are screened for ability to down-regulate cellular level of Rorl.

16. The method of claim 14, wherein the test compounds are screened for ability to inhibit the kinase activity of Rorl.

17. The method of claim 14, wherein (b) comprises testing the modulating compounds for ability to inhibit growth of a cultured lung cancer cell.

18. The method of claim 14, wherein the cultured lung cancer cell is A549 cell line or H460 cell line.

19. The method of claim 14, further comprising testing the identified modulating compounds for ability to modulate growth of a non-tumor control cell.

20. The method of claim 19, wherein (b) comprises comparing growth of a cultured lung cancer cell in the presence of a modulating compound to growth of the lung cancer cell in the absence of the modulating compound; wherein a significant inhibition of growth of the lung cancer cell in the presence of the modulating compound relative to growth of the lung cancer cell in the absence of the modulating compound identifies the modulating compound as an agent for treating lung cancer.
**FIGURE 2**

**growth of A549**

- % RLU of control
- control, Ror1, LRP6, β-catenin
- siRNA (50nM)

**growth of H460**

- % RLU of control
- control, Ror1, LRP6, β-catenin, polk.
- siRNA (50nM)
FIGURE 3

The figure shows a bar graph illustrating the percentage of apoptotic cells in H460 cells treated with different siRNAs at 50 nM. The categories include control, Ror1, LRP6, β-catenin, Polo kinase, and siRNA (50 nM). The graph indicates varying levels of apoptosis across these treatments.