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NOTCH PATHWAY SIGNALING INHIBITOR COMPOUNDS

Loss of the sensory hair cells in the inner ear through aging, exposure to noise, exposure to chemical agents, medications, disease, and genetic disorders cause hearing disorders in many people each year. Regardless of the etiology, the death or dysfunction of mechanosensory hair cells located within the organ of Corti of the cochlea is the primary cause of sensorineural hearing loss (SNHL). Prophylactic measures to inhibit hearing loss are primarily limited to peripheral protection, such as ear plugs or noise reduction or cancelling headphones. Current treatments for SNHL are primarily based on electronic technologies such as amplification of sound using hearing aids or bypassing the hair cells through electrical stimulation of the surviving spiral ganglion neurons using cochlear implants. Steroid administration, to afford systemic therapy or intratympanic therapy have also been suggested for sudden sensorineural hearing loss.

The cochlear sensory epithelium contains hair cells adapted for the detection of sound vibration, which is transduced by stereocilia at the hair cell's apical surfaces into electrical impulses and transmitted to the brain through the VIIIth cranial nerve. Auditory hair cells produced during development are post-mitotic and are not replaced after loss or as part of normal cell turnover in mammals. As a result, SNHL due to auditory hair cell loss is irreversible. Auditory hair cell development during the embryonic period includes a differentiation, in which prosensory epithelial cells acquire different fates, either hair cell or supporting cell, through a process of lateral inhibition which is mediated by Notch signaling. Prosensory epithelial cells are prevented from differentiating into hair cells by active Notch signaling stimulated by ligands on adjacent hair cells. These cells become supporting cells.

Notch signaling is an evolutionary conserved pathway that plays an integral role in development and tissue homeostasis in mammals. The Notch receptors and ligands contain single-pass transmembrane domains, are expressed on the cell surface and, for that reason, Notch signaling is particularly important in mediating communication between adjacent cells expressing the receptors and ligands. There are four known Notch receptors found in rodents and humans, termed Notch 1 to Notch 4. The Notch receptors are heterodimeric proteins composed of extracellular and intracellular domains that are

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initially synthesized as a single polypeptide. Receptor–ligand interaction triggers a series of proteolytic cleavages of the Notch receptor polypeptide in which γ -secretase activity is involved. γ -Secretase activity cleaves Notch intracellular domain from the internal side of the plasma membrane which translocates to the nucleus to form a transcription factor complex. Notch intracellular domain (NICD) is the active form of the protein. Various Notch signaling functions include proliferation, differentiation, apoptosis, angiogenesis, migration and self-renewal. These diverse roles of Notch signaling during the development and maintenance of normal tissues can be aberrantly activated in different forms of cancer. The oncogenic functions of Notch signaling include the inhibition of apoptosis and the promotion of cell proliferation.

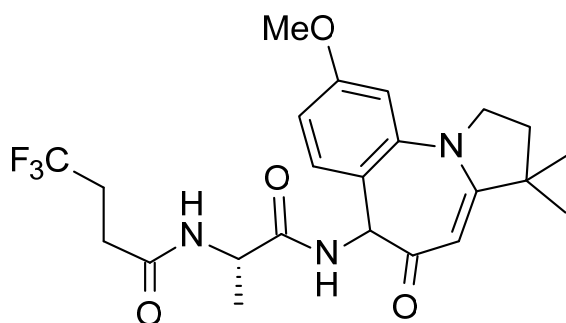
γ -Secretase plays a pivotal role in the Notch activation cascade. As a result, inhibitors of γ -secretase have been actively investigated for their potential to block Notch receptor activation for the treatment of cancer. No commercial Notch inhibitor chemotherapeutic drugs have emerged although clinical trials are continuing.

γ -Secretase, through the Notch signaling pathway, also plays a pivotal role in prosensory cell differentiation. As a result, inhibitors of γ -secretase have been actively investigated for their potential to block Notch receptor activation for the treatment of SNHL. Rather than administering a Notch inhibiting agent by various methodologies for entry into systemic circulation to induce atonal homolog 1 (*Atoh1* or *Math1*) expression at abnormally elevated levels and for extended periods of time, it would be useful and desirable to induce *Atoh1* expression, in the cochlear environment, at a more targeted expression level and period of time. The inability to modulate *Atoh1* remains an obstacle to research in auditory hair cells and the development of therapeutics for auditory hair cell regeneration. Although research continues, such as WO 2014/039781, no commercial Notch inhibitor for treating sensorineural hearing loss caused by auditory hair cell loss has emerged.

There is a need to find compounds having Notch pathway signaling inhibitory activity. There is a further need to find compounds which inhibit Notch pathway signaling by γ -secretase inhibitory activity. There is also a need to find compounds possessing distinct structural features that may contribute to Notch pathway signaling and γ -secretase inhibitory activity. A further need is to find compounds that induce

Atoh1 expression by inhibiting Notch pathway signaling. There is a further need to find compounds demonstrating desirable absorption, distribution, metabolism and excretion properties.

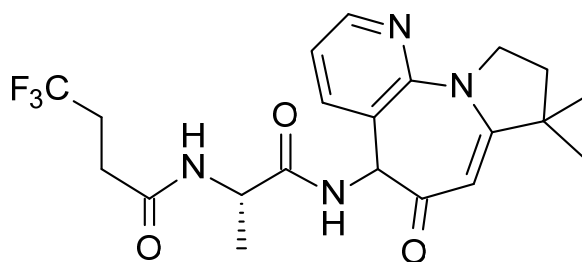
One aspect of the present disclosure is to provide a compound of the structure:



Compound 1

4,4,4-trifluoro-N-((2S)-1-((9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide, or a substantially diastereomerically pure isomer thereof, or a pharmaceutically acceptable salt of any of the above.

A further aspect of the present disclosure provides a compound of the structure:



Compound 2

N-((2S)-1-((8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide, or a substantially

diastereomerically pure isomer thereof, or a pharmaceutically acceptable salt of any of the above.

Another aspect of the present disclosure provides a pharmaceutical composition comprising either Compound 1, or a substantially pure diastereomer thereof; or

5 Compound 2, or a substantially pure diastereomer thereof; or a pharmaceutically acceptable salt of any of the above, and a pharmaceutically acceptable carrier.

The present disclosure also provides a method of treating a cancer which is T-cell acute lymphoblastic leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, erythroleukemia, 10 triple negative breast cancer, breast cancer, ovarian cancer, melanoma, lung cancer, non small cell lung cancer, pancreatic cancer, glioblastoma, colorectal cancer, head and neck cancer, cervical cancer, prostate cancer, liver cancer, oral squamous cell carcinoma, skin cancer, medulloblastoma, angiosarcoma, rhabdomyosarcoma, liposarcoma, malignant fibrous histiocyto

15 ma, hepatocellular carcinoma, intrahepatic and extrahepatic cholangiocarcinoma, and adenoid cystic carcinoma in a patient comprising administering to a patient in need thereof a therapeutically effective amount of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof.

20 The present disclosure further provides a method of treating lung cancer in a patient comprising administering to a patient in need thereof a therapeutically effective amount of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof.

25 The present disclosure also provides a method of treating sensorineural hearing loss caused by auditory hair cell loss in a patient in need thereof comprising administering a therapeutically effective amount of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, to said patient.

The present disclosure further provides a method of inducing auditory hair cell generation in a patient in need thereof, comprising administering a therapeutically effective amount of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a
5 pharmaceutically acceptable salt thereof, to said patient.

Another aspect of the present disclosure provides Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for use in therapy.

10 The present disclosure also provides Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for use in the treatment of T-cell acute lymphoblastic leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, erythroleukemia,
15 triple negative breast cancer, breast cancer, ovarian cancer, melanoma, lung cancer, non small cell lung cancer, pancreatic cancer, glioblastoma, colorectal cancer, head and neck cancer, cervical cancer, prostate cancer, liver cancer, oral squamous cell carcinoma, skin cancer, medulloblastoma, angiosarcoma, rhabdomyosarcoma, liposarcoma, malignant fibrous histiocytoma, hepatocellular carcinoma, intrahepatic or extrahepatic cholangiocarcinoma, or
20 adenoid cystic carcinoma.

The present disclosure also further provides Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for use in the treatment of lung cancer.

25 The present disclosure also provides Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof for use in the treatment of sensorineural hearing loss caused by auditory hair cell loss.

The present disclosure further provides Compound 1, a substantially pure
30 diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2,

a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for use in inducing auditory hair cell generation.

Another aspect of the present disclosure provides use of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for the treatment of T-cell acute lymphoblastic leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, erythroleukemia, triple negative breast cancer, breast cancer, ovarian cancer, melanoma, lung cancer, non small cell lung cancer, pancreatic cancer, glioblastoma, colorectal cancer, head and neck cancer, cervical cancer, prostate cancer, liver cancer, oral squamous cell carcinoma, skin cancer, medulloblastoma, angiosarcoma, rhabdomyosarcoma, liposarcoma, malignant fibrous histiocytoma, hepatocellular carcinoma, intrahepatic or extrahepatic cholangiocarcinoma, or adenoid cystic carcinoma.

The present disclosure also provides a use of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for the treatment of lung cancer.

Another aspect of the present disclosure provides use of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for the treatment of sensorineural hearing loss caused by auditory hair cell loss.

A further aspect of the present disclosure provides use of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for inducing auditory hair cell generation.

The present disclosure also provides a method of treating sensorineural hearing loss caused by auditory hair cell loss in a canine companion animal comprising administering a therapeutically effective amount of Compound 1, a substantially pure

diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, to said canine companion animal.

5 The present disclosure further provides a method of inducing auditory hair cell generation in a canine companion animal in need thereof, comprising administering a therapeutically effective amount of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, to said canine companion animal.

10 The phrase "Compound 1, a diastereomer thereof" means 4,4,4-trifluoro-N-((2S)-1-((9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide; or a diastereomer 4,4,4-trifluoro-N-((S)-1-(((S)-9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide or 4,4,4-trifluoro-N-((S)-1-(((R)-9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide. Similarly, the phrase "Compound 2, a diastereomer thereof" means N-((2S)-1-((8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide; or a diastereomer N-((S)-1-(((S)-8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide or N-((S)-1-(((R)-8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide.

25 The term "patient" means mammal and "mammal" includes, but is not limited to, a human after the postnatal period. The postnatal period in a human is the period beginning immediately after childbirth and extending for 30 days.

"Therapeutically effective amount" or "effective amount" means the dosage of either Compound 1, a substantially pure diastereomer thereof, which is Isomer 1 or Isomer 2, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, which is Isomer 1 or Isomer 2, or a pharmaceutically acceptable salt thereof, or pharmaceutical composition containing any of the above, necessary to inhibit Notch signaling in a cancer patient, and either destroy the target

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cancer cells or slow or arrest the progression of the cancer in a patient. Similarly, “therapeutically effective amount” or “effective amount” means the dosage of either Compound 1, a substantially pure diastereomer thereof, which is Isomer 1 or Isomer 2, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, which is Isomer 1 or Isomer 2, or a pharmaceutically effective salt thereof, or a pharmaceutical composition containing any of the above, necessary to inhibit Notch signaling in a sensorineural hearing loss patient caused by auditory hair cell loss or damage, or induce auditory hair cell generation.

A “substantially pure diastereomer of Compound 1, or Compound 2” means Isomer 1 or Isomer 2 substantially free of the other Isomer. Compound 1 or Compound 2 is “substantially diastereomerically pure” when the isomeric purity at the 6-position of Compound 1 or the 5-position of Compound 2, is greater than 90% enantiomeric excess. In another embodiment, the isomeric purity is greater than 95% enantiomeric excess at the 6-position of Compound 1 or the 5-position of Compound 2. In still another embodiment, the isomeric purity is greater than 98% enantiomeric excess at the 6-position of Compound 1 or the 5-position of Compound 2. In yet another embodiment, the isomeric purity is greater than 99% enantiomeric excess at the 6-position of Compound 1 or the 5-position of Compound 2. All stereoisomers, including diastereomeric mixtures of Compound 1 or Compound 2 are contemplated within the present invention.

Anticipated dosages of Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for treating cancer are in the range of 0.1 to 100 mg/patient/day. Preferred dosages are anticipated to be in the range of 1.0 to 75 mg/patient/day. Most preferred dosages are anticipated to be in the range of 2.0 to 50 mg/patient/day. Anticipated dosages of Compound 1, a diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a diastereomer thereof, or a pharmaceutically acceptable salt thereof, for treating sensorineural hearing loss caused by auditory hair cell loss or damage, or induce auditory hair cell generation are in the range of 0.01 to 100 mg/patient/day. Preferred dosages are anticipated to be in

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the range of 0.1 to 10 mg/patient/day. Most preferred dosages are anticipated to be in the range of 0.2 to 1.0 mg/patient/day.

For cancer, sensorineural hearing loss, or inducing auditory hair cell generation, the exact dosage required to treat a patient and the length of treatment time will be
5 determined by a physician in view of the stage and severity of the disease as well as the specific needs and response of the individual patient. Although expressed as dosage on a per day basis, the administration regimen may be adjusted to provide a more optimal therapeutic benefit to a patient and to manage and ameliorate any drug related toxicities.. In addition to daily administration, administration every other day (Q2D); every other day
10 over a five day period followed by two days without dosing (T.I.W.); every third day (Q3D); or once every week (Q.I.W.) over a 21-day dosing cycle; or other administration regimens may be appropriate.

The terms "treatment," "treat," and "treating," are meant to include the full spectrum of intervention for the cancer, sensorineural hearing loss caused by auditory hair
15 cell loss or damage, or induction of auditory hair cell generation, such as administration of the active compound to alleviate, slow or reverse one or more of the symptoms and to delay progression of the cancer or auditory hair cell loss or damage from which the patient is suffering, or induce auditory hair cell generation in the patient.

"Canine companion animals" means domesticated and domestic-bred canines
20 including, but not limited to, pets, service dogs, rescue dogs, herding dogs and livestock guardian dogs.

"Auditory hearing loss," "sensorineural hearing loss" or "hearing loss" in a human means the hearing threshold (softest sound heard (intensity) at a specific frequency) in a patient is 21 to 40 dB for mild; 41 to 55 dB for moderate; 56 to 70 dB for moderately-
25 severe; 71 to 90 dB for severe; and 91 dB and above for profound. Intensities tested typically range from 0 dB up to 120 dB. Frequencies tested are typically 250, 500, 1000, 2000, 4000 and 8000 Hz. Diagnostic hearing evaluations are carried out by routine testing known and used by those skilled in the art including pure tone audiometry including pure tone average, speech audiometry including speech reception threshold,
30 auditory brainstem response evaluations (ABR or BAER), transtympanic electrocochleography (ECOG), and otoacoustic emission testing (OAE). Pediatric and

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infant evaluations are also carried out by routine testing known and used by those skilled in the art including visual reinforcement audiometry, play audiometry, otoacoustic emissions (OAEs), and auditory brainstem response evaluations.

5 A compound of the present invention is preferably formulated as a pharmaceutical composition with a pharmaceutically acceptable carrier and administered by a variety of routes. Preferably, for treating cancer, such compositions are for oral administration.

For treating sensorineural hearing loss cause by auditory hair cell loss or damage, or inducing auditory hair cell generation a pharmaceutical composition suitable for oral or parenteral administration to afford local or systemic therapy may be formulated and
10 administered. Oral compositions include tablets, coated tablets, hard or soft gelatin capsules, solutions, emulsions or suspensions. Parenteral compositions may be formulated to permit administration including subcutaneous injections, intravenous, intramuscular intraperitoneal, intrapleural, intrasternal, transtympanic, intralabryinthine, or intracochlear injections, or infusion. The pharmaceutical compositions may be
15 formulated to permit a compound of the invention to be bioavailable upon administration of the composition to a patient, or formulated to afford controlled or sustained release of the active pharmaceutical ingredient. A pharmaceutical composition suitable for transtympanic administration to the middle ear cavity is preferred, and administration to the round window niche of the middle ear is also preferred. Intralabryrinthine injection
20 and intracochlear injection are also contemplated. The preferred route of administration for hearing loss treatment is local transtympanic injection, however this does not exclude pharmaceutical compositions suitable for alternate routes of administration to afford systemic delivery and may include compositions and dosing regimes alternative or in addition to local transtympanic administration. Also preferred is a sustained release local
25 delivery composition where the release interval may range from three (3) days to ninety (90) days depending on the delivery vehicle, or mixture of delivery vehicle agents, in which Compound 1, or a substantially pure diastereomer, or a pharmaceutically acceptable salt thereof, is associated.

Pharmaceutical compositions, processes for preparing, and delivery systems for
30 targeted delivery of drugs are well known in the art. *See, e.g.*, REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY (A. Gennaro, *et al.*, eds., 19th ed., Mack

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Publishing Co., 1995); Salt and Plontke, "Principles of Local Drug Delivery to the Inner Ear", *Audiol Neurotol*, 2009, (14); 350-360; Rhee, *et al.*, "Sustained-Release Injectable Drug Delivery," *Pharmaceutical Technology*, Special Issue Drug Delivery, 01 Nov. 2010. The pharmaceutical compositions may contain preservatives, solubilizers, stabilizers, wetting agents, emulsifiers, sweeteners, colorants, flavorants, salts for varying osmotic pressure, buffers, masking agents, or antioxidants.

A compound of the present invention is capable of reaction with a number of inorganic and organic acids to form pharmaceutically acceptable acid addition salts. Such pharmaceutically acceptable salts and common methodology for preparing them are well known in the art. *See, e.g.*, P. Stahl, *et al.*, HANDBOOK OF PHARMACEUTICAL SALTS: PROPERTIES, SELECTION AND USE, (VCHA/Wiley-VCH, 2002); S.M. Berge, *et al.*, "Pharmaceutical Salts," *Journal of Pharmaceutical Sciences*, Vol. 66, No. 1, January 1977.

Compound 1, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, may be prepared by a variety of procedures known in the art, as well as those described below. The specific synthetic steps may be combined in different ways to prepare Compound 1, a diastereomer thereof, or a pharmaceutically acceptable salt thereof, or Compound 2, a diastereomer thereof, or a pharmaceutically acceptable salt thereof.

Compound 1 is named: 4,4,4-trifluoro-N-((2S)-1-((9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide and may also be named 4,4,4-trifluoro-N-((1S)-2-[(9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-pyrrolo[1,2-a][1]benzazepin-6-yl)amino]-1-methyl-2-oxoethyl)butanamide; and may also be named: butanamide, 4,4,4-trifluoro-N-[(1S)-1-methyl-2-oxo-2-[(2,3,5,6-tetrahydro-9-methoxy-3,3-dimethyl-5-oxo-1H-pyrrolo[1,2-a][1]benzazepin-6-yl)amino]ethyl]-; and other names may be used to unambiguously identify Compound 1. The diastereomers are named 4,4,4-trifluoro-N-(((S)-1-(((S)-9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide and 4,4,4-trifluoro-N-(((S)-1-(((R)-9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-

yl)amino)-1-oxopropan-2-yl)butanamide. Other names may be used to unambiguously identify each of the diastereomers.

Compound 2 is named: N-((2S)-1-((8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide and may also be named N-((1S)-2-[(8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino]-1-methyl-2-oxoethyl)-4,4,4-trifluorobutanamide; and may also be named: butanamide, 4,4,4-trifluoro-N-[(1S)-1-methyl-2-oxo-2-[(6,8,9,10-tetrahydro-8,8-dimethyl-6-oxo-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino]ethyl]-; and other names may be used to unambiguously identify

Compound 2. The diastereomers are named N-((S)-1-(((S)-8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide and N-((S)-1-(((R)-8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide. Other names may be used to unambiguously identify each of the diastereomers.

It will be understood Compound 1 and Compound 2 are depicted with one of two chiral centers fixed. Herein, the Cahn-Ingold-Prelog designations of (R)- and (S)- are used to refer to specific isomers. Specific stereoisomers can be prepared by stereospecific synthesis using enantiomerically pure or enriched starting materials. The specific stereoisomers of either starting materials, intermediates, or racemic mixtures including Compound 1 or Compound 2 can be resolved by techniques well known in the art, such as those found in Stereochemistry of Organic Compounds, E. I. Eliel and S. H. Wilen (Wiley 1994) and Enantiomers, Racemates, and Resolutions, J., Jacques, A. Collet, and S. H. Wilen (Wiley 1991), including chromatography on chiral stationary phases, enzymatic resolutions, or fractional crystallization or chromatography of diastereomers formed for that purpose, such as diastereomeric salts. Where a chiral compound is isolated or resolved into its isomers, but absolute configurations or optical rotations are not determined, the isomers are arbitrarily designated as Isomer 1 and Isomer 2 corresponding to the order each elutes from chiral chromatography and if chiral chromatography is initiated early in the synthesis, the same designation is applied to subsequent intermediates and examples. While all mixtures containing the compounds of

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the present invention are contemplated within the present invention, the preferred embodiment is Compound 1, Isomer 2, or Compound 2, Isomer 2.

The compounds employed as initial starting materials in the synthesis of the compounds of the present invention are well known and, to the extent not commercially available, are readily synthesized using specific references provided, by standard
5 procedures commonly employed by those of ordinary skill in the art or are found in general reference texts.

Examples of known procedures and methods include those described in general reference texts such as Comprehensive Organic Transformations, VCH Publishers Inc,
10 1989; Compendium of Organic Synthetic Methods, Volumes 1-10, 1974-2002, Wiley Interscience; Advanced Organic Chemistry, Reactions Mechanisms, and Structure, 5th Edition, Michael B. Smith and Jerry March, Wiley Interscience, 2001; Advanced Organic Chemistry, 4th Edition, Part B, Reactions and Synthesis, Francis A. Carey and Richard J. Sundberg, Kluwer Academic / Plenum Publishers, 2000, etc., and references cited therein.

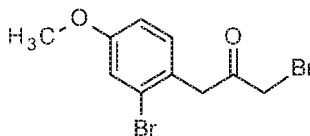
15 As used herein, the following terms have the meanings indicated: "mAtoh1" refers to murine atonal homolog 1 protein; "hAtoh 1" refers to human atonal homolog 1 protein; "Atoh1" refers to the human gene atonal homolog 1; "Basic Medium" refers to 500ml DMEM/F12 medium + 5ml N2 100X stock and 10ml B27 50X stock plus 500µl Ampicillin stock (1000X, 50mg/ml) and 1667µl Fungizone (300X); "BFGF" refers to
20 basic fibroblast growth factor; "DMEM" refers to Dulbecco's Modified Eagle's Medium; "DMSO" refers to dimethylsulfoxide; "EDTA" refers to ethylenediaminetetraacetic acid; "EGF" refers to epidermal growth factor; "EGTA" refers to ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra acetic acid; ES/MS refers to electrospray mass spectroscopy; "FBS" refers to fetal bovine serum; "GFP" refers to green fluorescent
25 protein; "h" refers to hour or hours; "HBSS" refers to Hank's Balanced Salt Solution; "HEK" refers to human embryonic kidney; "IC₅₀" refers to the concentration of an agent that produces 50% of the maximal inhibitory response possible for that agent; "IgG" refers to immunoglobulin G; "Math1" refers to the human gene atonal homolog 1; "Medium A" refers to 200ml Basic medium + EGF bFGF + IGF-1 + Heparan Sulfate at
30 20, 10, 50, and 50ng/ml respectively; "MEM" refers to minimum essential medium; "min" refers to minutes; "MS" refers to mass spectroscopy; "N1ICD" refers to Notch1

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Intracellular Domain; “nGFP” refers to nuclear green fluorescent protein; “OC” refers to organ of corti; “PBS” refers to phosphate buffered saline; “PBST” refers to Phosphate Buffered Saline + Tween®; “qPCR” refers to quantitative polymerase chain reaction; “qRT-PCR” refers to quantitative reverse transcription polymerase chain reaction; “rpm” refers to revolutions per minute; “RLT buffer” refers to RNeasyLysis buffer; “RT” refers to room temperature; “Tbp” refers to TATA-binding protein;

Preparation 1

10 1-Bromo-3-(2-bromo-4-methoxy-phenyl)propan-2-one



Add trimethylsilyldiazomethane (118.4 mL, 236.80 mmol, 2M in hexanes) dropwise to a stirred 0°C solution of 2-(2-bromo-4-methoxy-phenyl)acetyl chloride (52 g, 197.3 mmol) in tetrahydrofuran (197 mL) and acetonitrile (197 mL) under nitrogen.

15 After 15 minutes allow to warm to RT and stir for 2h under nitrogen. Concentrate to obtain a thick red oil (61 g). Add hydrogen bromide (36 mL, 197 mmol, 33% in acetic acid) dropwise to a stirred 5°C solution of 2-(2-bromo-4-methoxy-phenyl)acetyl azide from previous step in acetic acid (265 mL). After addition, allow to warm to RT under nitrogen. After 45 minutes, quench with ice/water, (500 mL) resulting in a brown

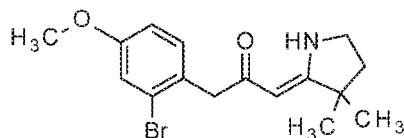
20 precipitate. Filter solids and wash with water (100 mL) and hexanes (100 mL) and dry under vacuum at 45°C for 2h to yield the title compound as an orange oil (63.0 g, 99%). ¹H NMR (300 MHz, CDCl₃): 7.17-7.12 (m, 2H), 6.85 (dd, J= 2.5, 8.5 Hz, 1H), 4.03 (s, 2H), 3.97 (s, 2H), 3.79 (s, 3H).

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Preparation 2

(3Z)-1-(2-Bromo-4-methoxy-phenyl)-3-(3,3-dimethylpyrrolidin-2-ylidene)propan-2-one

- 15 -

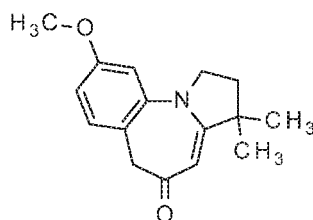


Add 3,3-dimethylpyrrolidine-2-thione (26.5 g, 205 mmol) to a suspension of the intermediate provided by Preparation 1, 1-bromo-3-(2-bromo-4-methoxy-phenyl)propan-2-one (60.00 g, 186 mmol), and potassium iodide (31 g, 86 mmol) in tetrahydrofuran (600 mL) at RT in one portion and stir for 1h. Add methyl tert-butyl ether (200 mL), filter out solids and rinse filter cake with methyl-tert-butyl ether (100 mL) to afford 1-(2-bromo-4-methoxy-phenyl)-3-[(4,4-dimethyl-2,3-dihydropyrrol-1-ium-5-yl)sulfanyl]propan-2-one iodide as a light yellow solid (100 g). To a suspension of the solid (100 g, 201 mmol) in acetonitrile (1 L), add triethylamine (56 mL, 401 mmoles) and triphenylphosphine (58 g, 221 mmol) and stir at 65°C for 2.5 h. Add methyl tert-butyl ether (200 ml) and filter out solids. Evaporate filtrate, triturate residue with methyl tert-butyl ether (20 mL) and filter out solids (twice). Evaporate combined filtrates to obtain 50 g crude material. Purify the residue via flash column chromatography on silica eluting with a 20-50% gradient of ethyl acetate in hexanes to obtain the title compound as an off-white solid (41 g, 70%).

MS (m/z): 338.0/340.0 (M⁺/M+2).

Preparation 3

9-Methoxy-3,3-dimethyl-2,3-dihydro-1H-pyrrolo[1,2-A][1]benzazepin-5(6H)-one



20

Degas/flush with nitrogen (x3) a mixture of the intermediate provided by Preparation 2, (3Z)-1-(2-bromo-4-methoxy-phenyl)-3-(3,3-dimethylpyrrolidin-2-ylidene)propan-2-one (40.0 g, 118 mmol), palladium acetate (2.7 g, 12 mmol), cesium carbonate (77 g, 237 mmol), 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene (13.7 g, 24 mmol), and N-methyl-2-pyrrolidone (1.2 L). Heat the suspension at 150°C for 4h.

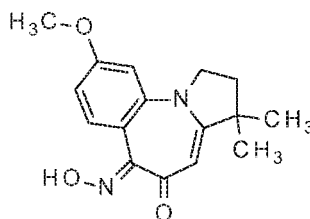
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- 16 -

Cool to RT, filter off solids, wash with ethyl acetate and discard solids. Add ethyl acetate (1 L) and water (500 mL) to the filtrate, filter off solids through diatomaceous earth and discard. Separate filtrate layers, extract from aqueous with ethyl acetate (2 x 20 mL), followed by dichloromethane (3 x 100 mL). Wash combined organic layers with 5% aqueous sodium bicarbonate solution twice, dry over sodium sulfate, filter and concentrate to obtain 100 mL dark brown oil. Filter material over a silica pad eluting with dichloromethane followed by 1% methanol/dichloromethane and concentrate filtrate. Partition residue between ethyl acetate and 5% aqueous sodium bicarbonate solution, backextract from aqueous with ethyl acetate, dry over sodium sulfate, filter and concentrate to obtain crude product as a dark brown foam. Dissolve resulting foam in ethyl acetate (250 mL), add SiliaBond Thiol® (80 g) and stir at RT overnight. Filter out solids, wash filter cake with ethyl acetate and dichloromethane, concentrate filtrate to obtain the title compound as a light brown solid (19.6 g, 65%). MS (m/z): 258.0 (M+H).

Preparation 4

6-Hydroxyimino-9-methoxy-3,3-dimethyl-2,3-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-5(6H)-one



Add potassium tert-butoxide (11 g, 98 mmol) in several portions to a stirred 0°C solution of the intermediate provided by Preparation 3, 9-methoxy-3,3-dimethyl-2,3-dihydro-1H-pyrrolo[1,2-A][1]benzazepin-5(6H)-one (16.8 g, 65 mmol), in tetrahydrofuran (336 mL) and stir for 15 minutes. Add amyl nitrite (12.2 mL, 91 mmol) dropwise and stir mixture for 30 minutes at 0°C. Pour reaction mixture onto ice/water (200 mL) and extract the mixture with ethyl acetate (2 x 200 mL). Filter out crystallized solids, extract from filtrate with dichloromethane (2 x 100 mL). Wash combined organics with brine (100 mL), dry over sodium sulfate, filter and concentrate to afford a light

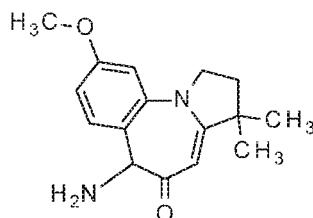
- 17 -

brown solid. Triturate solid with 1:1 methyl tert-butyl ether/hexane, combine with previously collected solid to obtain the title compound as a light yellow solid (16.5 g, 88%). MS (m/z): 287.1 (M+H).

5

Preparation 5

6-Amino-9-methoxy-3,3-dimethyl-2,3-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-5(6H)-one

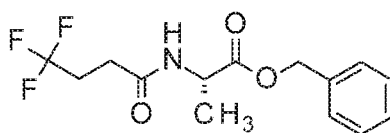


10 Add trifluoroacetic acid (17 mL, 231 mmol) dropwise over 10 minutes to a stirred 5°C-10°C suspension of the intermediate provided by Preparation 4, 6-hydroxyimino-9-methoxy-3,3-dimethyl-2,3-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-5(6H)-one (16.5 g, 58 mmol), and zinc dust (11.3 g, 173 mmol) in dichloromethane (248 mL) then allow to warm to RT. Filter mixture through a pad of diatomaceous earth, pour filtrate over a
15 mixture of ice and saturated aqueous sodium carbonate (1:1, 500 mL). Filter the resulting suspension through diatomaceous earth and rinse filter pad with dichloromethane. Extract from aqueous layer with dichloromethane (100 mL), dry combined organic layers over sodium sulfate, filter and concentrate to obtain the title compound as a light brown foam (14.7 g, 94%). MS (m/z): 273.1 (M+H).

20

Preparation 6

Benzyl (2S)-2-(4,4,4-trifluorobutanoylamino)propanoate



Add L-alanine benzyl ester hydrochloride (7 g, 32.5 mmol),
25 diisopropylethylamine (28 mL, 162 mmol), hydroxybenzotriazole hydrate (7.5 g, 49

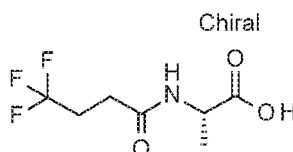
- 18 -

mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (9.3 g, 49 mmol) to a stirred solution of 4,4,4-trifluorobutyric acid (7.1 g, 49 mmol) in dichloromethane (162 mL) and stir at RT under N₂ for 20h. Quench with 20% aqueous citric acid solution (150 mL), stir mixture for 5 minutes, separate layers and extract from aqueous with dichloromethane (100 mL). Wash combined organic layers with saturated aqueous sodium bicarbonate solution (150 mL), dry over magnesium sulfate, filter and concentrate to obtain 10.6 g pale yellow solid. Purify the residue via flash column chromatography on silica eluting with a 25-50% gradient of ethyl acetate in hexanes to obtain the title compound as a white solid (9.2 g, 94%). MS (m/z): 304.2 (M+H).

10

Preparation 7

(2S)-2-(4,4,4-trifluorobutanoylamino)propanoic acid



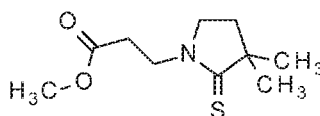
Add palladium (1.8 g, 0.8 mmol, 5% on C) to a stirred solution of the intermediate provided by Preparation 6, benzyl (2S)-2-(4,4,4-trifluorobutanoylamino)propanoate (8.8 g, 29 mmol), in methanol (88 mL) at RT. Degas the mixture and stir under hydrogen (balloon atmosphere) for 5 h. Filter the mixture over diatomaceous earth, wash pad with methanol and concentrate filtrate to obtain a white solid. Triturate solids with dichloromethane and dry under vacuum overnight at RT to yield the title compound as a white solid (6.11 g, 99% yield). MS (m/z): 214.1 (M+H).

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Preparation 8

Methyl 3-(3,3-dimethyl-2-thioxopyrrolidin-1-yl)propanoate

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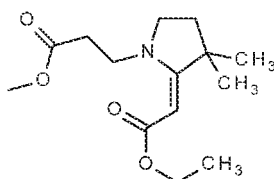
- 19 -

Dissolve 3,3-dimethylpyrrolidine-2-thione (15.7 g, 122 mmol) and methyl acrylate (12 mL, 134 mmol) in tetrahydrofuran (100 mL) and stir at RT under nitrogen. Add sodium hydroxide (0.8 g, 20 mmol) and stir overnight at RT under nitrogen. Dilute with brine, extract with ethyl acetate, separate layers, wash the organic layer with brine, dry over sodium sulfate, filter and concentrate to yield 27.5 g crude solids. Purify the residue via flash column chromatography on silica eluting with a 20-50% gradient of ethyl acetate in hexanes to obtain the title compound as a white solid (25.9 g, 99%). MS (m/z): 216.2 (M+H).

10

Preparation 9

Methyl 3-[(2Z)-2-(2-ethoxy-2-oxo-ethylidene)-3,3-dimethyl-pyrrolidin-1-yl]propanoate



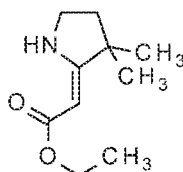
Add tetrakis(acetato)dirhodium(II) (3.74 g, 8.5 mmol) to a stirred solution of the intermediate provided by Preparation 8, methyl 3-(3,3-dimethyl-2-thioxopyrrolidin-1-yl)propanoate (41.4 g, 192 mmol), in toluene (156 mL) and heat to 110°C under nitrogen. Add ethyldiazoacetate (89 mL, 844 mmol) dropwise over approximately 18 h then heat overnight at 110°C. Concentrate and purify the residue via flash column chromatography on silica eluting with 30% ethyl acetate in hexanes to obtain the title compound as a yellow oil (34.5 g, 67%). MS (m/z): 270.2 (M+H).

20

Preparation 10

Ethyl (2Z)-2-(3,3-dimethylpyrrolidin-2-ylidene)acetate

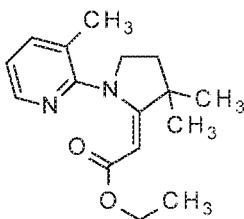
- 20 -



Add potassium hexamethyldisilazide (301 mL, 0.5 M in toluene, 151 mmol) dropwise over 45 minutes to a stirred solution of the intermediate provided by Preparation 9, methyl 3-[(2Z)-2-(2-ethoxy-2-oxo-ethylidene)-3,3-dimethyl-pyrrolidin-1-yl]propanoate (33.8 g, 126 mmol), in tetrahydrofuran (430 mL) under nitrogen using a water/ice bath to maintain temperature <30°C. Stir for 40 minutes after completion of addition. Quench with saturated aqueous sodium bicarbonate solution (250 mL) then concentrate. Partition between diethyl ether and brine solution, extract from aqueous with ethyl acetate (x 4), dry over sodium sulfate, filter and concentrate to obtain 37 g material. Purify the residue via flash column chromatography on silica eluting with 10% ethyl acetate in hexanes to obtain the title compound (9.1 g, 40%). MS (m/z): 184.2 (M+H).

Preparation 11

Ethyl (2Z)-2-[3,3-dimethyl-1-(3-methyl-2-pyridyl)pyrrolidin-2-ylidene]acetate



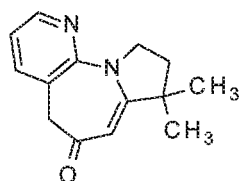
Combine 2-bromo-3-methylpyridine (1.5 g, 8.7 mmol), the intermediate provided by Preparation 10, ethyl (2Z)-2-(3,3-dimethylpyrrolidin-2-ylidene)acetate (1.6 g, 8.7 mmol), and sym-dimethylethylene diamine (0.77 mL, 8.7 mmol) in 1,4-dioxane (15 mL) in a reaction vessel. Bubble nitrogen through the solution with stirring for 10 minutes. Add potassium carbonate (3.6 g, 26 mmol) and copper (I) iodide (0.83 g, 4.4 mmol) all at once, seal and heat stirred mixture at 120°C for 2 days. Dilute with dichloromethane, filter through diatomaceous earth and concentrate filtrate. Purify the residue via flash column chromatography on silica eluting with a gradient of 5-100% ethyl acetate in

- 21 -

hexanes to obtain the title compound as a pale yellow oil (1.58 g, 66%). MS (m/z): 275.0 (M+H).

Preparation 12

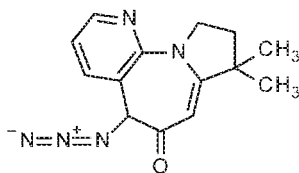
5 8,8-Dimethyl-9,10-dihydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-6(8H)-one



Add sodium bis(trimethylsilyl)amide (22 mL, 1M in tetrahydrofuran, 22 mmol) via syringe to a stirred solution of the intermediate provided by Preparation 11, ethyl
10 (2Z)-2-[3,3-dimethyl-1-(3-methyl-2-pyridyl)pyrrolidin-2-ylidene]acetate (2.88 g, 10.5 mmol), and tetrahydrofuran (60 mL) in a reaction vessel. Seal vessel and heat at 70°C with stirring for 3 days. Cool to RT, quench with brine, extract with ethyl acetate, dry over sodium sulfate, filter and concentrate to obtain a brown oil. Purify the residue via
15 in hexanes to obtain the title compound as a yellow solid (1.70 g, 71%). MS (m/z): 229.2 (M+H).

Preparation 13

20 6-Azido-10-aza-3,3-dimethyl-2,6-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-5-one



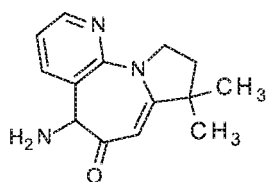
Add lithium diisopropylamide (7.0 mL, 10.5 mmol, 1.5 M in hexanes) over 10 minutes to a stirred -78°C solution of the intermediate provided by Preparation 12, 8,8-dimethyl-9,10-dihydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-6(8H)-one (1.85 g, 8.1

- 22 -

mmol), in tetrahydrofuran (50 mL) under nitrogen. After 30 minutes add acetic acid (2.3 mL, 41 mmol) via syringe then allow to warm to RT under nitrogen. Quench with saturated aqueous sodium bicarbonate solution. Partition mixture between brine and ethyl acetate, separate layers, wash ethyl acetate layer with brine, dry over sodium sulfate, filter and concentrate to obtain a brown solid. Purify the residue via flash column chromatography on silica eluting with a gradient of 5-60% ethyl acetate in hexanes to obtain the title compound (1.2 g, 55%). MS (m/z): 270.2 (M+H).

Preparation 14

10 5-Amino-3,3-dimethyl-9,10-dihydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-6(8H)-one



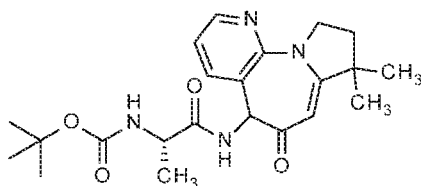
Add zinc dust (1.2 g, 18 mmol) followed by ammonium chloride (5 g, 66 mmol) to a stirred solution of the intermediate provided by Preparation 13, 6-azido-10-aza-3,3-dimethyl-2,6-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-5-one (1.2 g, 4.5 mmol), in ethanol (50 mL) and water (15 mL) at RT. After 1 h dilute with ethyl acetate, filter out solids and concentrate filtrate. Suspend residue between ethyl acetate and brine, separate layers, dry over sodium sulfate, filter and concentrate. Purify the residue via flash column chromatography on silica eluting with a gradient of a 5-40% [10% 2M ammonia in methanol/dichloromethane] in dichloromethane to obtain the title compound as a yellow solid (0.72 g, 67%). MS (m/z): 244.2 (M+H).

Preparation 15

tert-Butyl N-[(1S)-2-[(10-aza-3,3-dimethyl-5-oxo-2,6-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-6-yl)amino]-1-methyl-2-oxo-ethyl] carbamate

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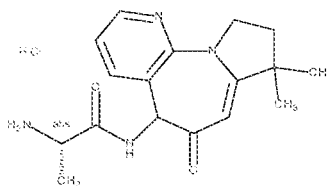
- 23 -



- Add (2S)-2-(tert-butoxycarbonylamino)propanoic acid (0.69 g, 3.6 mmol), 1-hydroxybenzotriazole hydrate (0.55 g, 3.6 mmol) and diisopropylethylamine (0.67 mL, 3.9 mmol) to a stirred 0°C solution of the intermediate provided by Preparation 14, 5-amino-3,3-dimethyl-9,10-dihydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-6(8H)-one (0.72 g, 3.0 mmol), in tetrahydrofuran (10 mL) under nitrogen. Add 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.68 g, 3.6 mmol) and allow mixture to warm to RT under nitrogen. After 2 h, dilute with water and extract with ethyl acetate. Wash organic layer with brine, dry over sodium sulfate, filter and concentrate.
- Purify the residue via flash column chromatography on silica eluting with a gradient of 50-100% ethyl acetate in hexanes to obtain the title compound as a yellow solid (1.04 g, 84%). MS (m/z): 415.0 (M+H).

Preparation 16

- (2S)-2-Amino-N-(10-aza-3,3-dimethyl-5-oxo-2,6-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-6-yl)propanamide hydrochloride

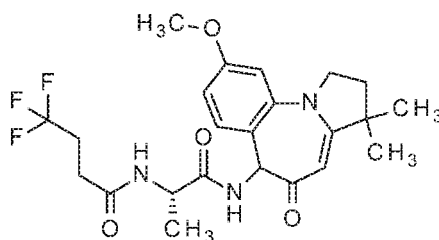


- Add hydrogen chloride (12.5 mL, 50 mmol, 4M in dioxane) to a stirred solution of the intermediate provided by Preparation 15 tert-butyl N-[(1S)-2-[(10-aza-3,3-dimethyl-5-oxo-2,6-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-6-yl)amino]-1-methyl-2-oxo-ethyl] carbamate (1.04 g, 2.5 mmol), in 1,4-dioxane (40 mL) and warm to 45°C with stirring. When LC/MS indicates complete reaction, concentrate to obtain the title compound as a yellow solid (0.93 g, crude). MS (m/z): 315.2 (M+1).

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Example 1

4,4,4-trifluoro-N-((2S)-1-((9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-
 5 benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide



Part 1

Diastereomeric 4,4,4-trifluoro-N-((2S)-1-((9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-
 10 tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide

Add 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (12.4 g, 65 mmol) to a stirred solution of the intermediate provided by Preparation 5, 6-Amino-9-methoxy-3,3-dimethyl-2,3-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-5(6H)-one, and the intermediate provided by Preparation 7, (2S)-2-(4,4,4-trifluorobutanoylamino)propanoic acid (10.9 g, 51 mmol), in dichloromethane (294 mL) at RT. Cool mixture to 5°C, add 1-hydroxybenzotriazole (8.75 g, 65 mmol) and continue stirring at RT for 10 minutes.

Wash mixture with water (3 x 100 mL), dry over sodium sulfate, filter and concentrate to afford a dark solid. Triturate with methyl tert-butyl ether to afford the title compound (mixture of diastereomers) as an off-white solid (22.0 g, 87%). MS (m/z): 468.1 (M+H).

20 Part 2

4,4,4-trifluoro-N-((2S)-1-((9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-
 benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide Isomers 1 and 2

Separate a mixture of diastereomers from Example 1, Part 1 on a Chiralpak AD column eluting with 10% acetonitrile/ethanol (0.2% dimethylethylamine)
 25 to obtain Isomer 1 compound (R_t =3.20 mins.) as a white solid (9.8 g, 38%) and Isomer 2

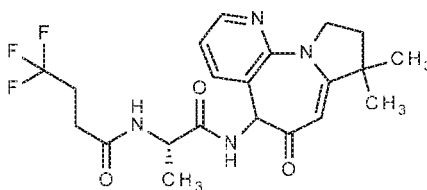
- 25 -

compound (Rt=7.37 mins.) as a white solid (9.0 g, 36%). MS (m/z): 468.2 (M+H) for both isomers.

5

Example 2

N-((2S)-1-((8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide



10

Part 1

Diastereomeric N-((1S)-2-[(8,8-Dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino]-1-methyl-2-oxoethyl)-4,4,4-trifluorobutanamide

Add 4,4,4-trifluorobutanoic acid (0.45 g, 3.2 mmol), 1-hydroxybenzotriazole hydrate (0.49 g, 3.2 mmol) and diisopropylethylamine (2.3 mL, 13.2 mmol) to a stirred 0°C solution of the intermediate provided by Preparation 16, (2S)-2-amino-N-(10-aza-3,3-dimethyl-5-oxo-2,6-dihydro-1H-pyrrolo[1,2-a][1]benzazepin-6-yl)propanamide hydrochloride (0.93 g, 2.6 mmol), in tetrahydrofuran (50 mL) under nitrogen. Add 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.61 g, 3.2 mmol) and allow mixture to warm to RT under nitrogen for 16 h. Dilute with water and extract with ethyl acetate. Wash organic layer with brine, dry over sodium sulfate, filter and concentrate. Purify the residue via flash column chromatography on silica eluting with a gradient of 75-100% ethyl acetate in hexanes to obtain title compound (mixture of diastereomers) as an off-white solid (0.82 g, 71%). MS (m/z): 439.2 (M+1).

25 **Part 2**

N-((2S)-1-((8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide Isomers 1 and 2

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Separate a mixture of diastereomers from Example 2, Part 1 on a Chiralpak AD-H column eluting with 15% MeOH/CO_{2(gas)} to obtain Isomer 1 (R_t=1.60 mins.) as a white solid (194 mg, 24%, epimerizes to 32% DE (diastereomeric excess)) and Isomer 2 (R_t=2.31 mins.) as a white solid (469 mg, 57%). MS (m/z): 439.0 (M+H) for both
5 isomers.

Cancer is increasingly recognized as a heterogeneous collection of diseases whose initiation and progression are induced by the aberrant function of one or more genes that
10 regulate DNA repair, genome stability, cell proliferation, cell death, adhesion, angiogenesis, invasion, and metastasis in cell and tissue microenvironments. Variant or aberrant function of the “cancer” genes may result from naturally occurring DNA polymorphism, changes in genome copy number (through amplification, deletion, chromosome loss, or duplication), changes in gene and chromosome structure (through
15 chromosomal translocation, inversion, or other rearrangement that leads to deregulated gene expression), and point mutations. Cancerous neoplasms may be induced by one aberrant gene function, and maintained by the same aberrant gene function, or maintenance and progression exacerbated by additional aberrant gene functions.

Beyond the genetic chromosomal aberrations mentioned above, each of the
20 cancers may also include epigenetic modifications of the genome including DNA methylation, genomic imprinting, and histone modification by acetylation, methylation, or phosphorylation. An epigenetic modification may play a role in the induction and/or maintenance of the malignancy.

Extensive catalogues of the cytogenetic aberrations in human cancer have been
25 compiled and are maintained and regularly updated online (see The Mitelman Database of Chromosome Aberrations in Cancer at the US National Cancer Institute (NCI) Cancer Genome Anatomy Project (CGAP). The database includes chromosomal aberrations for at least some of the malignancies of the present invention. The Wellcome Trust Sanger Institute Cancer Genome Project maintains a detailed online “Cancer Gene Census” of all
30 human genes that have been causally linked to tumorigenesis as well as the COSMIC (Catalogue of Somatic Mutations in Cancer) database of somatic mutations in human

cancer. A further source containing abundant information on cytogenetic changes causally linked to various cancers is the Atlas of Genetics and Cytogenetics in Oncology and Haematology.

Diagnosis of cancerous malignancies by biopsy, immunophenotyping and other tests are known and routinely used. In addition to high resolution chromosome banding and advanced chromosomal imaging technologies, chromosome aberrations in suspected cases of cancer can be determined through cytogenetic analysis such as fluorescence in situ hybridization (FISH), karyotyping, spectral karyotyping (SKY), multiplex FISH (M-FISH), comparative genomic hybridization (CGH), single nucleotide polymorphism arrays (SNP Chips) and other diagnostic and analysis tests known and used by those skilled in the art.

The oncogenic role of Notch was first reported in human T-cell leukemia involving a translocation of the Notch1 intracellular domain to the T-cell receptor- β promoter region, resulting in the over expression of Notch1 intracellular domain (Grabher et al. Nature Review Cancer, 2006(6):347-359; Weng et al. Science, 2004(306):269-271). Over expression of Notch1 intracellular domain in hematopoietic progenitor cells of mice caused the mice to exhibit T-cell acute lymphoblastic leukemia similar to humans. In addition to T-cell acute lymphoblastic leukemia, there is increasing evidence that Notch signals are oncogenic in other cancers through multiple mechanisms including receptor amplification and over expression of ligands and/or receptors including acute lymphoblastic leukemia, chronic lymphoblastic leukemia (Rosati et al, Blood, 2009(113): 856-865), acute myelogenous leukemia (Sliwa et al. Int J Clin Exp Pathol, 2014(7(3)): 882-889), chronic myelogenous leukemia (Nakahara et al. Blood, 2010(115(14)): 2872-2881), and erythroleukemia (Robert-Moreno et al, Leukemia, 2007(21): 1496-1503). Aberrant constitutive Notch signaling due to mutation or over expression of ligands and/or receptors is also implicated in a number of solid tumor malignancies including triple negative breast cancer (Stoeck et al, Cancer Discovery, 2014(4): 1154-1167), breast cancer, ovarian cancer (Park et al. Cancer Research, 2006(66):6312-6318), melanoma (Gast et al. Genes, Chromosomes & Cancer, 2010(49):733-745), lung cancer, non small cell lung cancer (Westhoff et al. PNAS, 2009(106):22293-22298), pancreatic cancer, glioblastoma, colorectal cancer, head and neck cancer, cervical cancer, prostate cancer,

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liver cancer, squamous cell carcinoma (oral), skin cancer and medulloblastoma (Rangathan et al., Nature Review Cancer, 2011(11):338-351 and Supplementary information S1 (table)). Aberrant constitutive Notch signaling due to mutation or over expression of ligands and/or receptors is also implicated in angiosarcoma (Ravi et al, J Clin Oncol, 2007, (25(18S, June 20 Supplement)): Abstract 10030), rhabdomyosarcoma (Belyea et al, Clin Cancer Res, 2011(17(23)): 7324-7336; Roma et al, Clin Cancer Res, 2011(17(3)): 505-513), liposarcoma (J Clin Oncol, 2009, (27(15S, Supplement)): Abstract 10526), malignant fibrous histiocytoma (Wang et al, Cancer Res, 2012, (72): 1013-1022), hepatocellular carcinoma (Villanueva et al, Gastroenterology, 2012, (143): 1660-1669), intrahepatic and extrahepatic cholangiocarcinoma (Wu et al, Int J Exp Pathol, 2014, (7(6)): 3272-3279; Sekiya et al, J Clin Invest, 2012, (122(11)): 3914-3918; Yoon et al, World J Gastroenterol, 2011, (17(35)): 4023-4030), and adenoid cystic carcinoma (Bell et al, Annals of Diagnostic Pathology, 2014, (18): 10-13; Stoeck et al, Cancer Discov, 2014, (4): 1154-1167). Inhibition of Notch signaling presents an attractive target to provide therapeutic benefits to cancer patients whose disease was induced by aberrant activation of constitutive Notch signaling pathway. Shih et al. Cancer Research, 2007(67)1879-1882.

One of the definitive genes for inner ear hair cell development is the mammalian homolog of the basic helix-loop-helix transcription factor atonal-1 (Atoh1). Expression of Atoh1 in cochlear cells is required for auditory hair cell genesis. Prosensory epithelial cells within the developing organ of Corti that express Atoh1 will differentiate into auditory hair cells (Helms et al, Development 2000, (127(6)): 1185-1196), and Atoh1 is one of the earliest markers of auditory hair cell differentiation. Supporting cells of the organ of Corti maintain the potential to develop hair cell characteristics including cilia formation (Zheng et al, Nature Neuroscience, 2000,(3(6)): 580-586 ; Kawamoto et al, J Neurosci, 2003, (23(11)): 4395-4400; Izumikawa et al, Nat Med, 2005, (11(3)):271-276; and proper hair cell function (Kawamoto et al, 2003).

Each hair cell in the cochlea is surrounded by non-sensory supporting cells that provide trophic and structural support for the hair cells and ganglion and are essential in maintaining proper ionic concentrations in the organ of Corti through gap junction intercellular communication. There are two types of hair cells: inner and outer hair cells.

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The cochlear hair cells in mammals including humans, consist of one row of inner hair cells and three rows of outer hair cells. The inner hair cells are the actual sensory receptors, and 95% of the fibers of the auditory nerve that project to the brain arise from this subpopulation. The terminations on the outer hair cells are almost all from efferent axons that arise from cells in the brain and these cells function as acoustical pre-amplifiers. Supporting cells play a key role in auditory hair cell generation after auditory hair cell loss or damage. During development, hair and supporting cells develop from a common progenitor and the appearance of a hair cell signals surrounding cells to develop into supporting cells through contact inhibition mediated by the Notch signaling pathway (Kelley, Nat Rev Neurosci, 2006, (11): 837-849.

Based on the ability of supporting cells to transdifferentiate into auditory hair cells, along with their shared developmental pathway, it has been postulated that supporting cells may function as hair cell progenitors (Parker et al, Audiology and Neurootology, 2004, (9(2)): 72-80). Several studies have demonstrated that bypassing cell cycle inhibition in supporting cells can result in auditory hair cell generation in mammals (Lowenheim et al, Proc Natl Acad Sci USA, 1999, (96): 4084-4088; Torchinsky et al, J Neurocytol, 1999, (28(10-11)): 913-924; Minoda et al, Hear Res, 2007, (232): 44-51). Therefore adult mammalian supporting cells maintain the ability to transdifferentiate into auditory hair cells once they are free to enter the cell cycle.

Drug therapy for restoration of auditory hair cells is a new approach and intratympanic administration to the middle ear fluid, preferably to the round window niche, without actual injection into the cochlea may effectively induce Atoh1 expression at a therapeutic dose and for a suitable length of time to cause auditory hair cell transdifferentiation of supporting cells in the cochlea. Mizutari et al, Neuron, 2013, (77(1)): 58-69 showed that middle ear delivery of a gamma secretase inhibitor (LY411575) may be employed to regenerate auditory hair cells lost to acoustic trauma in mice and that this generation of new hair cells from supporting cells resulted in significant, measurable, hearing recovery. This work provided conceptual evidence of a therapeutic effect of Notch pathway signaling inhibition on auditory hair cell generation and restoration of hearing in a mouse model, and provided a mechanistic (supporting cell differentiation to auditory hair cells) explanation for the physiological effect. Dose limiting toxicity was shown during systemic administration of LY411575.

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The following *in vitro* and *in vivo* studies demonstrate the Notch pathway signaling inhibitory activity and efficacy of Compounds 1 and 2, or a substantially pure diastereomer thereof, against a specific cancer cell line. These assays are generally recognized by those skilled in the art as indicative of human clinical chemotherapeutic activity. Inhibition of Notch intracellular domain cleavage by γ -secretase is believed to be effective against each of Notch 1, Notch 2, Notch 3 and Notch 4 receptors. Assays evidencing Notch pathway signaling inhibitory activity and efficacy may be carried out substantially as follows or by similar assays affording similar data.

10 Notch1 N1ICD Nuclear Accumulation Cellular Imaging Assay

HEK293ΔE12 cells (HEK293 cells are engineered to stably express mouse Notch1 cDNA coding for amino acid 1703-2183, NP_032740.3, (Full length mouse Notch 1 protein precursor: SEQ ID NO:1) with 23 amino acid signal peptide sequence, MPRLLTPLLCLTLLPALAARGLR (SEQ ID NO:2), at its N-terminus) are plated at 5000 cells/well in 96 well plates, incubated in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose with 5% fetal bovine serum (FBS) at 37 °C, 5 % CO₂ for 24 hours. Cells are treated with test compound, dosing at 10 points of 1:3 dilutions across the range of 1000 nM to 0.05 nM, and with final dimethyl sulfoxide (DMSO) concentration at 0.2%. After 24 hours treatment, cell plates are processed through following steps sequentially: fix cells with 100μl/well PREFER™ fixative for 30 minutes at room temperature (RT); permeablize cells with 100μl /well 0.1% TRITON® X100 in phosphate buffered saline (PBS) for 20 min at RT; wash 3 times with 100μl /well PBS each; add 50μl/well rabbit anti-N1ICD (Notch1 Intracellular Domain) antibody, at 1:2000 in PBS with 1 % bovine serum albumin and incubate 1.5 hours at 37 °C; wash 3 times with 100μl/well PBS each; incubate with 50μl/well goat anti-rabbit IgG (Immunoglobulin G) Alexa 488 at 1:1000 dilution in PBS with 1 % bovine serum albumin and incubate 1 hours at 37 °C ; wash 3 times with 100μl/well PBS each and add 100μl/well 15 μM propidium iodide with 50μg/ml RNase for 30 minutes to stain nuclei. Plates are scanned with ACUMEN EXPLORER™ Laser-scanning fluorescence microplate cytometer (TTP LABTECH LTD) to measure total cell nuclear count/well and total nuclear area/well with fluorescence at 655nm—705nm (emission of DNA bound propidium iodide) and

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fluorescence of antibody binding to N1ICD in nuclear region at 505nm—530nm. The main assay output is a ratio of total fluorescence of nuclear N1ICD to total nuclear area, the normalized nuclear N1ICD signal. A relative cytotoxicity profiling was collected as %cell number to 0.2% DMSO control cells. The antibody that recognizes cleaved Notch1 or N1ICD is raised to a human peptide corresponding to the amino terminal cleavage site of human Notch1 at Val1744. In untreated control cells, N1ICD generated from Notch1 will translocate and accumulate in nucleus. When cells are treated by a Notch 1 cleavage inhibiting compound, the signal of nuclear N1ICD will decrease. Concentration response and the IC₅₀ are determined by curve fitting to a four parameter logistic for the nuclear N1ICD signal, while the % cell number is plotted in the same graph for cytotoxicity profiling. Performing the assay essentially as described above, the average IC₅₀ for Compound 1, Isomer 2 is 0.37 nM (+/- 0.16; n=2) and Compound 2, Isomer 2 is 1.55 nM (+/- 1.12; n=2). Neither compound affects cell number up to 1000 nM concentration. These data evidence Compound 1, Isomer 2 and Compound 2, Isomer 2 each has affinity for Notch 1 and inhibits the intracellular accumulation of the Notch 1 intracellular domain cell signaling peptide.

In-vivo target inhibition studies

Animal studies

To evaluate *in vivo* effect of Compound 1, Isomer 2 and Compound 2, Isomer 2 on inhibition of Notch processing pharmacodynamics (PD), animal studies were conducted in non-tumor bearing Balb/C mice (Charles River). A total of 5 mice are used for each group. Mice are fed *ad libitum* on normal chow. Treatment is initiated with oral administration (gavage) of compound or vehicle (1% Na-CMC in 0.25% Tween-80) in 0.2 mL volume. At designated time points (4 or 8 hours after the dose) following treatment, animals are sacrificed by CO₂ asphyxiation and cervical dislocation. Tissues (Lungs) are removed and used for PD response analysis as measured by cleaved N1ICD.

N1ICD Analysis

To evaluate N1ICD levels in lung, approximately 75 mg is cut from the frozen tissue and minced prior to homogenization (actual mass recorded). Frozen tumor samples

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are transferred to Lysing Matrix-D™ tubes and re-suspended in ice-cold XY lysis buffer (25 mM Tris pH 7.5, 10 µg/ml Trypsin/Chymotrypsin inhibitor, 10 µg/ml Aprotinin, 60 mM Beta-glycerol phosphate, 1% Triton® X-100, 10 mM NaF, 2.5 mM pyrophosphate, 150 mM NaCl, 15 mM ethylene diamine tetra acetic acid (EDTA) pH 8.0, 5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetra acetic acid (EGTA) pH 8.0, 1 mM Na Vanadate, 10 µg/ml Leupeptin, 1 mM dithiothreitol, 1 µM microcystin LR, 10 µg/ml N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 2 mM Nα-p-tosyl-L-arginine methyl ester hydrochloride (TAME), 15 mM 4-nitrophenyl phosphate di(tris) salt (PNPP), 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 5 mM benzamidine, 1 µM Okadaic Acid) containing 1X Complete tablet (Roche Complete™ No. 11697 498 001) and 1X Protease Inhibitor cocktail (Sigma-Aldrich P8340) at a mass: volume ratio of 75 mg/ml buffer. Tissues are homogenized in a Fast Prep FP120 homogenizer (Thermo Scientific, Rockford, IL) at a speed of 6.0 for 30 seconds at 4°C, followed by 15 minute incubation on ice. This is repeated for a total of 2-3 cycles until homogenization is complete. Lysates are spun in a 4°C eppendorf centrifuge at 30,000 rpm for 15 minutes to remove debris. 400 µl of supernatant is removed and transferred to a new eppendorf tube and subjected to a freeze/thaw cycle. Samples are re-spun in a 4°C eppendorf centrifuge at 30,000 rpm for 30 minutes and 120 µl of supernatant is collected for analysis. Total protein concentration is determined using Pierce BCA Protein Assay Kit™ (Thermo Scientific, Rockford, IL) using a Thermomax™ plate reader (Molecular Devices, Sunnyvale, CA). NIICD levels are determined using a custom NIICD ELISA. Analyte is captured with a cleaved Notch1(Val1744)-specific custom rabbit monoclonal antibody and detected with a C-terminal Notch1 SULFO-TAG™ (Meso Scale Discovery, Gaithersburg, Maryland) polyclonal sheep antibody (R&D Systems, Minneapolis, MN). Lysates are diluted to 2 µg/µl in ice-cold ELISA tris lysis buffer (R6OTX) (Meso Scale Discovery, Gaithersburg, Maryland) containing 1X Complete tablet (Roche Complete™ mini No. 11836 153 001) and 1X Protease Inhibitor cocktail (Sigma-Aldrich P8340), and 25µl is added to the ELISA plate. Incubation of 50 µg protein lysate is done at RT for one hour each to capture analyte and with detection antibody. Plates are read on a Sector Imager 6000™ (Meso Scale Discovery, Gaithersburg, Maryland). Background subtracted NIICD is normalized to total protein and presented as % inhibition relative to the vehicle-treated

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group. N1ICD % inhibition and statistical significance (p value) as measured by Dunett's method in tumors harvested 4 hours after last dose for Compound 1, Isomer 2 or Compound 2, Isomer 2 is analyzed essentially as described above and summarized in Table 1.

5

Table 1.

Compound	Dose (mg/kg)	Time (hours) following treatment	% N1ICD inhibition (Ave $\pm$ SD; n=1)	p Value
1, Isomer 2	10	4	70 $\pm$ 12	< 0.0001
1, Isomer 2	10	8	65 $\pm$ 11	< 0.0001
1, Isomer 2	100	8	92 $\pm$ 3	< 0.0001
1, Isomer 2	30	8	87 $\pm$ 3	< 0.0001
1, Isomer 2	10	8	52 $\pm$ 31	0.0054
1, Isomer 2	3	8	11 $\pm$ 13	Not Significant
1, Isomer 2	1	8	6 $\pm$ 15	Not Significant
1, Isomer 2	0.3	8	-7 $\pm$ 26	Not Significant
2, Isomer 2	10	4	65 $\pm$ 11	< 0.0001
2, Isomer 2	10	8	15 $\pm$ 14	Not Significant

The data in Table 1 evidences the inhibition of N1ICD cleavage by Compound 1, Isomer 2 and Compound 2, Isomer 2 in mouse lung tissue. The data in Table 1 further provides an *in vivo* correlation to the functional activity data described above.

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Induction of mAtoh1 Expression in Otic Spheres

Generally, organ of Corti cell isolation is carried out on Day 0, largely by the method in Oshima et al., Methods Mol Biol., 2009; 493: 141–162. Cell culture and propagation are carried out for 3-4 days. Cell plating and test compound treatment takes place on Day 3 or 4. Attachment and differentiation is allowed to take place over the next 7 days. On Days 10 or 11, cell lysis and RNA isolation is carried out. TaqMan qRT-PCR is carried out on Day 12 and the results analyzed on Days 13-15 for Atoh1 and Tbp1 expression.

Isolation of cells

Surrounding tissue from the petrous portion of the temporal bone are removed from 1 to 4 day-old postnatal mice (transgenic strain: mAtoh1-driven nGFP; Lumpkin EA, et al., Gene Expr Patterns, 2003; (36): 389-95 of both sexes. The conch shaped cochlea is separated from the vestibular system using forceps. At this stage of development, the bony labyrinth is not completely calcified and is easily dissected using forceps. Carefully open the bony labyrinth of the cochlea and remove the spiral ligament and attached organ of Corti coiled together along the spiral of the modiolus by unwinding apically from the modiolus. Starting at the base, separate the spiral ligament from the organ of Corti using fine forceps.

Transfer the dissected organ of Corti (OC) into individual 1.5 ml tubes filled with 850 µl ice cold HBSS. Transfer up to 12 OC tissues into the same tube. Quick-spin down OC tissues and remove the HBSS. Disassociate the cells by adding 100 µl pre-warmed TrypLE™ Select (Life Technologies) and incubate at 37°C for 13 min. Quick spin down dissociated OC tissues. Remove TrypLE™ Select. Add 100 µl of Medium A (DMEM/F12, N2 supplement (LifeTechnologies), B27 supplement (LifeTechnologies), ampicillin (50µg/ml) and Fungizone (LifeTechnologies), EGF (20 ng/ml), bFGF (10 ng/ml), IGF-1 50 ng/ml), and heparan sulfate (50 ng/ml)). The dissected and digested tissue is then triturated with a P200 pipette tip. Remove any cell aggregates and debris; transfer the cell suspension and wash through a pre-wetted 70 µm cell strainer to pool the cell suspension in a single fresh culture tube. Rinse dissociation tubes and the cell strainer with sufficient Medium A and pool with the cell suspension. Count cell density

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with hemocytometer or Countess® (Life Technologies). Add fresh Medium A to cell suspension to achieve a final plating density of 1.0×10^5 cell/ml and plate into ultra-low attachment cultureware, T-75 (Greiner).

Culturing in Suspension to Propagate OC spheres

- 5 The dissociated, single cells are cultured in an ultra-low attachment cultureware, (Greiner) in Medium A for 3 to 4 days in a 5% CO₂ humidified incubator at 37°C to obtain clonally grown spheres, termed first generation spheres, from the floating cells. If cells stick to the non-adherent dishes during the culture period they can be dislodged by gentle mixing.

10

Attachment Culture and Treatment

- After culturing the spheres for 3-4 days, visually count the spheres by microscope to standardize the seeding density of otic spheres per ml for this step. Collect the cells by centrifugation and re-suspend spheres in Basic Medium at a concentration of approximately 2000 spheres per ml. Seed the spheres in a volume of 150 µl per well of a cell culture-treated 96 well plate to obtain approximately 300 spheres per well. Treat the spheres with experimental agents in quadruplicate at a final volume of 200 µl in Basic Medium (DMEM/F12, N2 supplement (LifeTechnologies) B27 supplement (LifeTechnologies), ampicillin (50 µg/ml) and Fungizone (LifeTechnologies). Culture the treated spheres for 7 days in a humidified temperature controlled incubator at 5% CO₂, 37°C. Remove medium and proceed with RNA extraction.
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RNA Extraction

- Add buffer RLT plus (Qiagen) supplemented with 1% Beta-mercaptoethanol, 175 µl per well. Add 2.5 µl of 4 ng/µl of RNA carrier (RNeasy® Plus Micro Kit; Qiagen) diluted in Buffer RLT Plus into each well before RNA extraction. Total RNA is extracted using the RNeasy® Plus Micro Kit (Qiagen) according to the manufacturer's instructions.
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cDNA Synthesis

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cDNA synthesis is carried out using the SuperScript® III First-Strand Synthesis System (LifeTechnologies, catalogue 18080-051) following the manufacturer's protocol using random hexamers for cDNA synthesis. Dilute cDNA 50% by adding 21 μ l of ultra-pure nuclease-free water. 5 μ l of diluted cDNA is used for qPCR.

5 qPCR

To quantify the expression of hair cell markers from the treated sphere cultures, qPCR with probes to detect a gene of interest *mAtoh1* and an endogenous control gene is carried out in a single, two-color multiplex reaction. TaqMan Gene Expression Master Mix (LifeTechnologies, 4369016) and a probe of interest (*Atoh1*; Mm00476035_s1 and an endogenous control probe (*Tbp1*; Mm00446971_m1, LifeTechnologies) is used according to the manufacturer's instructions. Conditions are kept constant for each probe. Relative gene expression between treatment groups is analyzed using the $\Delta\Delta C_T$ method and replicate measurements are averaged. Experiments are carried out in triplicate, at a minimum, and reported as a grand mean.

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Table 2

Compound	RQ at 10 nM	RQ at 100 nM	RQ at 1 μ M
1, Isomer 2	1.43 $\pm$ 0.17*	1.76 $\pm$ 0.17*	1.64 $\pm$ 0.13*
2, Isomer 2	1.14 $\pm$ 0.07	1.21 $\pm$ 0.13	1.57 $\pm$ 0.11*

The data in Table 2 shows relative quantitation values (RQ) of *Atoh1* expression, a marker of hair cell generation, in otic spheres treated with each of Compound 1, Isomer 2 and Compound 2, Isomer 2 at three concentrations (10nM, 100nM and 1 μ M). Data ($\pm$ standard error of the mean, SEM) represent the gene expression fold-induction relative to negative control (DMSO carrier)-treated samples. (*, significant difference from negative control, $p < 0.05$).

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Induction of hAtoh1 Expression in Human Tumor Cell Lines

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To evaluate potency of Compounds 1, Isomer 2 and 2, Isomer 2 in their ability to induce hAtoh1 expression, a human tumor cell line is utilized. Endogenous Notch signaling regulation for this cell line is similar to that in the inner ear. Generally, this assay is carried out following principles in Kazanjian et al., Gastroenterology, 2010, 139(3): 918-928 and Supplementary Materials and Methods.

Generally, standard cell culture techniques are used. Low passage human colorectal adenocarcinoma cell line LS174T (ATCC CL-188) maintained in Growth Medium (MEM 10% FBS) is plated 10,000 cells in 100 μ l Assay Medium (MEM 1% FBS) per well into 96-well tissue culture treated plates.

The following day, half-log serial dilutions of 10 mM stock test compounds (diluted in 100% DMSO) were carried out to obtain eleven decreasing doses of drug compound. Final concentrations are to span 10 μ M to 0.127 nM. A 3.16-fold serial dilution is used, with DMSO concentrations in each well of 100%. A further 1:10 dilution of the initial DMSO dilution is carried out into Assay Medium. Another dilution of 1:100 again into Assay Medium is carried out.

100 μ l of the test compound dilutions are added to each well containing cells and Growth Media and grown for 72 hours at 37°C in 5% CO₂.

Cells are collected from the plates on day 4, and the RNA extracted and purified using RNeasy® Plus 96 RNA (Qiagen) following the manufacturer's protocol.

cDNA synthesis is carried out using the SuperScript® III First-Strand Synthesis System (LifeTechnologies, 18080-051) following the manufacturer's protocol using random hexamers for cDNA synthesis. The cDNA is diluted 50% by adding 21 μ l of ultra-pure nuclease-free water. 5 μ l of the diluted cDNA is used for qPCR.

qPCR

To quantify the expression of hair cell markers from the treated sphere cultures, qPCR probes to detect hAtoh1 or TBP expression in a single two-color multiplex reaction is carried out. To measure relative gene expression for each sample, TaqMan Gene

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Expression Master Mix (LifeTechnologies, 4369016) and a probe of interest (human Atoh1; LifeTechnologies Assay Id Hs00944192_s1) plus an endogenous control (human TBP; LifeTechnologies Assay Id Hs00427620_m1) is used according to the manufacturer's instructions. Conditions are kept constant for each probe. Relative gene expression between treatment groups is analyzed using the $\Delta\Delta C_T$ method and replicate measurements are averaged.

Four separate assays are carried out to obtain IC_{50} values for each test compound. The IC_{50} value is determined by fitting concentration response data to "log(antagonist) vs. response (three parameter)" model using GraphPad Prism® software for 12 doses across a concentration range of 4.0E-11 to 1.0E-6 M with 108 total points analyzed for each compound. The IC_{50} value for Compounds 1, Isomer 2 and 2, Isomer 2 are shown in Table 3.

Table 3

Compound	IC_{50} nM
1, Isomer 2	16.64
2, Isomer 2	54.34

The data in Table 3 shows IC_{50} values of Compound 1, Isomer 2 and Compound 2, Isomer 2 as calculated by the relative expression of hAtoh1, a marker of auditory hair cell generation in this assay.

Response to Compounds in an Organ of Corti Explant Assay

Generally, organ of Corti organotypic explant culture is carried out on Day 0 according to the method described in Parker et al., Journal of Visualized Experiments, 2010, (36), e1685. Explant culture is carried out for 4-7 days at which point the culture is either prepared for cellular lysis for qRT-PCR, or tissue fixation for immunohistochemistry.

Isolation and Culture of Organ of Corti Explants

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The bulla and surrounding tissue from the petrous portion of the temporal bone are removed from 1 to 4 day-old postnatal mice (transgenic strain: *Atoh1*-driven nGFP; Lumpkin EA, et al., *Gene Expr Patterns*, 2003, (36):389-95) of both sexes. Organs of Corti are dissected in Hanks solution, the spiral ligament is removed and the explants are placed on cover slips coated with poly-L-ornithine (0.01%, Sigma) and laminin (50 mg/ml, Becton Dickinson) to obtain a preparation with a flat cochlear surface. Each cochlear explant is then placed in a single well of a 24 well plate (Falcon) and cultured in DMEM (Invitrogen) with 5% fetal bovine serum, 5% horse serum, and penicillin-streptomycin (LifeTechnologies). Experimental compounds are added with medium on Day 0 and replaced every 2-3 days. All cultures are maintained in a 5% CO₂ humidified incubator at 37°C.

Gene Expression Analysis by qRT-PCR

RNA Extraction is carried out by lysing the explant tissue with 350µl RLT Plus buffer (Qiagen) per well, and total RNA is purified by RNeasy® Plus Mini Kit (Qiagen). cDNA synthesis is carried out with the SuperScript® III First-Strand Synthesis System (LifeTechnologies) using random hexamers. To quantify the induction of hair cell marker expression, qPCR is performed with TaqMan Gene Expression Master Mix (LifeTechnologies, 4369016) and probes to detect genes of interest (eg. *hAtoh1*; Assay ID Mm00476035_s1, *Pou4f3*; Assay ID Mm04213795_s1 and *Myo7a*; Assay ID Mm01274015_m1, LifeTechnologies) and an endogenous control gene, *Tbp* (Assay ID Mm00446971_m1, LifeTechnologies). Relative gene expression between treatment groups is analyzed using the $\Delta\Delta C_T$ method and replicate measurements are averaged. Experiments are carried out in triplicate, at a minimum, and reported as a grand mean.

Table 4

Compound	<i>Atoh1</i>	<i>Pou4f3</i>	<i>Myo7a</i>
1, Isomer 2	3.72±0.54	2.04±0.35	1.73±0.33
2, Isomer 2	3.10±0.69	1.65±0.25	1.49±0.22

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The data in Table 4 shows relative quantitation values (RQ) of expression of the hair cell generation markers; *Atoh1*, *Pou4f3*, and *Myo7a*, in organ of Corti explants treated with Compound 1, Isomer 2 and Compound 2, Isomer 2 at 1 μ M. Data ($\pm$ SEM) represent the fold-induction in gene expression relative to negative control-treated samples.

Immuno-Staining of New Atoh1-Expressing Hair Cells

Following the explant culture, tissues are fixed in the 24 well plate with 200 μ l Cytifix/Cytoperm (BD) for 30 min, rinsed with PBS containing TritonX100 (PBST), and stained with 200 μ l of primary antibody solution, consisting of 4% normal donkey serum in PBST with goat anti-GFP (1:2500; AbCAM, ab5450) and rabbit anti-myosin VIIa (1:1000; Proteus Bioscience #25-6790) for 1 hour. The tissues are then thoroughly washed with PBST and stained with a secondary antibody solution, consisting of Alexa Fluor 488 donkey anti-goat IgG (1:1000; Invitrogen, A-11055) and Alexa Fluor 568 donkey anti-rabbit IgG (1:1000; Invitrogen, A10042) for 1 hour. They are then thoroughly washed with PBST and mounted on microscope slides. Individual hair cells are identified by expression of the Atoh1-surrogate marker GFP (Green) and myosin VIIa (red), imaged, and then counted within a 100 μ M length at the mid-apex region in order to examine the formation of nascent hair cells in experimental, compound-treated samples in contrast to hair cell numbers in untreated or negative control (biological inactive compound)-treated samples. Blinded cell counts are measured in five separate experiments.

Table 5

Compound	Number of Outer Hair Cells (per100 μ M)	Percent Increase
1, Isomer 2	50.4 $\pm$ 4.9	14.15 $\pm$ 1.9%

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2, Isomer 2	55.8±3.1	17.26±1.2%
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The data in Table 5 shows outer hair cell numbers in organ of Corti explants cultured for four days in the presence of Compound 1, Isomer 2 (n=13) or Compound 2, Isomer 2 (n=15) at 1 μ M, and immunostained for GFP (a surrogate Atoh1 marker) and myosin VIIa. Percent increase in hair cells are relative to negative control-treated samples (n=18).

Gene Expression Analysis After Ototoxic Damage

A variation of the organ of Corti organotypic explant culture is used to model hair cell regeneration in response to subsequent compound treatment after ototoxic damage. This damage model is carried out according to the method described in Korrapati et al., PLOSOne, 2013, 8(8), e73276 where a 95% reduction in mid-apical hair cells was reported. On Day 0, to damage and selectively kill hair cells within the organ of Corti explant, aminoglycoside treatment (gentamicin (100 μ M)) is added for 18-24 hours. On Day 1, gentamicin is removed and treatment with Compound 1, Isomer 2 and Compound 2, Isomer 2 at 5 μ M began. Explant culture continues for 4 days total at which point the culture is prepared for the qRT-PCR protocol described above. Visible confirmation of gentamicin-induced damage is easily observed between cultures treated with negative control compounds. However, quantitative measurement for the response to Compound 1, Isomer 2 and Compound 2, Isomer 2 treatment following ototoxic damage obtained by qRT-PCR measured increases in gene expression for prosensory hair cell markers Atoh1 and Pou4f3 and the hair cell marker Myo7A.

Table 6

	% Induction Over Control-treated Damaged Organ of Corti (Mean $\pm$ SEM; p Value) n = 11		
Compound (5 μ M)	Atoh1	Pou4f3	Myo7A

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1, Isomer 2	242±16; <0.0005*	114±25; <0.05*	102±18; 0.0522
2, Isomer 2	169±24; <0.05*	28±11; 0.0633	52±15; not signif.

The data in Table 6 shows relative increases in gene expression by qRT-PCR of the hair cell generation markers *Atoh1*, *Pou4f3*, and *Myo7a*, in organ of Corti explants treated with Compound 1, Isomer 2 and Compound 2, Isomer 2 at 5 μ M after gentamicin (100 μ M) damage. Data represent the percent increase in gene expression relative to negative control-treated samples with the gentamicin-induced hair cell damage. (*, significant difference from negative control, $p < 0.05$)

Hair cell marker induction in Whole otic capsule culture

The otic capsule (otic capsule explant), comprised of the cochlear bony labyrinth and vestibular systems, is dissected from 1 to 4 day-old postnatal mice (transgenic strain: *Atoh1*-driven nGFP; Lumpkin EA, et al., Gene Expr. Patterns, 2003, 3(4): 389-95) of both sexes generally according to the method described in Parker et al., Journal of Visualized Experiments, 2010, (36), e1685. The entire otic capsule is isolated from the parietal bone and maintained *ex-vivo* in a roller tube culture system for up to 3 days in medium containing DMEM, high glucose, 5% FBS, 5% horse serum, 1 μ g/ml ampicillin.

The otic capsule explant is used to evaluate test compound, in various delivery vehicles, penetrate into the inner ear. Tests are carried out with compositions of Compound 1, Isomer 2, (7.2 mM) in various delivery vehicles. Small volumes (100-200 nl) are directly applied to the round window niche of the cochlea. The cochlea is dissected, treated, incubated for 1-2 hrs, then entered into the roller culture. After 48 hrs. the otic capsule explant is opened to remove the organ of Corti from the bony labyrinth of the cochlea and assayed by RT-PCR.

The composition is allowed to remain for 1-2 hours then washed away with culture medium. The otic capsule explant is then cultured for 48 hours in untreated medium. The cochlea is broken open and the entire organ of Corti is then dissected out and prepared for quantitative RT-PCR to measure *Atoh1* hair cell marker expression

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levels (as described in organ of Corti explant assay, above). Table 7 shows Atoh1 induction in the specified delivery vehicle. Up-regulation of Atoh1 by Compound 1, Isomer 2, in all tested compositions (2-3 fold, n of 12-17) is measured in comparison to vehicle-only control-treated otic capsule explants after 48 hours in culture.

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Table 7: Atoh1 Gene Expression (RT-PCR)

	45-70% PEG (Polyethylene glycol 400)	10% Poloxamer (Pluronic® F108)	20% Poloxamer (Pluronic® F108)	1-2% Hyaluronic Acid
Liquid state	Viscous liquid	Viscous liquid	Gel	Gel
Test compound solubility	Clear solution	Suspension	Suspension	Suspension
Mean Fold-Induction	2.933	2.398	2.612	2.459
SEM	0.3193	0.2653	0.4441	0.2567
P value	<0.0001	0.0002	0.0019	0.0001

In Vivo perilymph test compound uptake into inner ear

Female albino Hartley guinea pigs (Charles River, France) are anesthetized with a mixture of ketamine/xylazine prior to 70 µl of Compound 1, Isomer 2, in compositions as described above for Whole otic capsule culture assay are injected by transtympanic route to completely fill the middle ear. Perilymph (inner ear fluid) samples are extracted at 0.5, 1, 6, 24 and 48 hours post-injection and assayed by LCMS (n of 5) for concentration of test compound. Table 8 shows test compound is taken up into the inner ear following transtympanic administration to the middle ear with these delivery vehicles.

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Table 8: *IN VIVO* Perilymph Levels

	70% PEG (Polyethylene glycol 400)	10% Poloxamer (Pluronic® F108)	20% Poloxamer (Pluronic® F108)	1% Hyaluronic Acid
Cmax (µM)	327.1	753.0 ±	1926.2 ±	187.3 ±

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±SEM	± 93	306	493	113
Time Cmax	1 hr	30 min	30 min	1 hr
% Applied Test Compound in perilymph	1.04	2.39	6.11	0.59
R squared	0.5262	0.7640	0.5600	0.4229
AUC ($\mu\text{mol.hr.L}^{-1}$)	1451	7002	8634	2711

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Amino Acid Sequences

SEQ ID NO:1 (Mouse Notch 1 protein precursor; full length)

MPRLLTPLLCLTLLPALAARGLRCSQPSGTCLNGGRCEVANGTEACVCSGAFVG
5 QRCQDSNPCLSTPCKNAGTCHVVDHGGTVDYACSCPLGFSGPLCLTPLDNACLA
NPCRNGGTCDLLTLTEYKCRCPGWSGKSCQQADPCASNPCANGGQCLPFESSYI
CRCPPGFHGPTCRQDVNECSQNPGLCRHGGTCHNEIGSYRCACRATHGTGPHCELP
YVPCSPSPCQNGGTCRPTGDTTHEACLPGFAGQNCENVDDCPGNNCKNGGAC
VDGVNTYNCRCPEWTGQYCTEDVDECQLMPNACQNGGTCHNTHGGYNCVVCV
10 NGWTGEDCSENIDDCASAACFQGATCHDRVASFYCECPHGRTGLLCHLNDACIS
NPCNEGSNCDTNPVNGKAICTCPSGYTGPACSQDVDECALGANPCEHAGKCLNT
LGSFECQCLQGYTGPRCEIDVNECISNPCQNDATCLDQIGEFQCICMPGYEGVYCE
INTDECASSPCLHNGHCMDKINEFQCQCPKGFNGHLCQYDVDECASTPCKNGAK
CLDGPNTYTCVCTEGYTGTHCEVDIDECDPDPCHYGSKDGVATFTCLCQPGYT
15 GHHCETNINECHSQPCRHHGGTCQDRDNSYLCLCLKGTTGPNCEINLDDCASNPCD
SGTCLDKIDGYECACEPGYTGSMCNVNIDEACAGSPCHNGGTCEDGIAGFTCRCPE
GYHDPTCLSEVNECNSNPCIHGACRDGLNGYKCDCAPGWSGTNCDINNNECESN
PCVNGGTCKDMTSGYVCTCREGFSGPNCQTNINECASNPCLNQGTCIDDVAGYK
CNCPLPYTGATCEVVLAPCATSPCKNSGVCKESEDYESFSCVCPTGWQGGTCEV
20 DINECVKSPCRHGASCQNTNGSYRCLCQAGYTGRNCESDIDDCRPNPCHNGGSC
TDGINTAFCDCLPGFQGAFCCEEDINECASNPCQNGANCTDCVDSYTCTCPVGFNG
IHCENNTPDCTESSCFNGGTCVDGINSFTCLCPPGFTGSYCQYDVNECDSRPCLHG
GTCQDSYGTYKCTCPQGYTGLNCQNLVRWCDSAPCKNNGGRCWQTNTQYHCEC
RSGWTGVNCDVLSVSCEVAAQKRIGDVTLLCQHGGGLCVDEGDKHYCHCQAGY
25 TGSYCEDEVDECSNPNCQNGATCTDYLGGFSCCKCVAGYHGSNCSEEINECLSQPC
QNGGTCIDLTSYKCS CPRGTQGVHCEINVDDCHPPLDPASRSPKCFNNGTCVDQ
VGGYTCTCPPGFVGERCEGDVNECLSNPCDPRGTQNCVQVRVNDFFHCECRAGHT
GRRCESVINGCRGKPKCKNGGVCASANTARGFICRCPAGFEGATCENDARTCGS
LRCLNGGTCISGPRSPTCLCLGSFTGPECQFPASSPCVGSNPCYNQGTCEPTSENPF
30 YRCLCPAKFNGLLCHILDYSFTGGAGRDIPPPQIEEACELPECQVDAGNKVCNLQ
CNNHACGWDGGDCSLNFNDPWKNCTQSLQCWKYFSDGHCDSDQNSAGCLFDG
FDCQLTEGQCNPPLYDQYCKDHFSGDGHCDQGCNSAECEWDGLDCAEHVPERLAA
GTLVLVLLPPDQLRNNSFHFLRELSHVLHTNVVFKRDAQGQQMIFPYYGHEEE
LRKHPIKRSTVGWATSSLLPGTSGGRQRRELDPMDIRGSIVYLEIDNRQCQVSSSQ
35 CFQSATDVAAFLGALASLGS LNIPYKIEAVKSEPVEPPLPSQLHLMYVAAAFAVL
LFFVGCGVLLSRKRRRQHGGQLWFPEGFKVSEASKKKRREPLGEDSVGLKPLKNA
SDGALMDDNQNEWGDEDLETKKFRFEPPVLPDLSQTDHRQWTQQHLDAAD
LRMSAMAPTPPQGEVDADCMDVNVVRGPDGFTPLMIASCSGGGLETGNSEEEEDA
PAVISDFIYQGASLHNQTDRTGETALHLAARYSRSDAAKRLLEASADANIQDNM
40 GRTPLHAAVSADAQGVFQILLRN RATDL DARMHDGTTPLILAA RLAVEGMLEDL
INSHADVNAVDDLGSALHWAAAVNNVDAAVVLLKNGANKDMQNNKEETPLF
LAAREGSYETAKVLLDHFANRDITDHMDRLPRDIAQERMHHDIVRLLD EYNLVR
SPQLHG TALGGTPTLSPTLCSPNGYLG NLKSATQGKKARKPSTKGLACGSKEAK
DLKARRKKSQDGKGCLLDSSSMLSPVDSLES PHGYLSDVASPPLLPSPFQQSPSM
45 PLSHLPGMPDTHLGISHLNVA AKPEMAALAGGSRLAFEP PPPRLSHLPVASSASTV
LSTNGTGAMNFTV GAPASLNGQCEWL PRLQNGMVPSQYNPLRPGVTPGTLSTQA

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AGLQHSMMGPLHSSLSTNTLSPIIYQGLPNTRLATQPHLVQTQQVQPQNLQLQPQ
NLQPPSQPHLSVSSAANGHLGRSFLSGEPSQADVQPLGPSSLPVHTILPQESQALPT
SLPSSMVPPMTTQFLTPPSQHSSSPVDNTPSHQLQVPEHPFLTSPESPQWSS
SSPHSNISDWSEGISSPPTTMPSQITHIPEAFK

5

SEQ ID NO:2 (signal peptide)

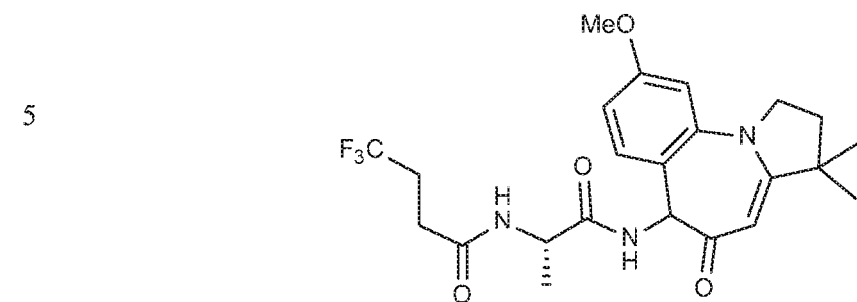
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We claim:

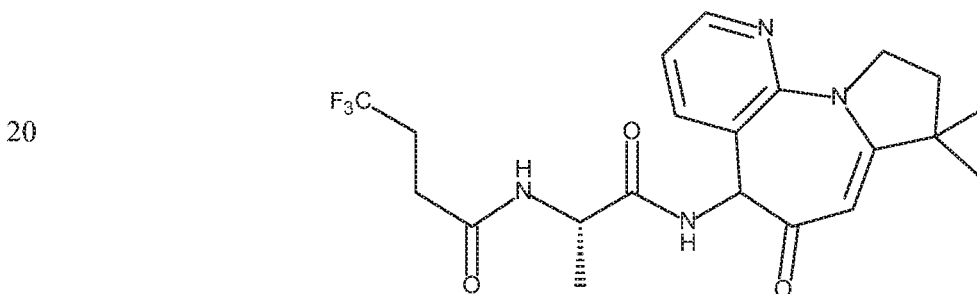
1. A compound of the structure:



4,4,4-trifluoro-N-((2S)-1-((9-methoxy-3,3-dimethyl-5-oxo-2,3,5,6-tetrahydro-1H-benzo[f]pyrrolo[1,2-a]azepin-6-yl)amino)-1-oxopropan-2-yl)butanamide, a substantially diastereomerically pure isomer thereof, or a pharmaceutically acceptable salt of any of the above.

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2. A compound of the structure:



25 N-((2S)-1-((8,8-dimethyl-6-oxo-6,8,9,10-tetrahydro-5H-pyrido[3,2-f]pyrrolo[1,2-a]azepin-5-yl)amino)-1-oxopropan-2-yl)-4,4,4-trifluorobutanamide, a substantially diastereomerically pure isomer thereof, or a pharmaceutically acceptable salt of any of the above.

3. A pharmaceutical composition comprising either a compound of claim 1 or
 30 2, or a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

4. A compound of claim 1 or 2, or a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for use in therapy.

5. Use of a compound of claim 1 or 2, or a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for the treatment of T-cell acute lymphoblastic leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, erythroleukemia, triple negative breast cancer, breast cancer, ovarian cancer, melanoma, lung cancer, non small cell lung cancer, pancreatic cancer, glioblastoma, colorectal cancer, head and neck cancer, cervical cancer, prostate cancer, liver cancer, oral squamous cell carcinoma, skin cancer, medulloblastoma, angiosarcoma, rhabdomyosarcoma, liposarcoma, malignant fibrous histiocytoma, hepatocellular carcinoma, intrahepatic or extrahepatic cholangiocarcinoma, or adenoid cystic carcinoma.
6. The use of claim 5, for the manufacture of a medicament for the treatment of lung cancer.
7. Use of a compound of claim 1 or 2, or a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for the treatment of sensorineural hearing loss caused by auditory hair cell loss.

8. Use of a compound of claim 1 or 2, or a substantially pure diastereomer thereof, or a pharmaceutically acceptable salt thereof, for the manufacture of a medicament for inducing auditory hair cell generation.