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(54) Title: METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF VENTILATOR-INDUCED DIAPHRAGMATIC DYSFUNCTION

(57) Abstract: The present invention relates to methods and pharmaceutical compositions for the treatment of ventilator-induced diaphragmatic dysfunction. In particular, the present invention relates to agents capable of stabilizing the RyR1-calstabin1 interaction and thus capable of reducing SR Ca^{2+} leak via RyR1 channel for use in the treatment of ventilator-induced diaphragmatic dysfunction.

METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF VENTILATOR-INDUCED DIAPHRAGMATIC DYSFUNCTION

FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of ventilator-induced diaphragmatic dysfunction.

BACKGROUND OF THE INVENTION:

The need for artificial respiratory support by mechanical ventilation (MV) is one of the most common reasons for admission to the intensive care unit (ICU). Although it is life saving in the short term, human and animal studies have previously shown that MV results in a progressive reduction in diaphragmatic force-generating capacity, together with diaphragm muscle fiber injury and atrophy. These findings comprise a condition termed Ventilator-Induced Diaphragmatic Dysfunction (VIDD) .VIDD, which is common in ICU settings,, can interfere with the ability to discontinue MV, with a major negative impact on subsequent patient outcomes and subsequently increased health care costs. The precise cellular pathways involved in MV-induced diaphragm weakness remain poorly understood. Animal models strongly suggest that oxidative stress plays a major role in VIDD. Moreover, recent studies have identified mitochondria as an essential source of reactive oxygen species (ROS) implicated in VIDD. Additionally, proteolytic systems such as caspases and calpains activation, which play significant roles in degrading cytoskeletal proteins in muscle and can also be activated by ROS, are suggested to be involved in the development of MV-induced diaphragm muscle fiber atrophy and injury (*9, 20-22*).

Although many of the processes implicated in VIDD have been associated with increased oxidative stress, the potential role of Ca^{2+} homeostasis disruption in these pathological changes has not been addressed. Maes et al. (Respiratory Research, 2010, 11:178) discloses that indirect evidence suggests that prolonged controlled mechanical ventilation (CMV) results in an increase in intracellular calcium levels in the diaphragm such that protection against CMV-induced increases in intracellular calcium levels and/or increasing calpastatin binding to calpain could be potential mechanisms by which corticosteroids prevent activation of the protease calpain and protect against VIDD. VIDD has not been known to be linked to excitation-contraction coupling, which refers to a succession of cellular events leading to muscle contraction.

Briefly, in skeletal muscle membrane depolarization activates voltage-sensing Ca^{2+} channels in the transverse tubules that in turn activate RyR1, leading to sarcoplasmic reticulum (SR) Ca^{2+} release. The subsequent rise in cytoplasmic Ca^{2+} concentration triggers actin-myosin cross-bridge formation, sarcomere shortening and muscle contraction. RyR1 is a homotetrameric macromolecular protein complex that includes four RyR1 monomers (~565 kDa each), kinases, a phosphatase (PP1), a phosphodiesterase (PDE4D3), and calmodulin. In addition, the RyR1 channel-stabilizing subunit calstabin1 (Cal1 or FK506 binding protein 12, FKBP12) is critical to the proper function of the channel. Maladaptive cAMP-dependent protein kinase A (PKA)-mediated phosphorylation and redox-dependent modifications (cysteine-nitrosylation and oxidation) of the RyR1 have been linked to a loss of the normal association between calstabin1 and the rest of the RyR1 complex .. This in turn results in impaired Ca^{2+} handling and contractile dysfunction in conditions as diverse as heart failure, chronic muscle fatigue, muscular dystrophy and ageing.

Accordingly, there is a need for improved treatment of VIDD that this is now provided by the present invention.

SUMMARY OF THE INVENTION:

The present invention relates to an agent capable of stabilizing the RyR1-calstabin1 interaction for use in a method for the treatment of ventilator-induced diaphragmatic dysfunction in a subject in need thereof. In particular, these agents are capable of stabilizing the RyR1-calstabin1 interaction and thus capable of reducing SR Ca^{2+} leak via RyR1 channel. These agents are generally known as Rycals. Specific compounds and general formulae covering the agents that are suitable for this invention are provided in the detailed description.

The present invention also relates to a method for screening a plurality of test substances useful for treatment of ventilator-induced diaphragmatic dysfunction comprising the steps consisting of (a) testing each of the test substances for its ability stabilize the RyR1-calstabin1 interaction and/or to reduce SR Ca2+ leak via RyR1 channel and (b) and positively selecting the test substances capable of restoring said integrated stress response.

Another embodiment of the invention is a method for the treatment of ventilator-induced diaphragmatic dysfunction in a subject in need thereof which comprises administering to the subject one of the agents disclosed herein that is capable of stabilizing RyR1-calstabin1 interaction. The agent may be identified from the screening method disclosed herein.

Yet another embodiment of the invention relates to the use of an agent capable of stabilizing RyR1-calstabin1 interaction for the treatment of ventilator-induced diaphragmatic dysfunction in a subject in need thereof. The useful agents are those described herein as well as those identified from the screening method disclosed herein.

BRIEF DESCRIPTION OF THE FIGURES:

Figures 1A to 1C show that VIDD is associated with defective RyR1 in human diaphragm muscle, with Figure 1A including immunoblots of immunoprecipitated RyR1 of human diaphragm samples collected from short term (control) and long term (MV) mechanically ventilated patients, Figure 1B including bar graphs that show quantification of the immunoblots of Figure 1A, and Figure 1C including single channel traces of single RyR1 incorporated in planar lipid bilayers.

Figures 2A to 2D show that VIDD is associated with defective RyR1 in porcine diaphragm muscle, with Figure 1A providing graphs of diaphragmatic contractile function as a function of stimulation frequency; Figure 2B providing single channel traces of single RyR1 incorporated in planar lipid bilayers, Figure 2C providing representative immunoblots of immunoprecipitated RyR1 of pig diaphragm samples collected from control and mechanically ventilated groups after 72 hours of anesthesia, and Figure 2D providing bar graphs that show quantification of the immunoblots relative to total RyR1 immunoprecipitated.

Figure 3 illustrates that VIDD is associated with early impairment of RyR1 in murine diaphragm muscle, with Figure 3A providing representative records of specific diaphragmatic force production measured *ex vivo* in muscle bundles under isometric conditions in control and mechanically ventilated (MV) mice, Figure 3B showing averaged force – frequency relationships recorded in control and MV mice, Figure 3C providing representative immunoblots of immunoprecipitated RyR1, and Figure 3D providing bar graphs showing the quantification of immunoblots relative to total RyR1 immunoprecipitated of murine diaphragm samples.

Figures 4A to 4E show that RyR1 stabilization prevents VIDD, with Figure 4A, with Figure 4A providing representative records of specific diaphragmatic force production measured *ex vivo* at 1, 30 and 100Hz in muscle under isometric conditions in wild-type mice, Figure 4B providing averaged specific force–frequency relationships recorded in various

wild-type MV, Figure 4C providing a comparison of mean Ca^{2+} sparks frequency between MV and MV_107, Figure 4D providing representative records of specific diaphragmatic force production in calstabin1 knockout mice, and Figure 4E providing averaged specific force–frequency relationships recorded in various mice.

Figure 5Aprovides a graph that illustrates RyR1 open probability in human and pig diaphragm muscles in control condition and after MV.

Figure 5B provides bar graphs that show that Ventilation in Continuous Positive Airway Pressure (Cpap) mode does not affect RyR1 remodeling.

Figure 5C provides graphs that illustrate that Ventilation in Continuous Positive Airway Pressure (Cpap) mode does not affect RyR1 function.

Figure 5C provides graphs that show that force frequency relationships of control and cal1-/- diaphragm muscle in resting condition.

DETAILED DESCRIPTION OF THE INVENTION:

Ventilator-induced diaphragmatic dysfunction (VIDD) refers to the diaphragm muscle weakness following prolonged controlled mechanical ventilation (MV). The presence of VIDD impedes recovery from respiratory failure, but the pathophysiological mechanisms accounting for VIDD are still not fully understood. Here the inventors show in human subjects and animal models of VIDD (pigs and mice) that MV is associated with rapid remodeling of the sarcoplasmic reticulum (SR) calcium release channel/ryanodine receptor (RyR1) in the diaphragm. The RyR1 macromolecular complex was oxidized, S-nitrosylated, Ser-2844 phosphorylated and depleted of the stabilizing subunit calstabin1, following MV. These post-translational modifications of RyR1 were mediated by oxidative stress and resulted in abnormal resting SR Ca^{2+} leak and reduced contractile function. Treatment with S107, a small molecule drug that stabilizes the RyR1-calstabin1 interaction despite these RyR1 complex modifications effectively prevented VIDD. Diaphragm dysfunction is common in MV patients and is a major cause of failure to wean patients from artificial respiratory support. This study provides the first evidence of RyR1 alterations as a proximal mechanism underlying VIDD and a potential therapeutic target.

Accordingly, the present invention relates to a method for the treatment of ventilator-induced diaphragmatic dysfunction in a subject in need thereof comprising by administering to the subject an agent capable of stabilizing the RyR1-calstabin1 interaction and thus capable of reducing SR Ca^{2+} leak via RyR1 channel.

In some embodiments, the subject needs artificial respiratory support because he suffers from respiratory failure and/or heart failure which can be aggravated by sepsis, metabolic disorder, neuromuscular diseases, or surgery along with post-surgical recovery. Typically, the subject suffers from a disease for which the worsening of the symptoms has led the subject to need artificial respiratory support (i.e. mechanical ventilation). For example, some lung diseases, such as Chronic Obstructive Pulmonary Disease (COPD), pneumonia, sepsis (including severe sepsis and septic shock), Acute Respiratory Distress Syndrome (ARDS), Severe Acute Respiratory Syndrome (SARS) and cystic fibrosis (CF) usually require some form of ventilation assistance in order to clinically improve the subject. In some embodiments the subject suffers from a trauma. Pulmonary dysfunction in trauma patients is multifactorial and may be the result of direct contusion of the lung tissue, lung injury by fractured ribs, loss of chest wall function, fat embolism to the lung from long bone fractures, aspiration of blood or gastric contents and the consequences of the activation of the systemic inflammatory response syndrome (SIRS) of shock, reperfusion, and transfusion therapy.

As used herein, the expression "ventilator-induced diaphragmatic dysfunction" or "VIDD" has its general meaning in the art and refers to the condition wherein diaphragmatic atrophy and contractile dysfunction occur after prolonged controlled mechanical ventilation (Powers SK, Wiggs MP, Sollanek KJ, Smuder AJ. Invited Review: Ventilator-induced diaphragm dysfunction: cause and effect. Am J Physiol Regul Integr Comp Physiol. 2013 Jul 10.). Ventilator-induced diaphragmatic dysfunction may result from prolonged controlled mechanical ventilation (MV), e.g., greater than 12 hours. However, such prolonged MV is not limited to any specific time-length. For example, in some embodiments, prolonged MV includes a time from at least about 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 50, or 100 hours, to from at least about 1, 10, 20, 50, 75, 100 or greater hours, days, or years. In another embodiment, prolonged MV includes a time from at least about 5, 6, 7, 8, 9 or 10 hours, to from at least about 10, 20 or 50 hours. In some embodiments, prolonged MV is from about at least 10-12 hours to any time disclosed herein that is greater than the 10-12 hour period.

As used herein, the terms “treating” or “treatment” or “alleviation” refers to therapeutic treatment, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. A subject is successfully “treated” for ventilator-induced diaphragmatic dysfunction, if after receiving a therapeutic amount of the agent according to the invention, the subject shows observable and/or measurable reduction in or absence of one or more signs and symptoms of ventilator-induced diaphragmatic dysfunction, such as, e.g., a significant reduction in diaphragmatic contractile function, or a decrease of diaphragm atrophy evaluated by CT scan or ultra sound. In a particular embodiment the treatment is a prophylactic treatment. The term "prophylactic treatment" as used herein, refers to any medical or public health procedure whose purpose is to prevent a disease. As used herein, the terms "prevent", "prevention" and "preventing" refer to the reduction in the risk of acquiring or developing a given condition, or the reduction or inhibition of the recurrence or said condition in a subject who is not ill, but who has been or may be near a subject with the disease. It is also to be appreciated that the various modes of treatment or prevention of medical conditions as described are intended to mean “substantial,” which includes total but also less than total treatment or prevention, and wherein some biologically or medically relevant result is achieved.

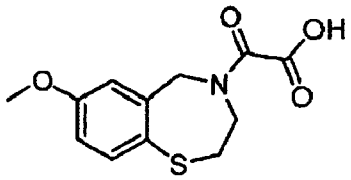
In some embodiments, the agent is administered before MV, immediately after MV initiation, during MV, and/or immediately after MV. In some embodiments, administration of the agent according to the invention is provided at any time during MV.

The agent according to the invention is also suitable for preventing risks associated with ventilator-induced diaphragmatic dysfunction. Risks associated with ventilator dependence include increased discomfort and risk of secondary diseases for the patient (such as pneumonia, pulmonary fibrosis, aspiration, acute renal failure, cardiac arrhythmias, sepsis, vocal fold dysfunction, and acute lung injury secondary to barotrauma or volotrauma), increased morbidity and mortality, high health care costs, and longer treatment duration times. Although patients with chronic ventilator dependency (CVD) comprise only 5% to 10% of patients in intensive care units, they consume approximately 50% of all ICU resources, as measured in staff time and equipment usage. Specifically, it has been estimated that weaning patients consumed about 41% of total ventilation time in intensive care unit patients. The economic cost of long term MV dependence is enormous. Episodes of long term MV

dependency can financially devastate families and health care institutions and are a financial drain on private insurers and government health care resources.

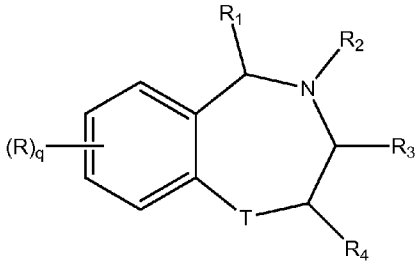
Typically the agents of the invention are named Rycals which are well known in the art (e.g. Andersson DC, Marks AR. Fixing ryanodine receptor Ca leak - a novel therapeutic strategy for contractile failure in heart and skeletal muscle. Drug Discov Today Dis Mech. 2010 Summer;7(2):e151-e157.; Marks AR. Calcium cycling proteins and heart failure: mechanisms and therapeutics. J Clin Invest. 2013 Jan 2;123(1):46-52. doi: 10.1172/JCI62834. Epub 2013 Jan 2. Review.).

One example of Rycal is S36 which has the formula I:



(I)

In certain embodiments, the Rycal is selected from the group consisting of compounds of the general Formula II:



(II)

wherein,

- T is O, CH2, NH, or $S=(O_2)_n$;
- n is 0, 1, or 2;
- q is 0, 1, 2, 3, or 4;
- each R is independently selected from the group consisting of H, halogen, -OH, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-SO_3H$, $-S(=O)_2$alkyl, -S(=O)alkyl, $-OS(=O)_2CF_3$, acyl, -O-acyl, alkyl, alkoxyl, alkylamino, alkylarylamino, alkylthio, cycloalkyl, alkylaryl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio, and (hetero-)arylamino; wherein each acyl, -O-

acyl, alkyl, alkoxyl, alkylamino, alkylarylamino, alkylthio, cycloalkyl, alkylaryl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-) arylthio, and (hetero-)arylamino may be optionally substituted;

- R_1 is selected from the group consisting of H, oxo, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted;
- R_2 is selected from the group consisting of H, $-C(=O)R_5$, $-C(=S)R_6$, $-SO_2R_7$, $-P(=O)R_8R_9$, $-(CH_2)m-R_{10}$, alkyl, aryl, alkylaryl, heteroaryl, cycloalkyl, cycloalkylalkyl, and heterocyclyl; wherein each alkyl, aryl, alkylaryl, heteroaryl, cycloalkyl, cycloalkylalkyl, and heterocyclyl may be optionally substituted and wherein m is 0, 1, 2, 3, or 4;
- R_3 is selected from the group consisting of H, $-CO_2Y$, $-C(=O)NHY$, acyl, -O-acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted; and wherein Y is selected from the group consisting of H, alkyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl, and wherein each alkyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted;
- R_4 is selected from the group consisting of H, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted;
- R_5 is selected from the group consisting of $-NR_{15}R_{16}$, $-(CH_2)zNR_{15}R_{16}$, $-NHNR_{15}R_{16}$, -NHOH, $-OR_{15}$, $-C(=O)NHNR_{15}R_{16}$, $-CO_2R_{15}$, $-C(=O)NR_{15}NR_{16}$, $-CH_2X$, acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted, and wherein z is 1, 2, 3, 4, 5, or 6;
- R_6 is selected from the group consisting of $-OR_{15}$, $-NHNR_{15}R_{16}$, -NHOH, $-NR_{15}R_{16}$, $-CH_2X$, acyl, alkenyl, alkyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted;
- R_7 is selected from the group consisting of $-OR_{15}$, $-NR_{15}R_{16}$, $-NHNR_{15}R_{16}$, -NHOH, alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl,

heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted;

- R_8 and R_9 independently are selected from the group consisting of OH, acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted;
- R_{10} is selected from the group consisting of $-NR_{15}R_{16}$, OH, $-SO_2R_{11}$, $-NHSO_2R_{11}$, $C(=O)(R_{12})$, $NHC=O(R_{12})$, $-OC=O(R_{12})$, and $-P(=O)R_{13}R_{14}$;
- R_{11}, R_{12}, R_{13}, and R_{14} independently are selected from the group consisting of H, OH, NH_2, $-NHNH_2$, -NHOH, acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted; X is selected from the group consisting of halogen, $-CN$, $-CO_2R_{15}$, $-C(=O)NR_{15}R_{16}$, $-NR_{15}R_{16}$, $-OR_{15}$, $-SO_2R_7$ and $-P(=O)R_8R_9$ and
- R_{15} and R_{16} independently are selected from the group consisting of H, acyl, alkenyl, alkoxyl, OH, NH_2, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted; and optionally R_{15} and R_{16} together with the N to which they are bonded may form a heterocycle which may be substituted; the nitrogen in the benzothiazepine ring may optionally be a quaternary nitrogen;

and enantiomers, diastereomers, tautomers, pharmaceutically acceptable salts, hydrates, solvates, complexes, and prodrugs thereof, or any combination thereof.

The term "alkyl" as used herein refers to a linear or branched, saturated hydrocarbon and preferably one having from 1 to 6 carbon atoms. Representative alkyl groups include, but are not limited to, methyl, ethyl, propyl, isopropyl, butyl, sec-butyl, tert-butyl, pentyl, isopentyl, neopentyl, hexyl, isohexyl, and neohexyl. The term "C_1-C_4 alkyl" refers to a

straight or branched chain alkane (hydrocarbon) radical containing from 1 to 4 carbon atoms, such as methyl, ethyl, propyl, isopropyl, n-butyl, t-butyl, and isobutyl.

The term "alkenyl" as used herein refers to a linear or branched hydrocarbon and preferably one having from 2 to 6 carbon atoms and having at least one carbon-carbon double bond. In one embodiment, the alkenyl has one or two double bonds. The alkenyl moiety may exist in the E or Z conformation and the compounds of the present invention include both conformations.

The term "alkynyl" as used herein refers to a linear or branched hydrocarbon and preferably one having from 2 to 6 carbon atoms and having at least one carbon-carbon triple bond.

The term "aryl" as used herein refers to an aromatic group and preferably one containing 1 to 3 aromatic rings, either fused or linked.

The term "cyclic group" as used herein includes a cycloalkyl group and a heterocyclic group.

The term "cycloalkyl group" as used herein refers to a three- to seven-membered saturated or partially unsaturated carbon ring. Any suitable ring position of the cycloalkyl group may be covalently linked to the defined chemical structure. Non-limiting examples of cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and cycloheptyl.

The term "halogen" as used herein refers to fluorine, chlorine, bromine, and iodine.

The term "heterocyclic group" or "heterocyclic" or "heterocyclyl" or "heterocyclo" as used herein refers to fully saturated, or partially or fully unsaturated, including aromatic (i.e., "heteroaryl") cyclic groups (for example, 4 to 7 membered monocyclic, 7 to 11 membered bicyclic, or 10 to 16 membered tricyclic ring systems) which have at least one heteroatom in at least one carbon atom-containing ring. Each ring of the heterocyclic group containing a heteroatom may have 1, 2, 3, or 4 heteroatoms selected from nitrogen atoms, oxygen atoms and/or sulfur atoms, where the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatoms may optionally be quaternized. The heterocyclic group may be

attached to the remainder of the molecule at any heteroatom or carbon atom of the ring or ring system. Non-limiting examples of heterocyclic groups include, but are not limited to, azepanyl, azetidinyl, aziridinyl, dioxolanyl, furanyl, furazanyl, homo piperazinyl, imidazolidinyl, imidazolinyl, isothiazolyl, isoxazolyl, morpholinyl, oxadiazolyl, oxazolidinyl, oxazolyl, oxazolidinyl, pyrimidinyl, phenanthridinyl, phenanthrolinyl, piperazinyl, piperidinyl, pyranyl, pyrazinyl, pyrazolidinyl, pyrazolinyl, pyrazolyl, pyridazinyl, pyridooxazolyl, pyridoimidazolyl, pyridothiazolyl, pyridinyl, pyrimidinyl, pyrrolidinyl, pyrrolinyl, quinuclidinyl, tetrahydrofuranyl, thiadiazinyl, thiadiazolyl, thienyl, thienothiazolyl, thienooxazolyl, thienoimidazolyl, thiomorpholinyl, thiophenyl, triazinyl, and triazolyl. Non- limiting examples of bicyclic heterocyclic groups include indolyl, isoindolyl, benzothiazolyl, benzoxazolyl, benzoxadiazolyl, benzothienyl, quinuclidinyl, quinolinyl, tetrahydroisoquinolinyl, isoquinolinyl, benzimidazolyl, benzopyranyl, indolizinyl, benzofuryl, benzofurazanyl, chromonyl, coumarinyl, benzopyranyl, cinnolinyl, quinoxalinyl, indazolyl, pyrrolopyridyl, furopyridinyl (such as furo[2,3-c]pyridinyl, furo[3,2-b]pyridinyl] or furo[2,3-b]pyridinyl), dihydroisoindolyl, dihydroquinazolinyl (such as 3,4-dihydro-4-oxo-quinazolinyl), triazinylazepinyl, tetrahydroquinolinyl and the like. Non-limiting examples of tricyclic heterocyclic groups include carbazolyl, benzidolyl, phenanthrolinyl, acridinyl, phenanthridinyl, xanthenyl and the like.

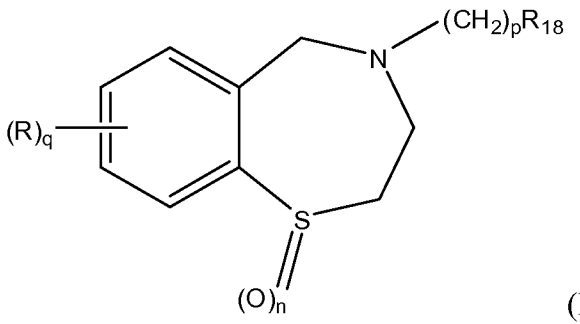
The term "phenyl" as used herein refers to a substituted or unsubstituted phenyl group.

The aforementioned terms "alkyl," "alkenyl," "alkynyl," "aryl," "acyl," "phenyl," "cyclic group," "cycloalkyl," "heterocyclyl," "heterocyclo," and "heterocycle" may further be optionally substituted with one or more substituents. Non- limiting examples of substituents include but are not limited to one or more of the following groups: hydrogen, halogen, CF_3, OCF_3, cyano, nitro, N_3, oxo, cycloalkyl, alkenyl, alkynyl, heterocycle, aryl, alkylaryl, heteroaryl, OR_a, SR_a, $S(=O)R_e$, $S(=O)_2R_e$, $P(=O)_2R_e$, $S(=O)_2OR_a$, $P(=O)_2OR_a$, NR_bR_c, $NR_bS(=O)_2Re$, $NR_bP(=O)2Re$, $S(=O)2\ NR_bRc$, $P(=O)2NR_bNR_c$, $C(=O)OR_a$, $C(=O)R_a$, $C(=O)NR_bR_c$, $OC(=O)R_a$, $OC(=O)NR_bR_c$, $NR_bC(=O)OR_a$, $NR_dC(=O)NR_bR_c$, $NR_dS(=O)_2NR_bR_c$, $NR_dP(=O)_2NR_bR_c$, $NR_bC(=O)R_a$, or $NR_bP(=O)_2R_e$, wherein R_a is hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, alkylaryl, heteroaryl, heterocycle, or aryl; R_b, R_c and R_d are independently hydrogen, alkyl, cycloalkyl, alkylaryl, heteroaryl, heterocycle, aryl, or said R_b and R_c together with the N to which they are bonded optionally form a heterocycle; and R_e is alkyl, cycloalkyl, alkenyl, cycloalkenyl, alkynyl, alkylaryl, heteroaryl, heterocycle,

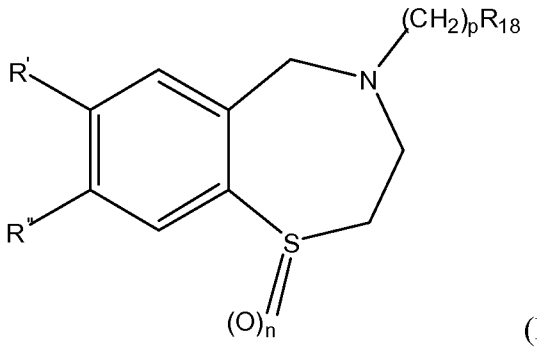
or aryl. In the aforementioned representative substitutents, groups such as alkyl, cycloalkyl, alkenyl, alkynyl, cycloalkenyl, alkylaryl, heteroaryl, heterocycle and aryl can themselves be optionally substituted.

The term "quaternary nitrogen" refers to a tetravalent positively charged nitrogen atom including, for example, the positively charged nitrogen in a tetraalkylammonium group (e.g., tetramethylammonium, N-methylpyridinium), the positively charged nitrogen in protonated ammonium species (e.g., trimethyl-hydroammonium, N-hydropyridinium), the positively charged nitrogen in amine N-oxides (e.g., N-methyl-morpholine-N-oxide, pyridine-N-oxide), and the positively charged nitrogen in an N-amino-ammonium group (e.g., N-aminopyridinium).

In other embodiments, the Rycal is selected form the group consisting of compounds of formula II-k or II-k-1:



(II-k)



(II-k-1)

wherein:

- R, R' and R" are independently selected from the group consisting of H, halogen, -OH, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-SO_3H$, $-S(=O)_2$alkyl, -S(=O)alkyl, $-OS(=O)_2CF_3$, acyl, alkyl, alkoxyl, alkylamino, alkylthio, cycloalkyl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio, and (hetero-)arylamino; and wherein each acyl, alkyl, alkoxyl, alkylamino, cycloalkyl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio may be substituted or unsubstituted;

- R_{18} is selected from the group consisting of H, $-NR_{15}R_{16}$, $-C(=O)NR_{15}R_{16}$, $-(C=O)OR_{15}$, $-OR_{15}$, alkyl, aryl, cycloalkyl, heterocyclyl, and at one labeling group; wherein each alkyl, aryl, cycloalkyl, and heterocyclyl may be substituted or unsubstituted;

and further wherein:

- q is 0, 1, 2, 3, or 4;
- p is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- n is 0, 1, or 2;

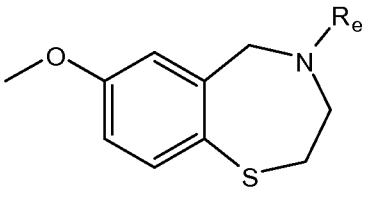
-and enantiomers, diastereomers, tautomers, pharmaceutically acceptable salts, hydrates, solvates, complexes and prodrugs thereof.

In certain embodiments, the present invention provides compounds of formula II-k, wherein each R is independently selected from the group consisting of H, halogen, -OH, -OMe, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-S(=O)_2C_1$-C_4alkyl, $-S(=O)C_1$-C_4alkyl, $-S$-C_1-C_4alkyl, $-OS(=O)_2CF_3$, Ph, $-NHCH_2Ph$, -C(=O)Me, -OC(=O)Me, morpholinyl and propenyl; and n is 0, 1 or 2. In some cases, R is H or is OMe at position 7 of the benzothiazepine ring.

In certain embodiments, the present invention provides compounds of formula II-k-1, wherein R' and R" are independently selected from the group consisting of H, halogen, -OH, -OMe, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-S(=O)_2C_1$-C_4alkyl, $-S(=O)C_1$-C_4alkyl, $-S$-C_1-C_4alkyl, $-OS(=O)_2CF_3$, Ph, $-NHCH_2Ph$, -C(=O)Me, -OC(=O)Me, morpholinyl and propenyl; and n is 0, 1 or 2. In some cases, R' is H or OMe, and R" is H.

In other embodiments, the present invention provides compounds of formula II-k or II-k-1, wherein R_{18} is selected from the group consisting of H, $-NR_{15}R_{16}$, $-(C=O)OR_{15}$, $-OR_{15}$, alkyl, aryl, and at one labeling group; and wherein each alkyl and aryl may be substituted or unsubstituted. In some cases, m is 1, and R_{18} is Ph, C(=O)OMe, C(=O)OH, aminoalkyl, NH_2, NHOH, or NHCbz. In other cases, m is 0, and R_{18} is C_1-C_4 alkyl, such as Me, Et, propyl, and butyl. In yet other cases, m is 2, and R_{18} is pyrrolidine, piperidine, piperazine, or morpholine. In some embodiments, m is 3, 4, 5, 5, 7, or 8, and R_{18} is a fluorescent labeling group selected from bodipy, dansyl, fluorescein, rhodamine, Texas red, cyanine dyes, pyrene, coumarins, Cascade Blue™, Pacific Blue, Marina Blue, Oregon Green, 4',6-Diamidino-2-phenylindole (DAPI), indopyra dyes, lucifer yellow, propidium iodide, porphyrins, arginine, and variants and derivatives thereof.

In certain other embodiments, the invention provides compounds of Formula II-o:

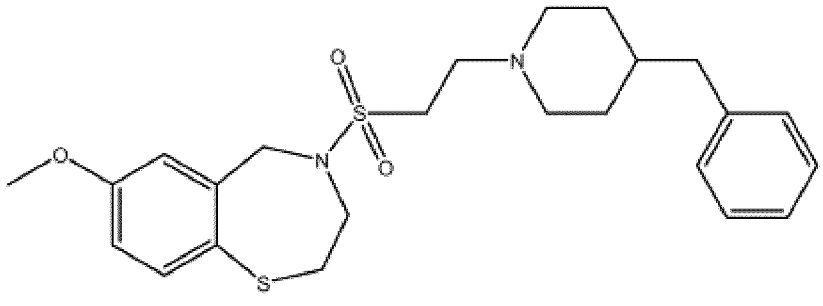


(II-o)

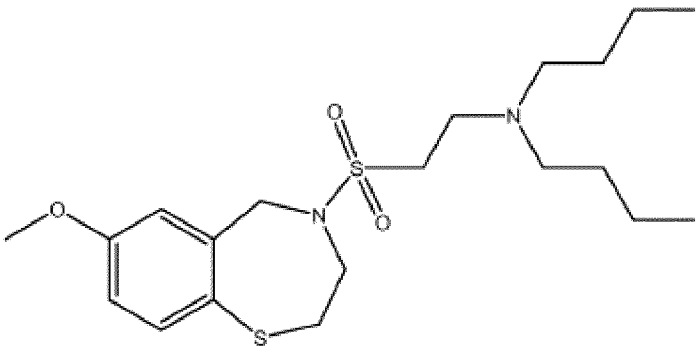
wherein:

- R_e is -(C_1-C_6 alkyl)-phenyl, -(C_1-C_6 alkyl)-C(O)R_b, or substituted or unsubstituted -C_1-C_6 alkyl; and
- R_b is -OH or -O-(C_1-C_6 alkyl), and
- and wherein the phenyl or substituted alkyl is substituted with one or more of halogen, hydroxyl, -C_1-C_6 alkyl, -O-(C_1-C_6 alkyl), -NH_2, -NH(C_1-C_6 alkyl), -N(C_1-C_6 alkyl)$_2$, cyano, or dioxolane.

In some embodiments, the Rycal of Formula II, II-k, II-k-1 or II-o is selected from the group consisting of SI, S2, S3, S4, S5, S6, S7, S9, S11, S12, S13, S14, S19, S20, S22, S23, S24, S25, S26, S27, S36, S37, S38, S40, S43, S44, S45, S46, S47, S48, S49, S50, S51, S52, S53, S54, S55, S56, S57, S58, S59, S60, S61, S62, S63, S64, S66, S67, S68, S69, S70, S71, S72, S73, S74, S75, S76, S77, S78, S79, S80, S81, S82, S83, S84, S85, S86, S87, S88, S89, S90, S91, S92, S93, S94, S95, S96, S97, S98, S99, S100, S101, S102, S103, S104, S107, S108, S109, S110, S111, S112, S113, S114, S115, S116, S117, S118, S119, S120, S121, S122, S123, S136, S137, S138, S139, S140, S146, S147, S148, S149, S150, S151, S152, S153, S156, S157, S159, S160, S161, S166, S167, S182, S186, S189, S203, S217, S251, S252, S258, S277, S279, S282, S291, S293, S296, S301, S302, S306, S311, S312, S313, S318, S322, S324, S326, S331, S335, S337, S351, S352, S353, S354, S397, S398, S399, S423, S454, S463, S466, S470, S473, S477 and salts thereof. The structures of these agents are provided in the Detailed Description of the International Patent Application WO2012/037105 and as follows:.



S1



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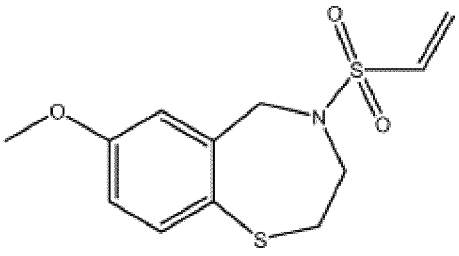

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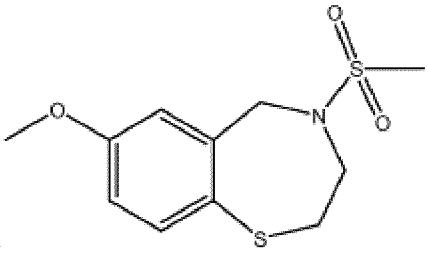

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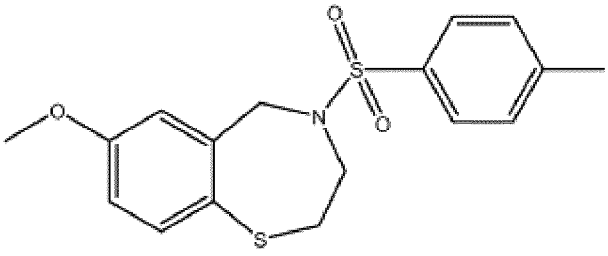

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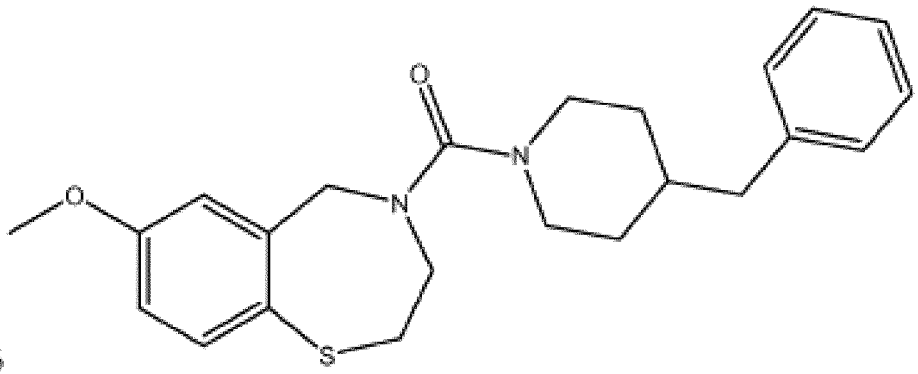

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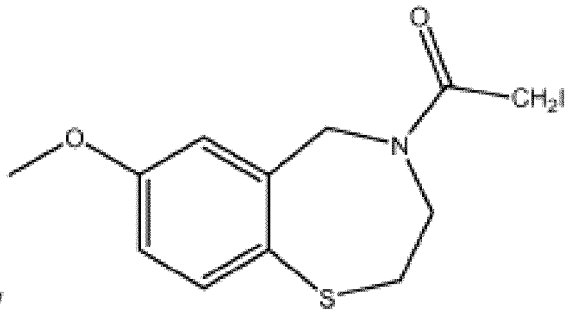

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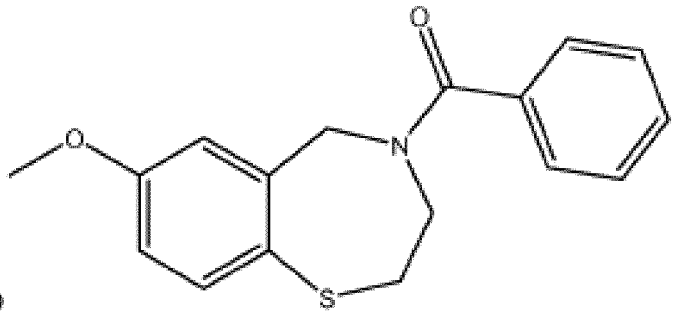

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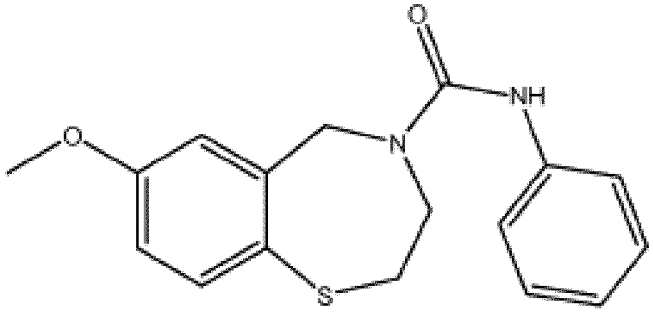

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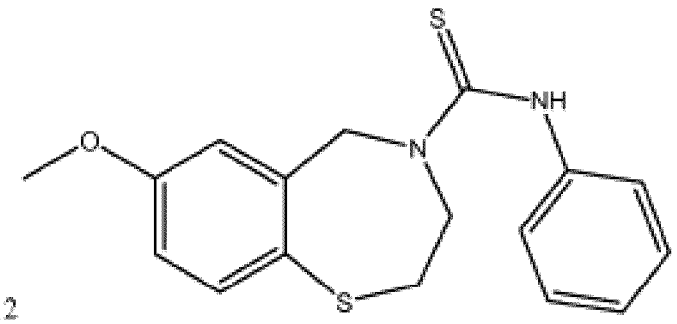

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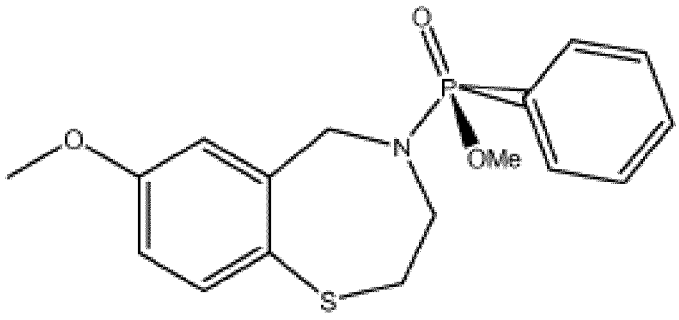

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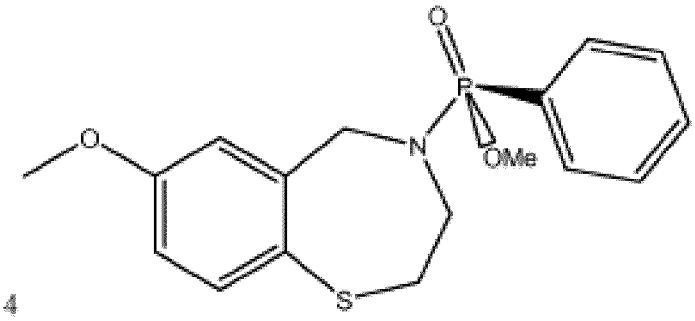

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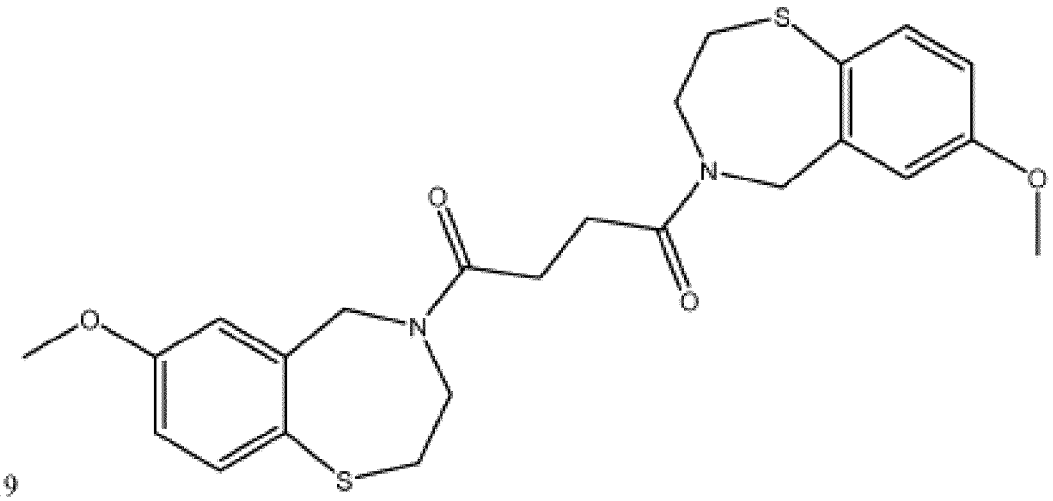

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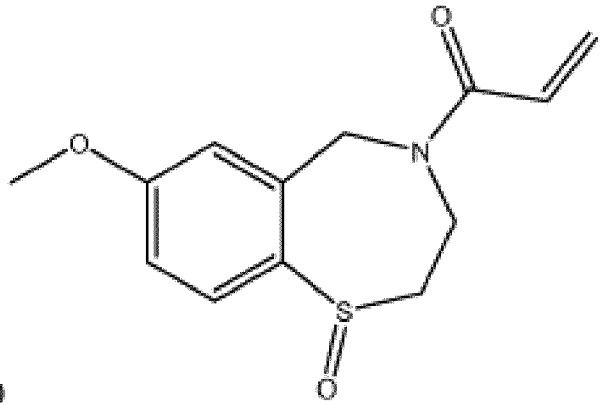

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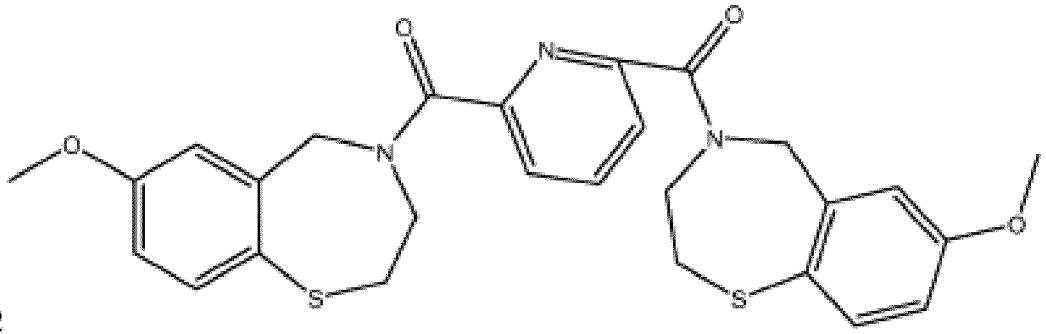

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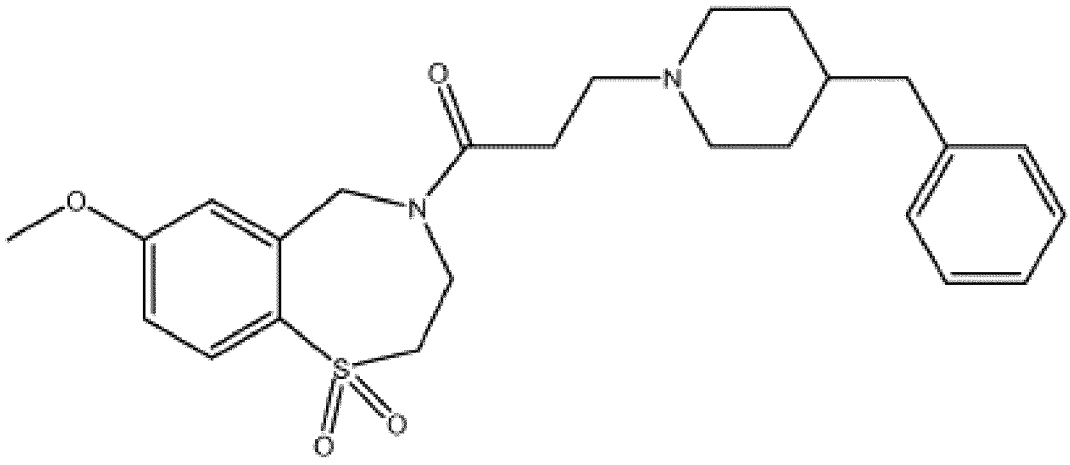
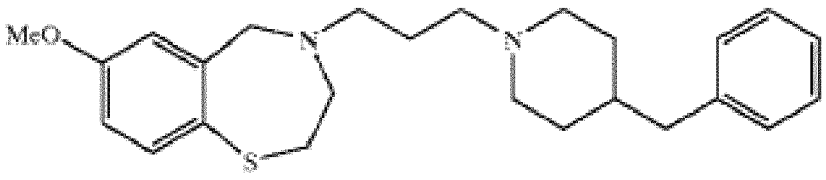


S23

S-24



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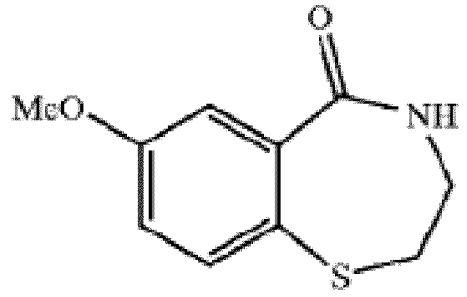

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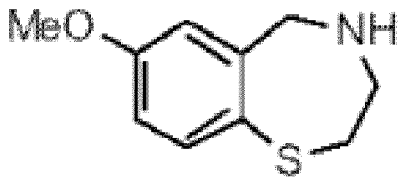

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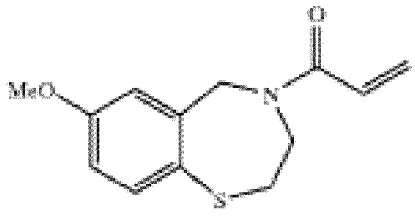

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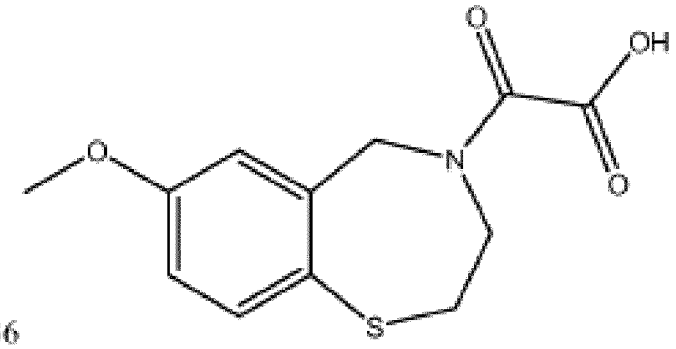

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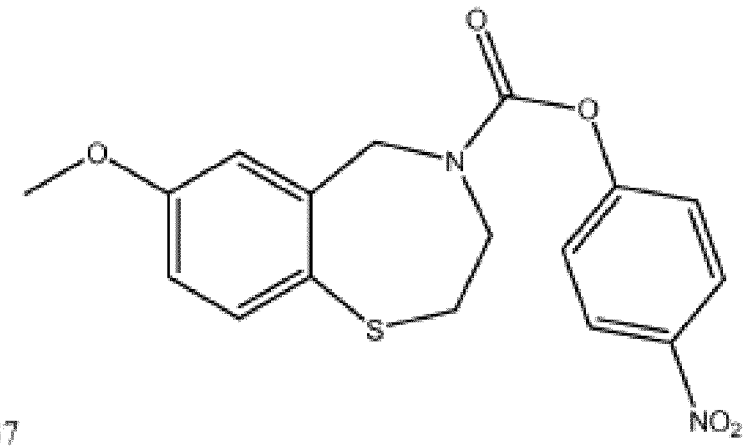

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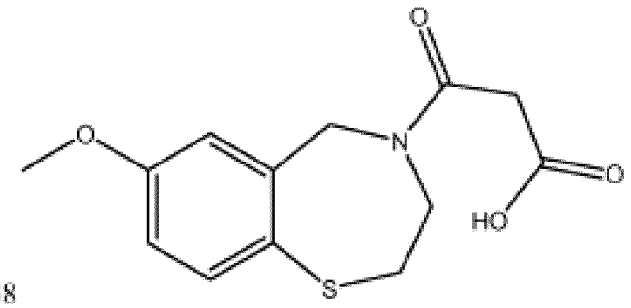

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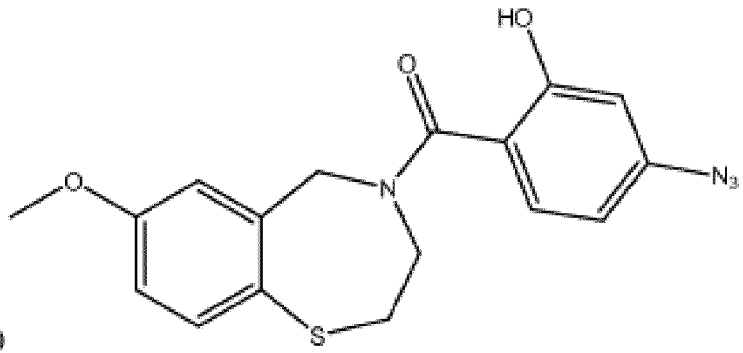

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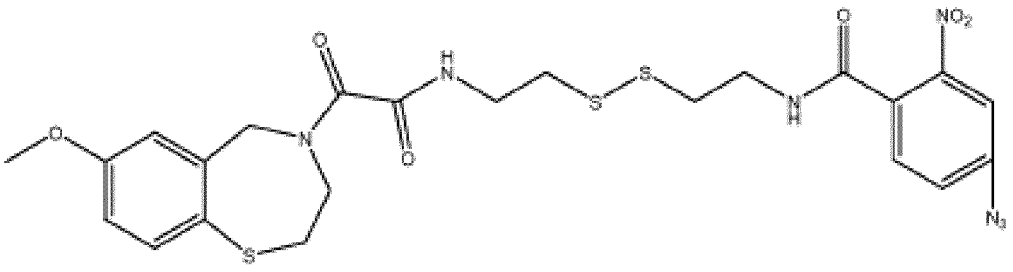

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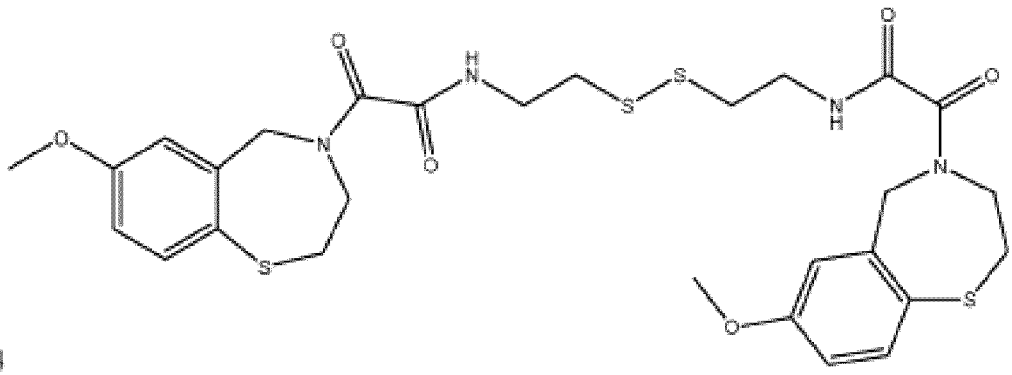

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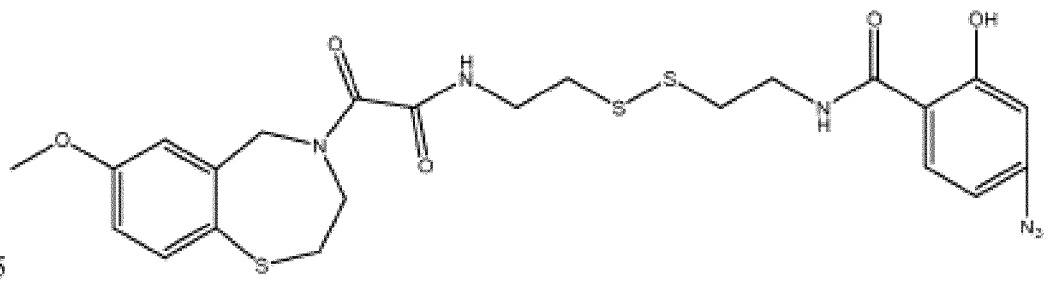

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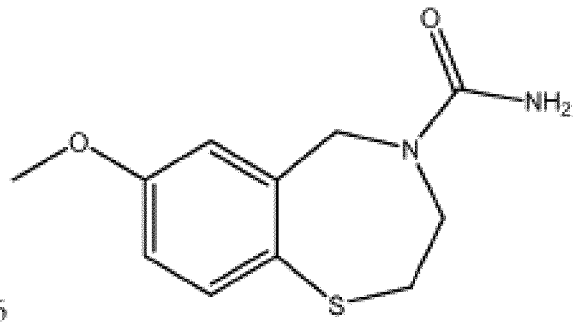

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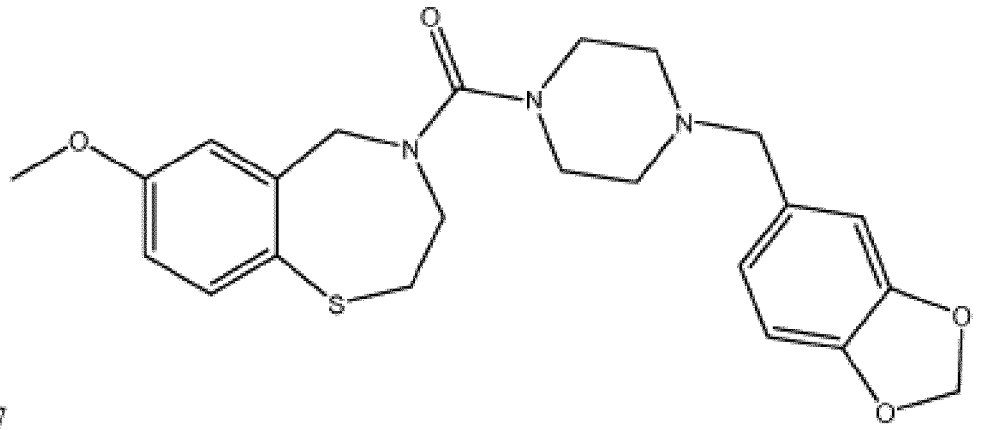

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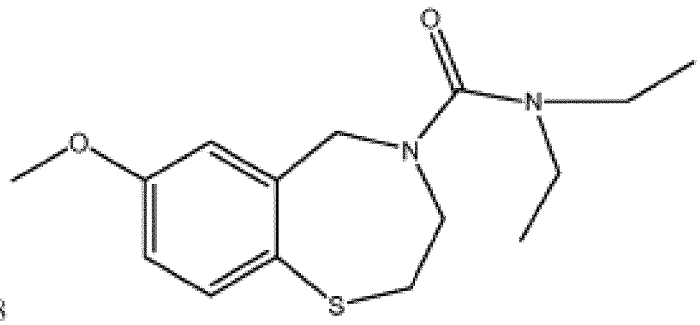

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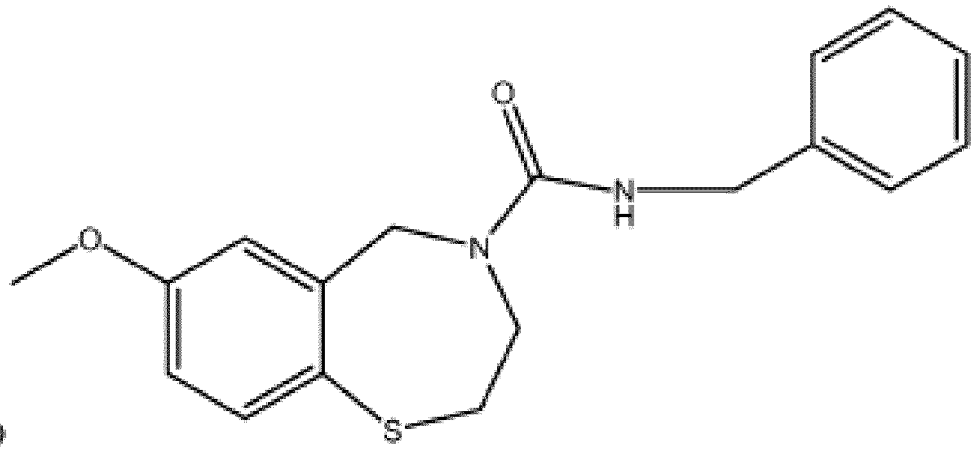

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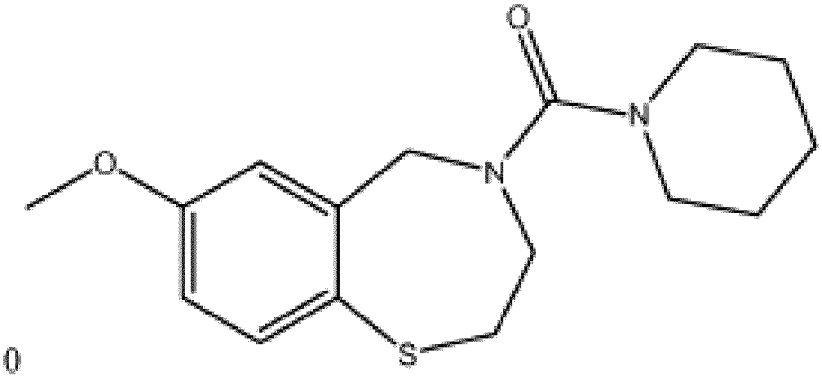

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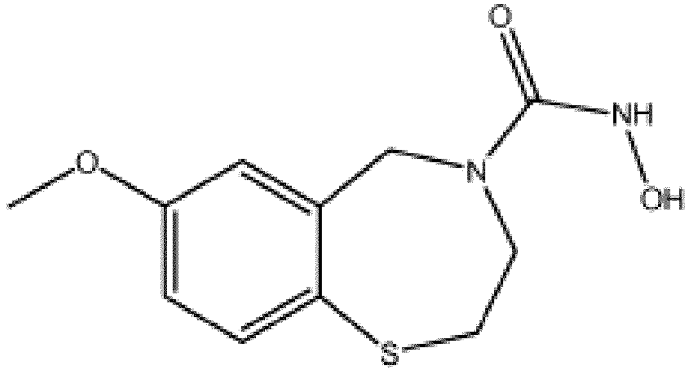

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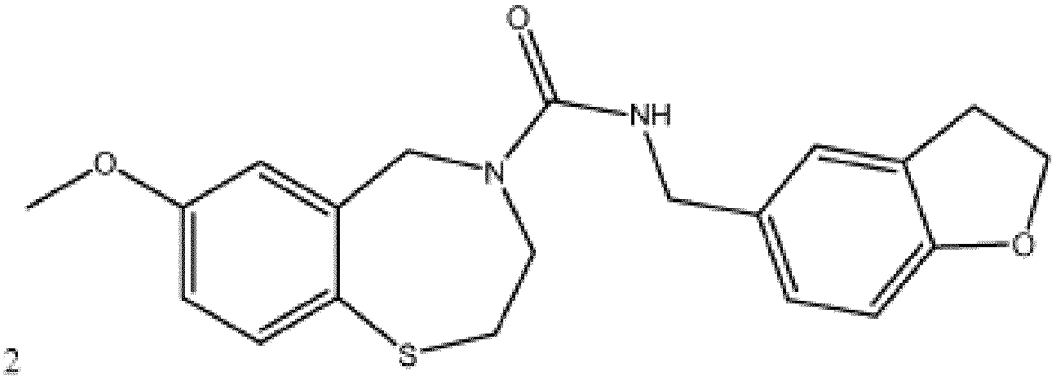
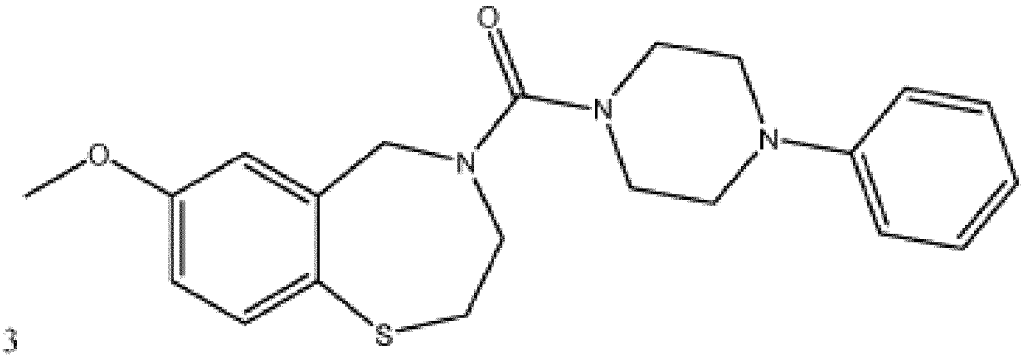


S52



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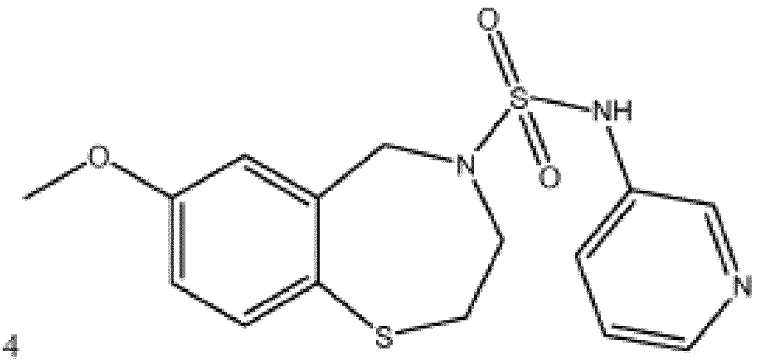

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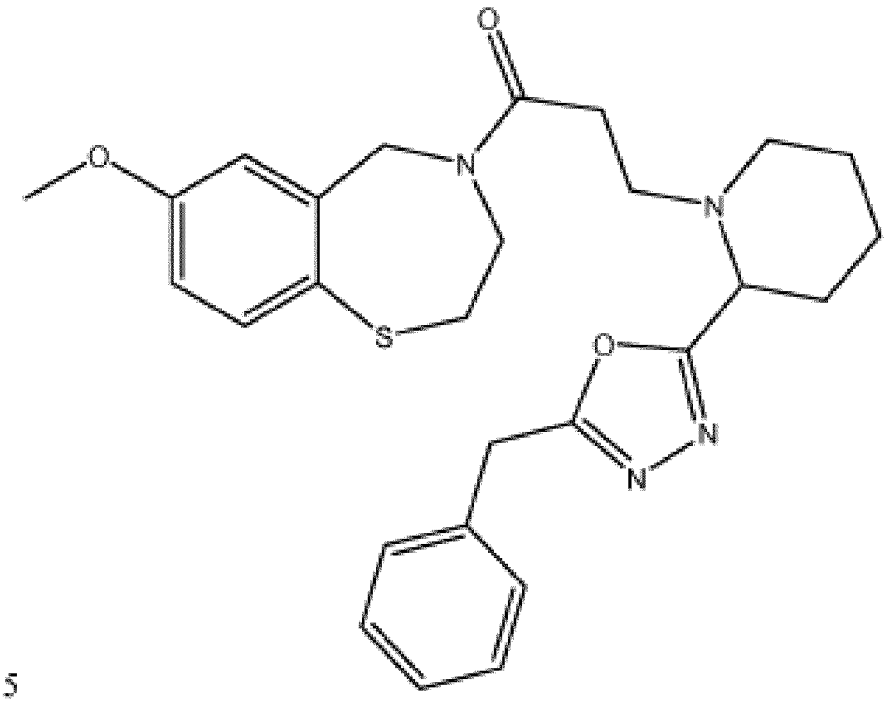

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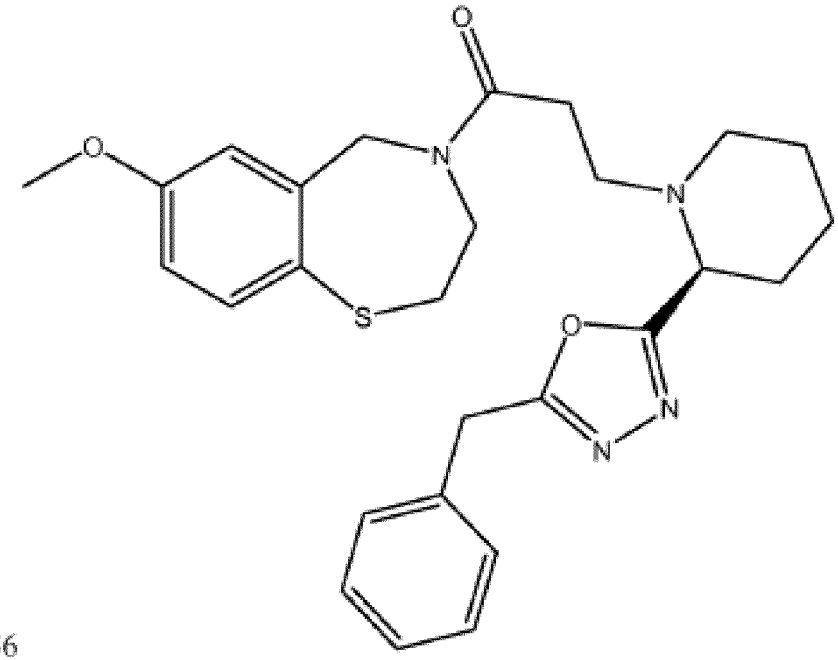

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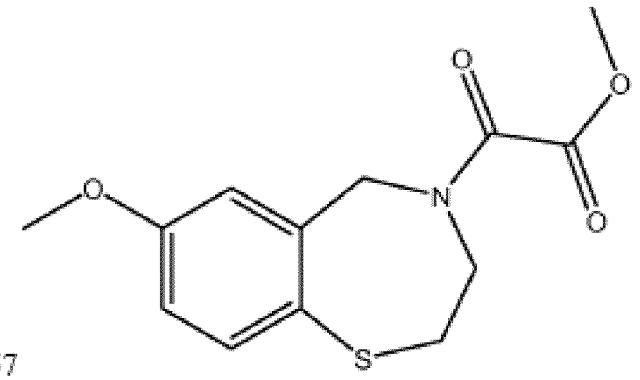

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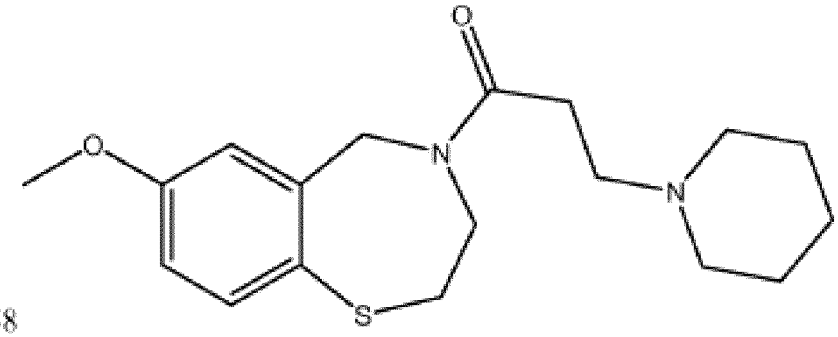

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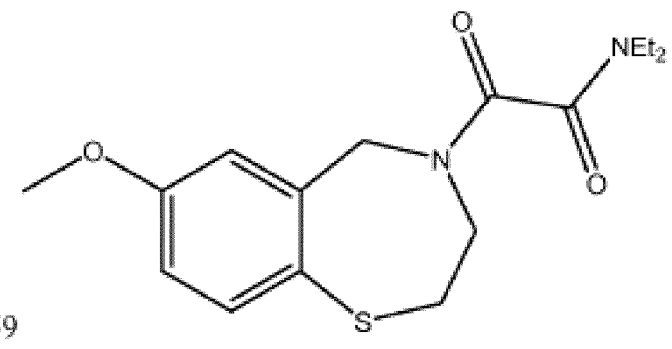

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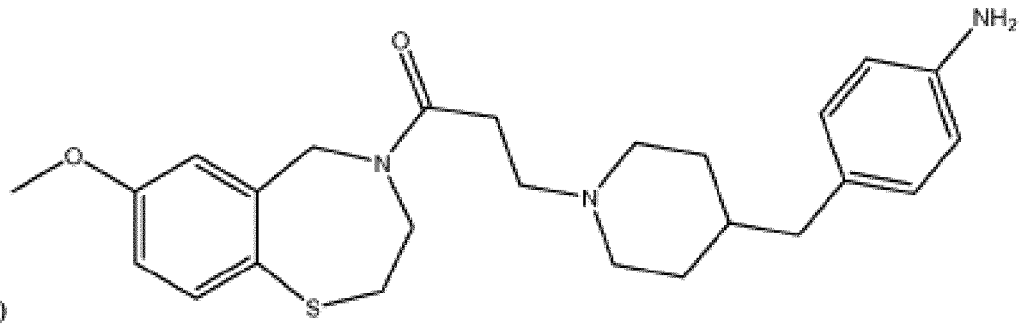

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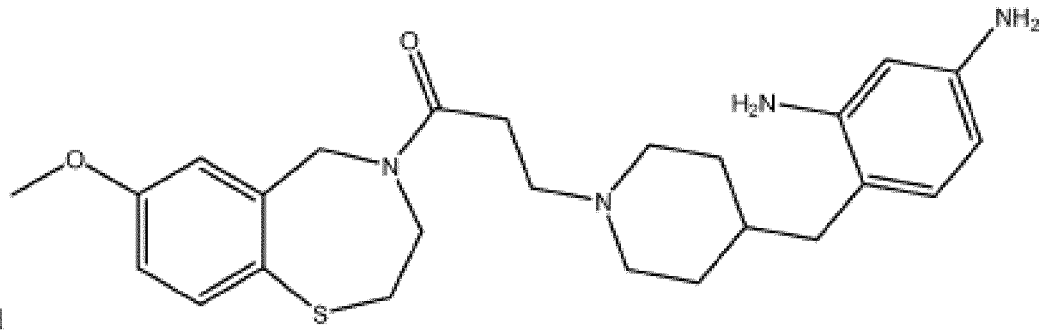

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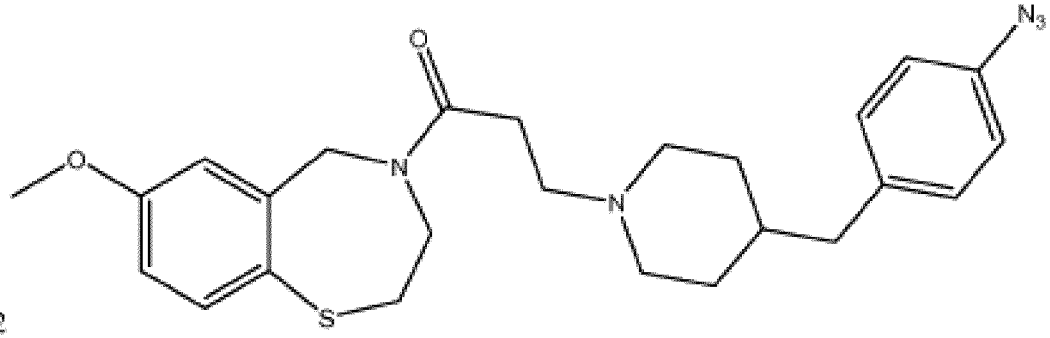

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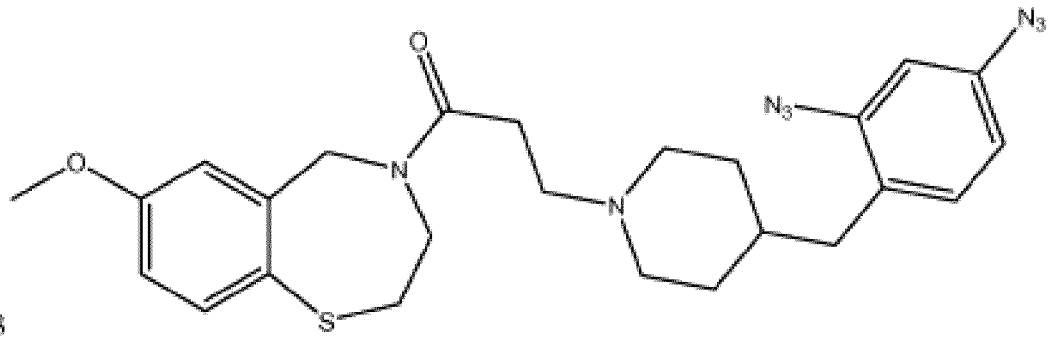

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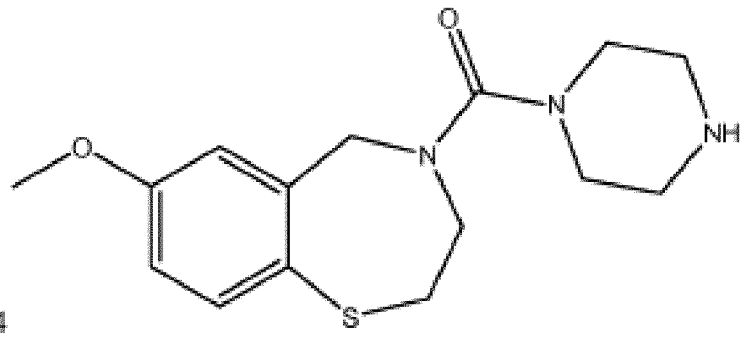

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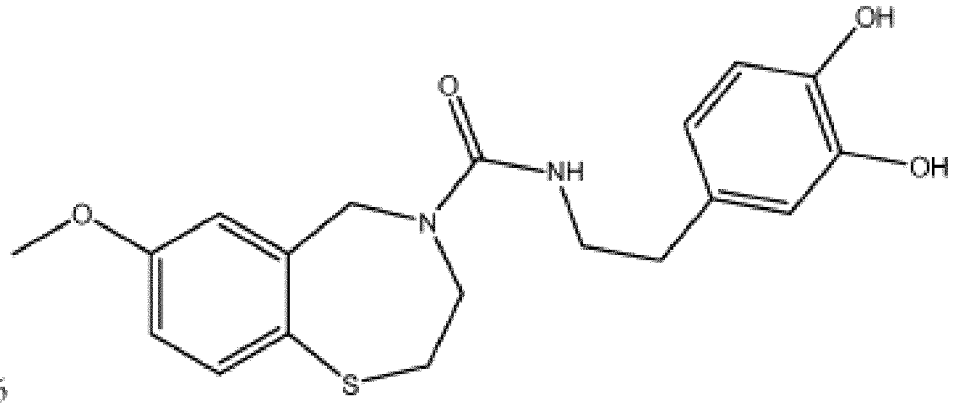

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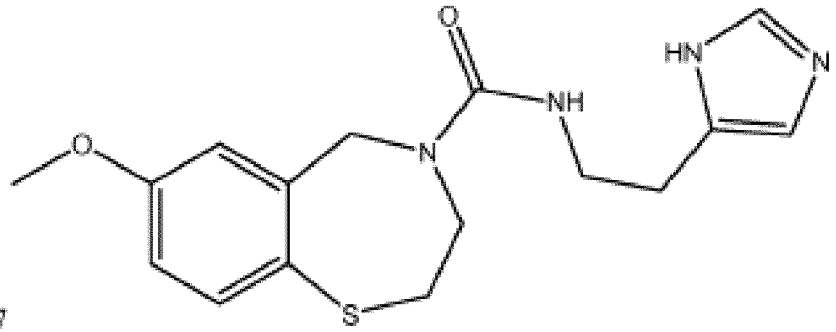

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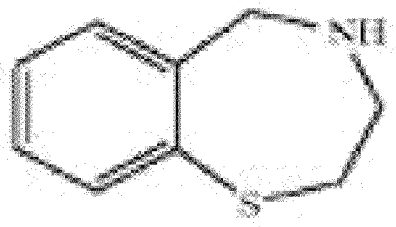

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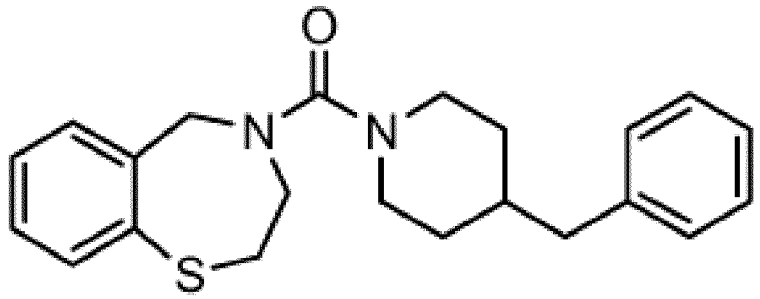

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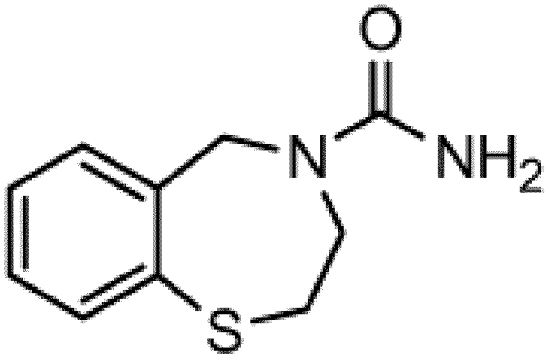

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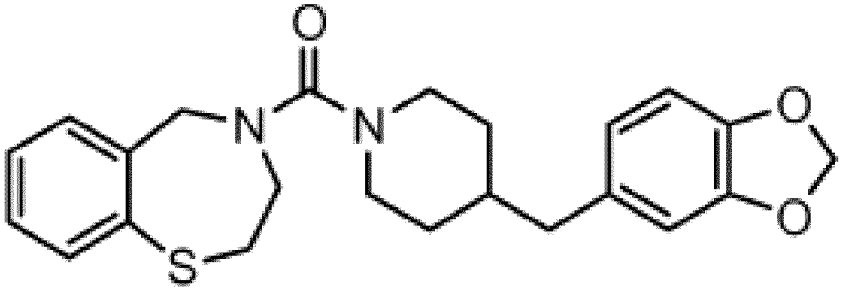

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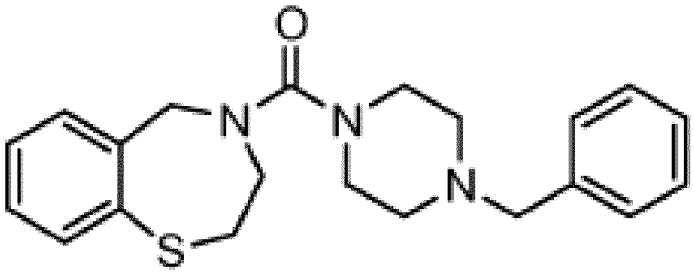

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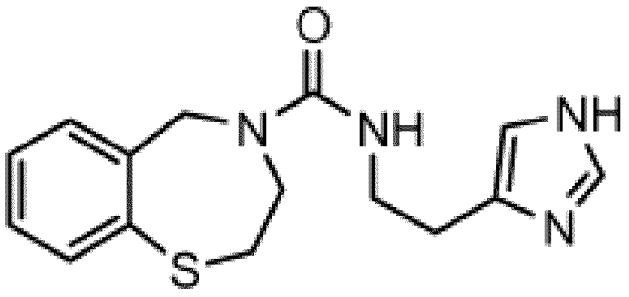

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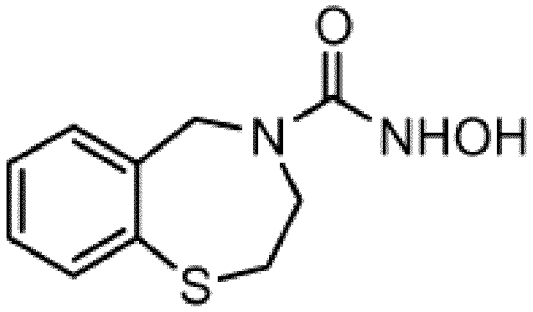

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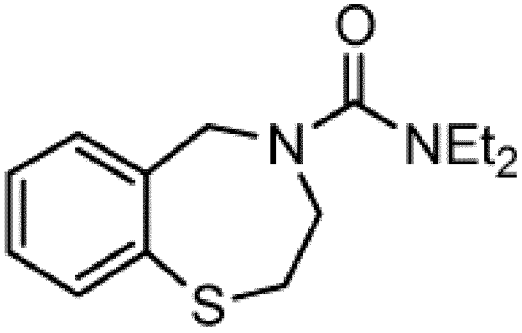

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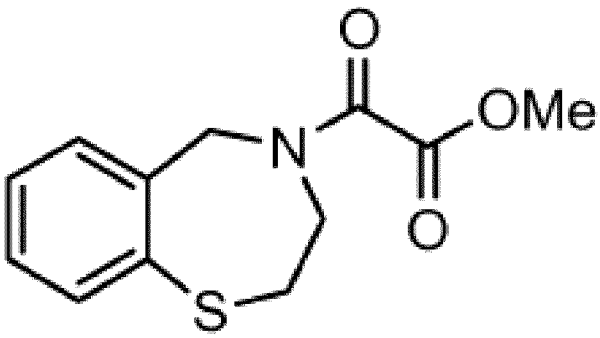

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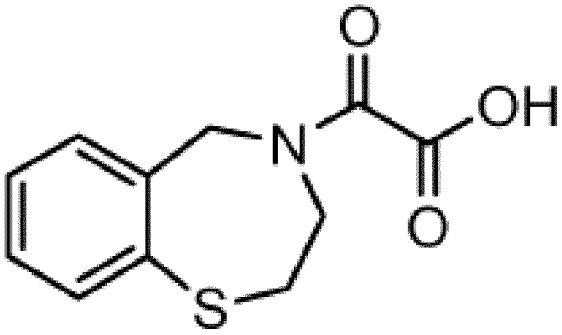

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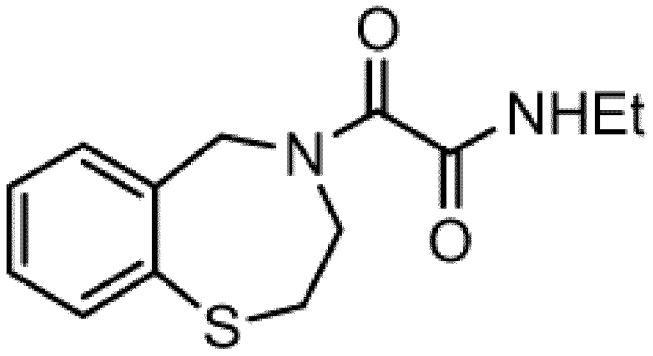

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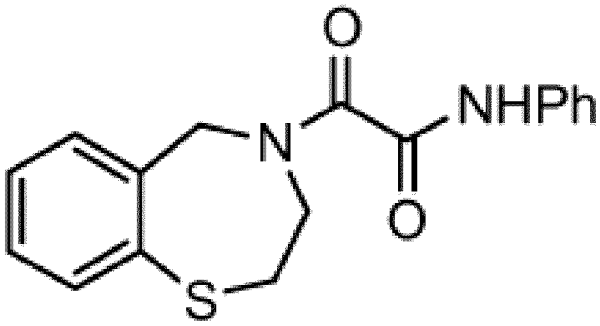

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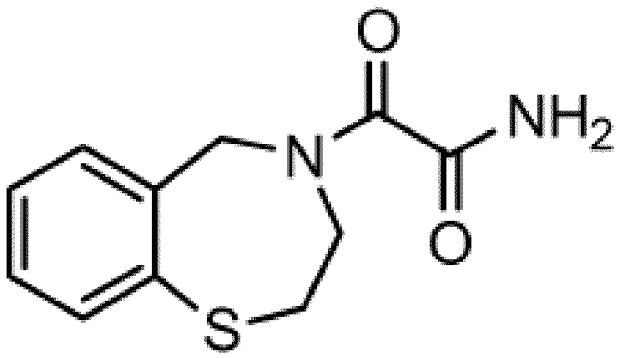

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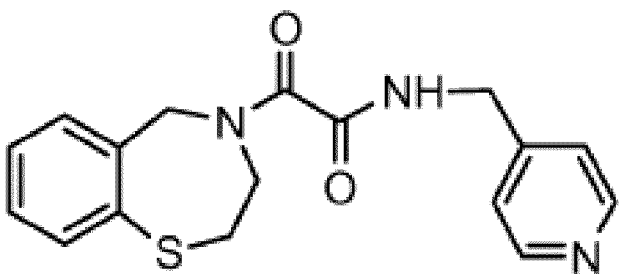

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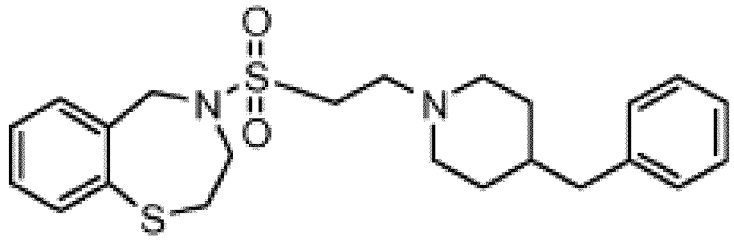

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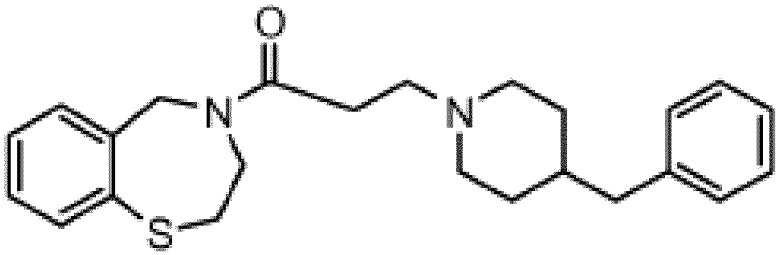

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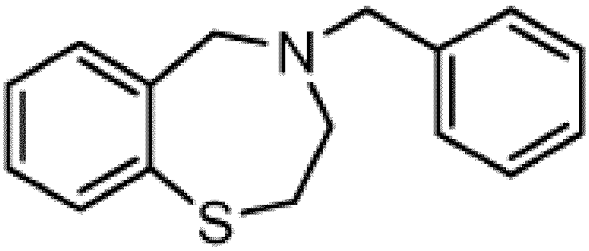

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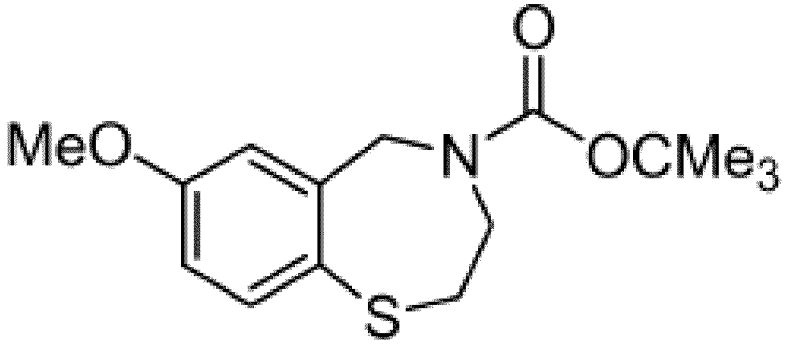

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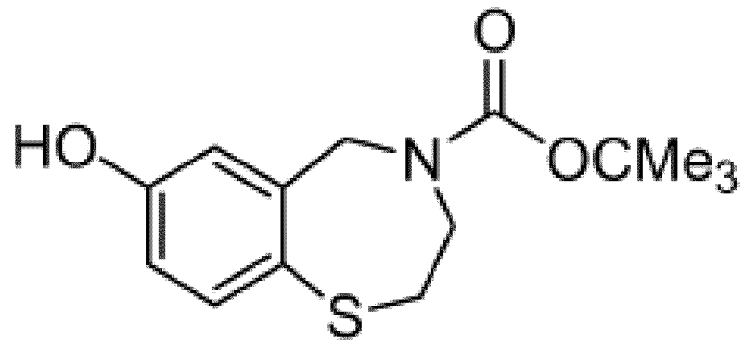

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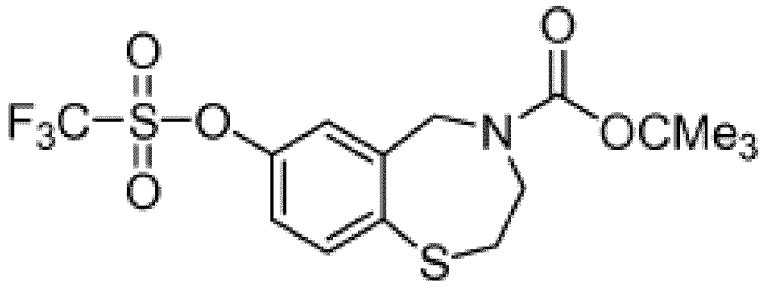

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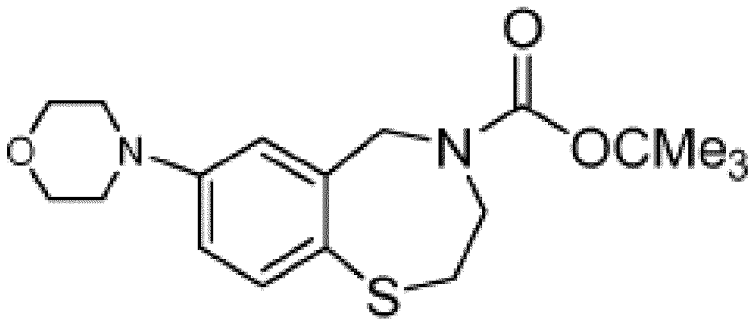

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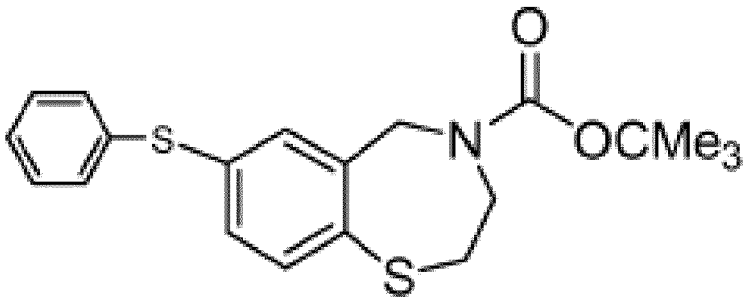

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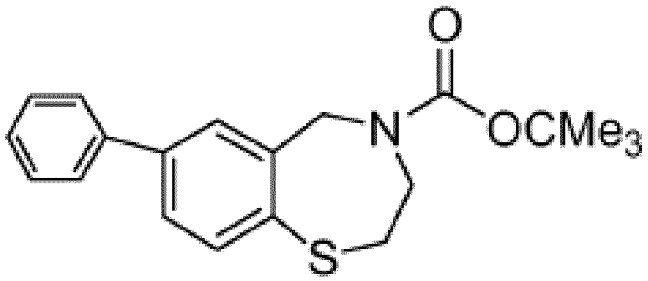

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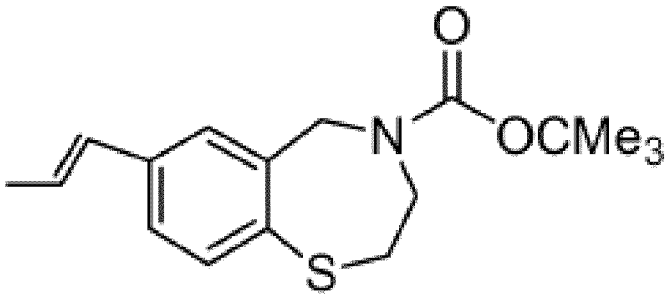

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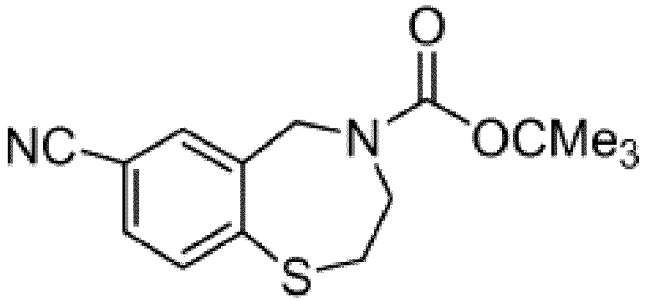

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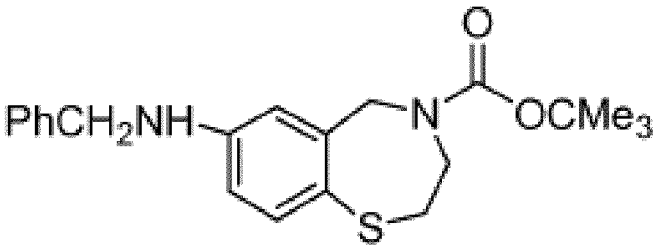

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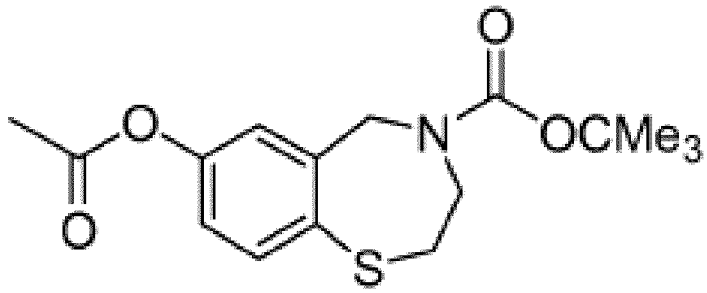

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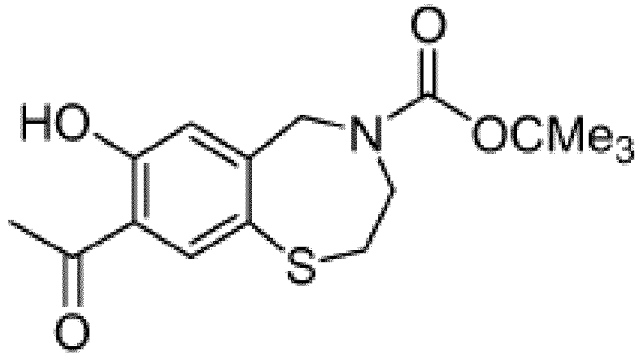

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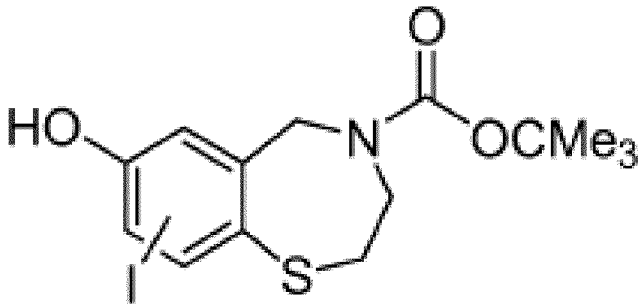

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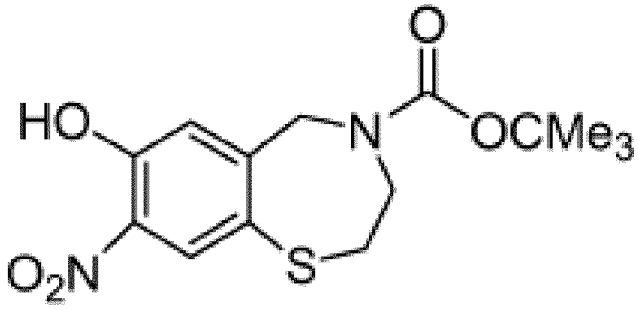

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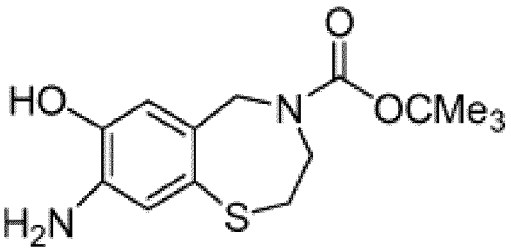

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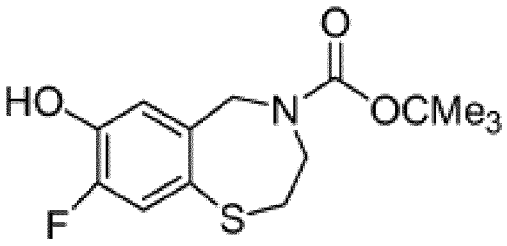

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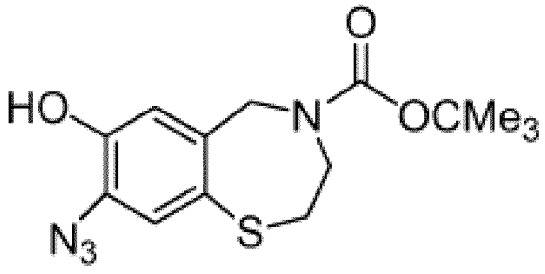

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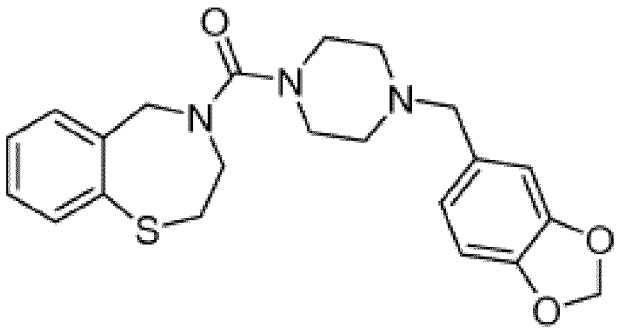

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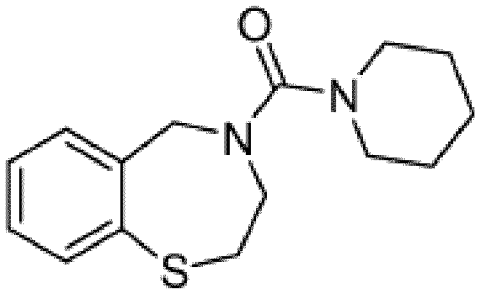

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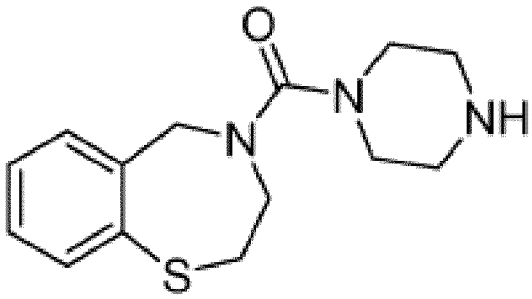

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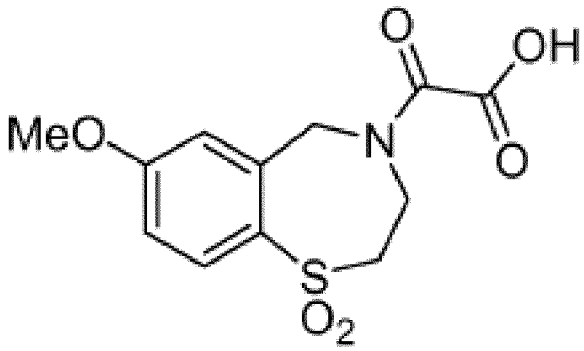

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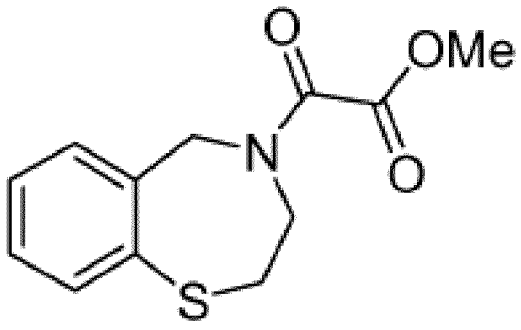

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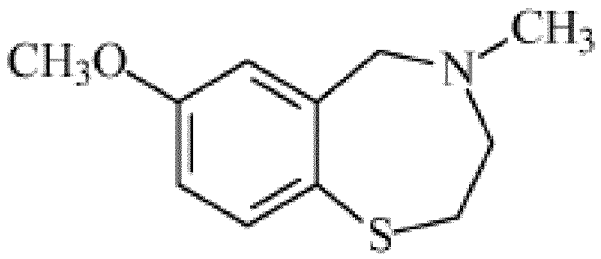

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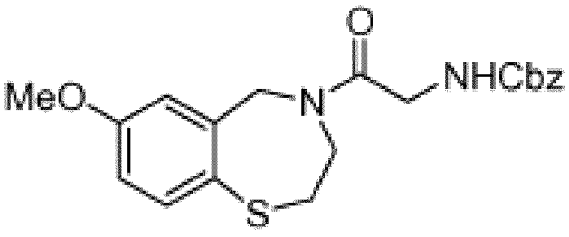

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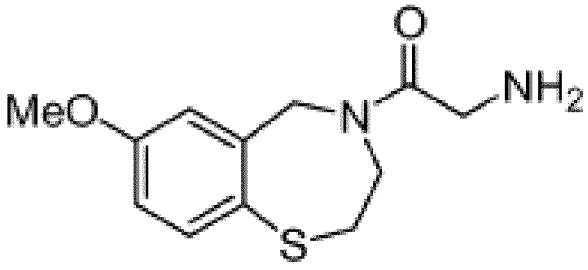

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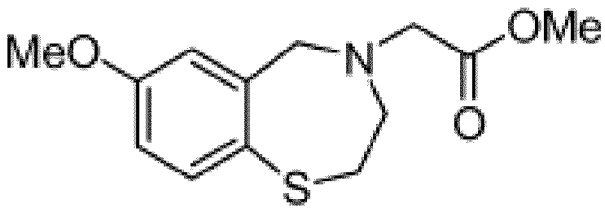

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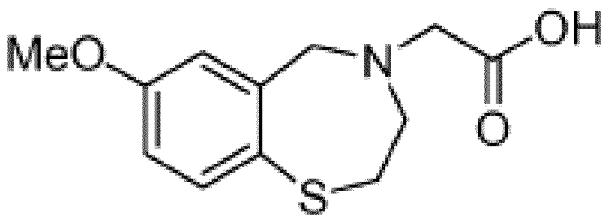

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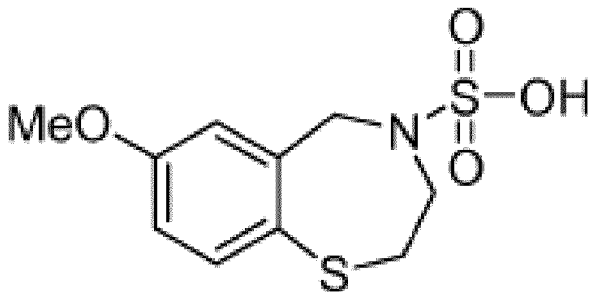

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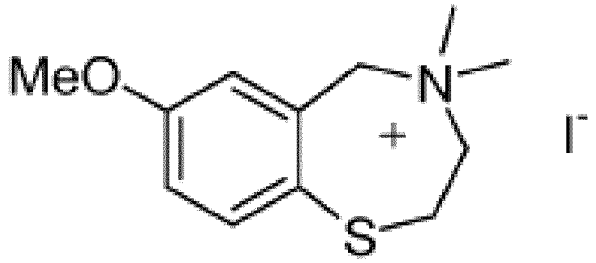

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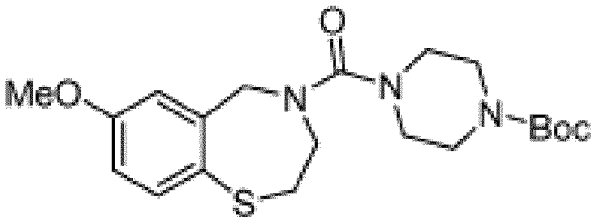

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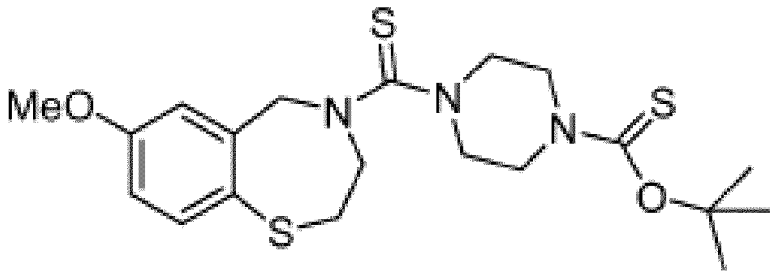

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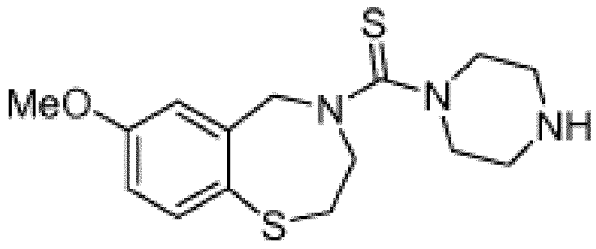

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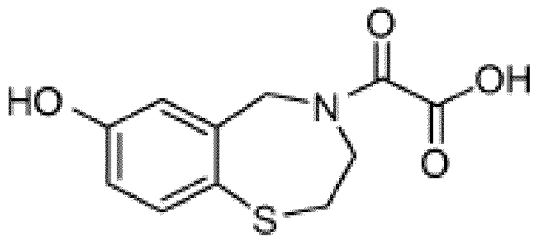

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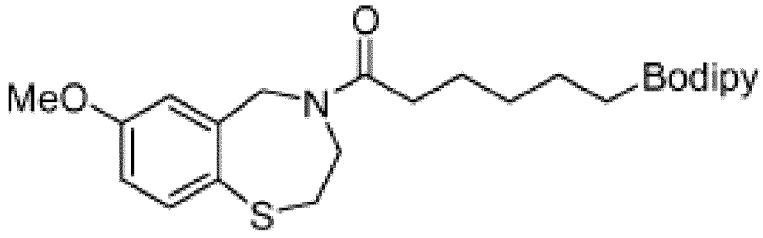

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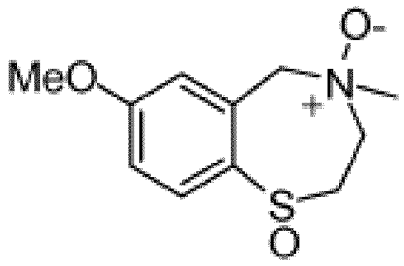

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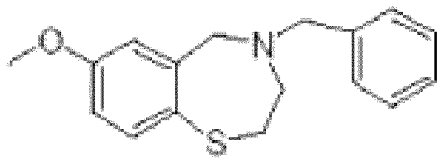

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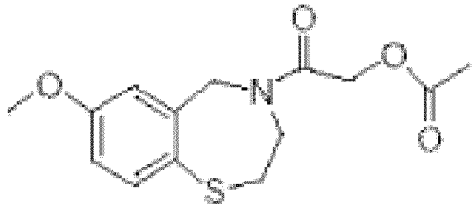

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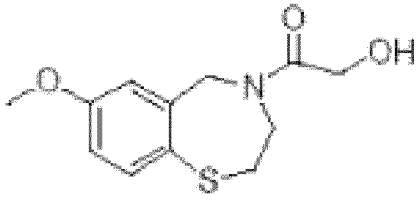

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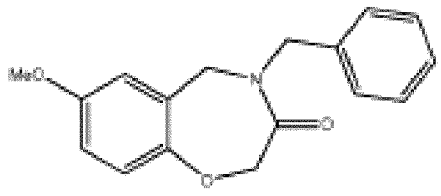

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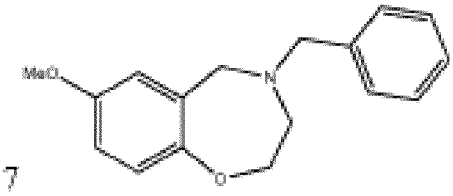

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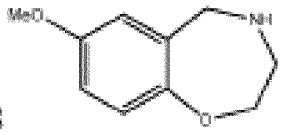

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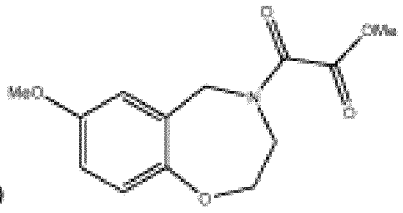

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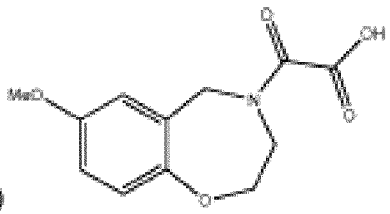

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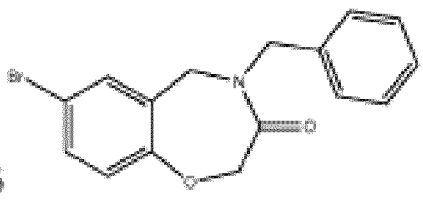

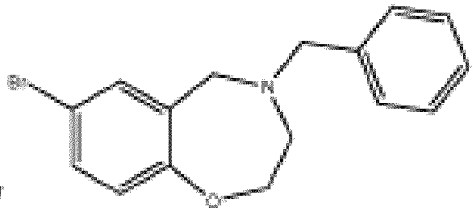

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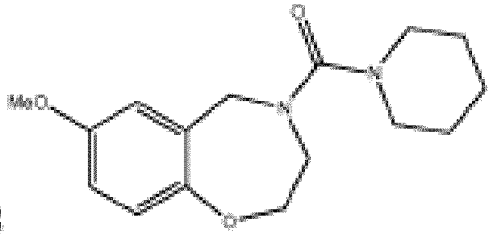

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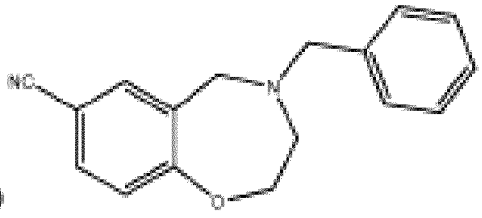

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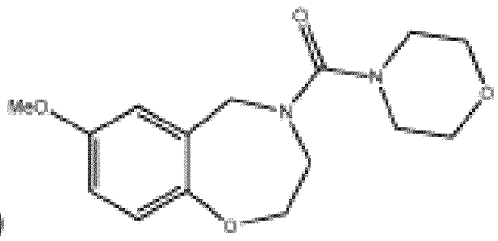

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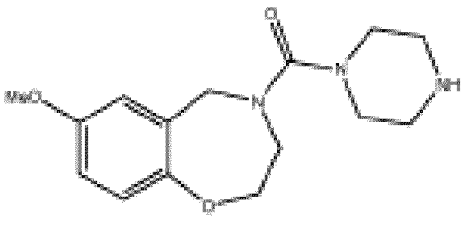

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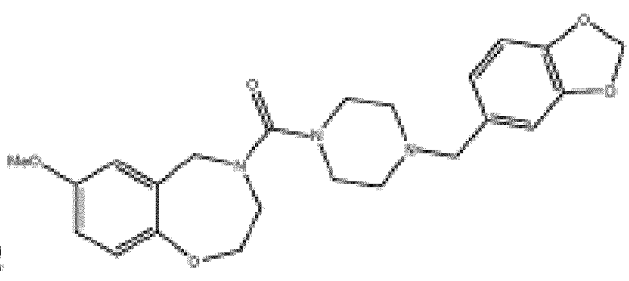

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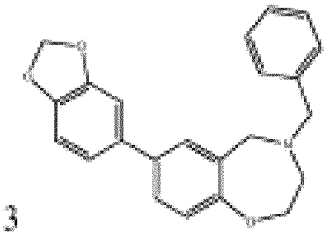

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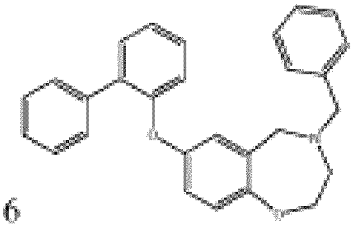

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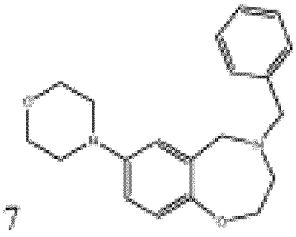

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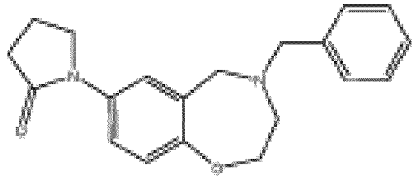

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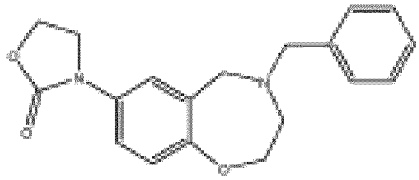

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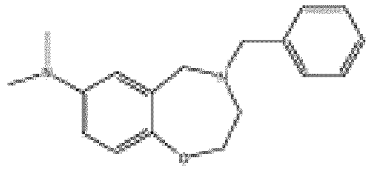

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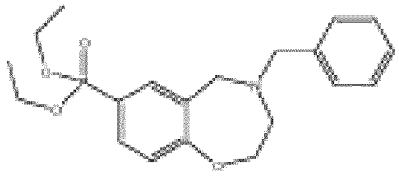

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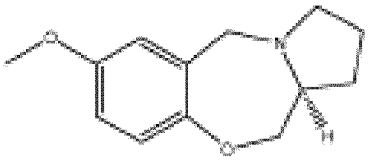

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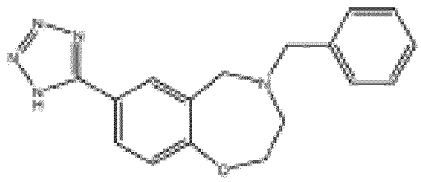

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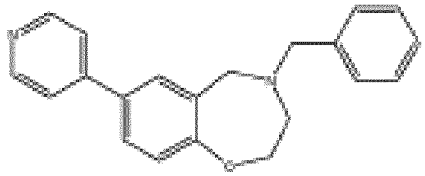

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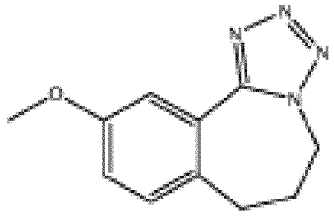

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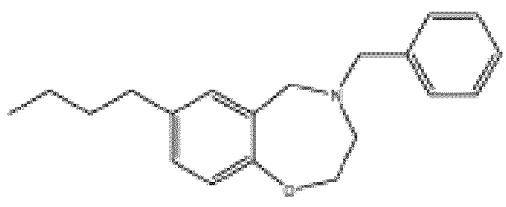

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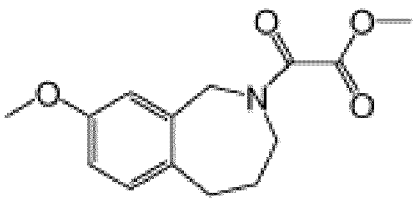

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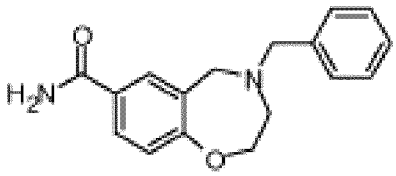

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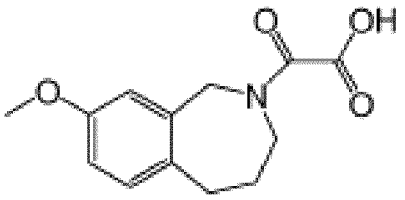

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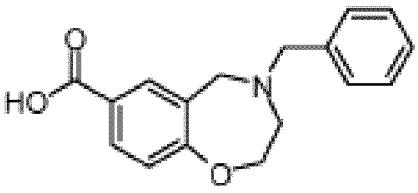

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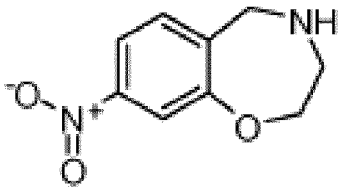

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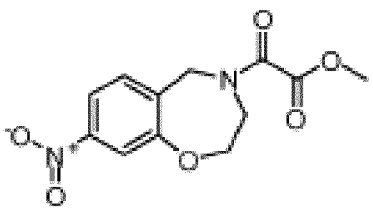

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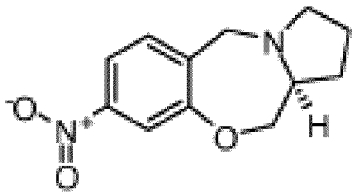

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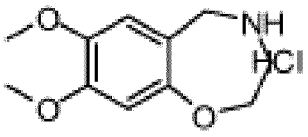

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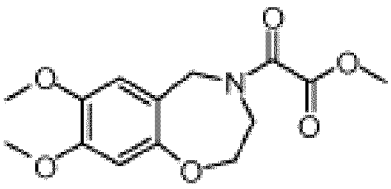

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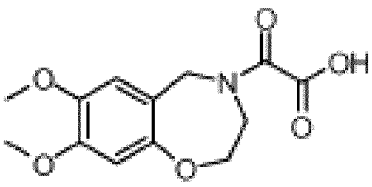

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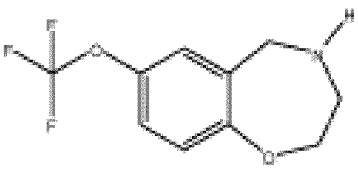

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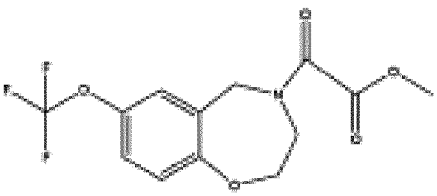

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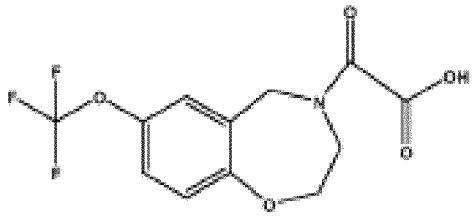

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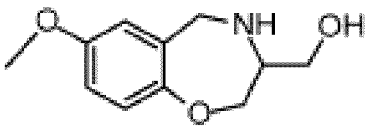

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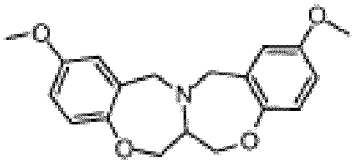

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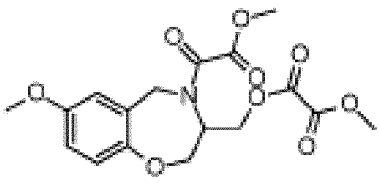

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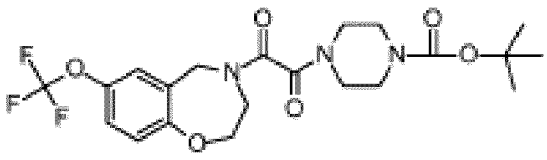

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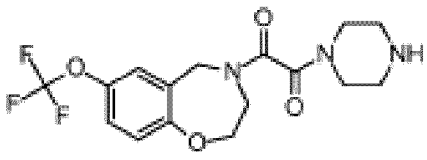

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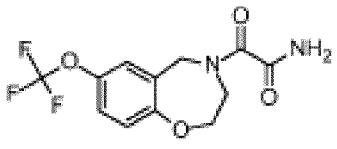
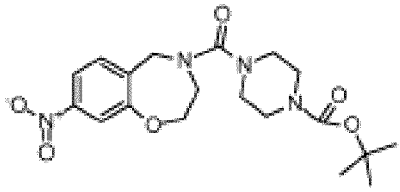
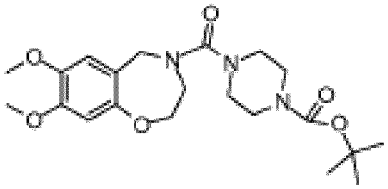


S313



S318



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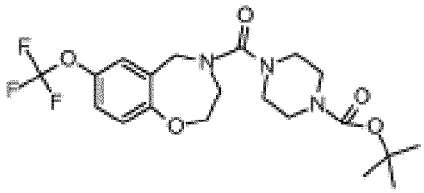

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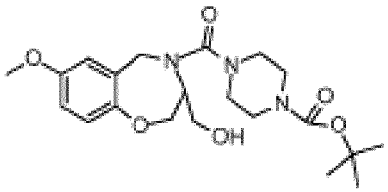

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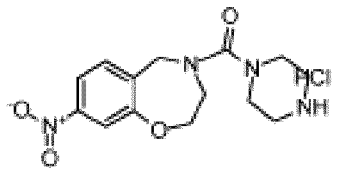

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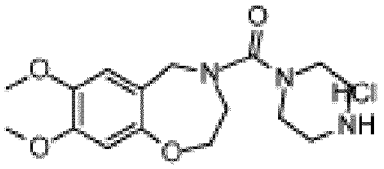
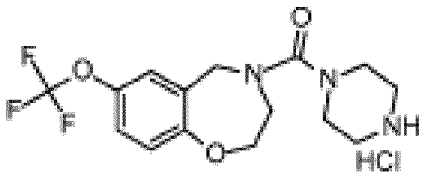
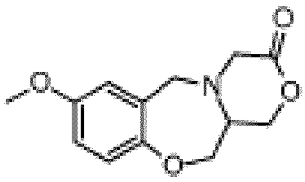


S335



S337



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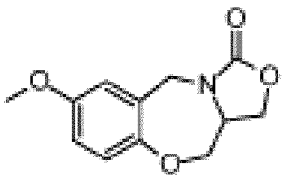

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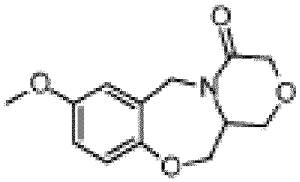

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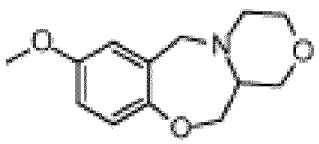

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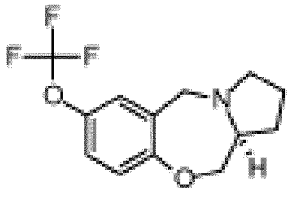

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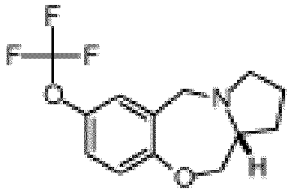

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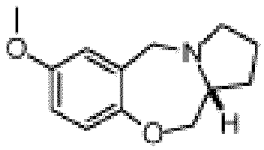

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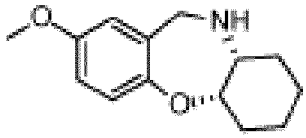

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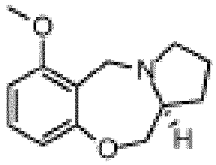

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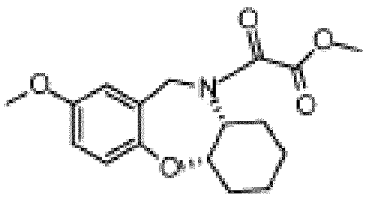

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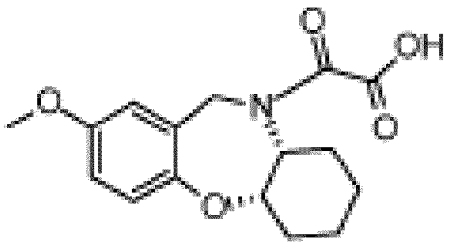

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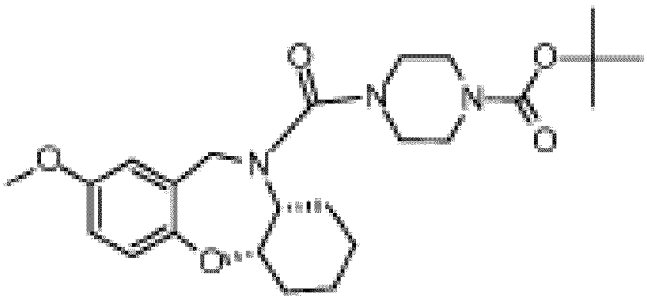

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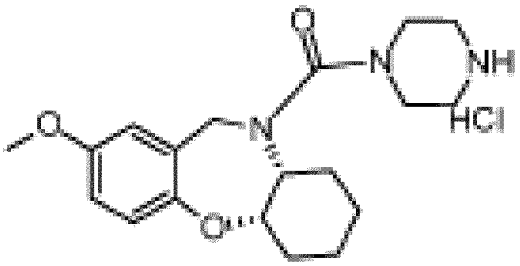

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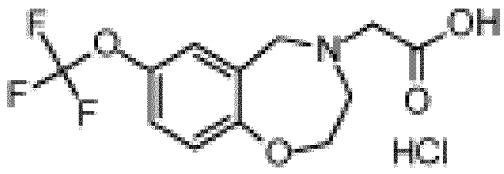
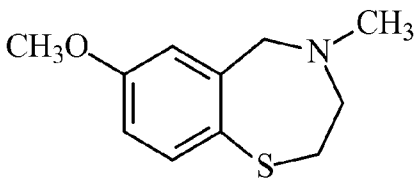


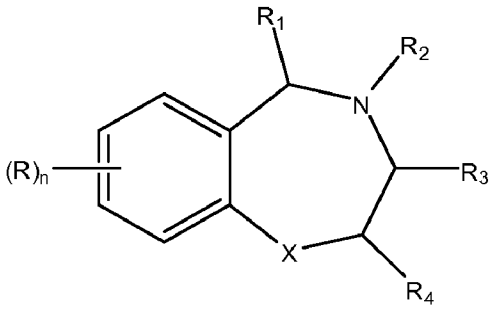
S477

Representative compounds of Formula II-o include without limitation S107, S110, S111, S120, and S121. In a particular embodiment, the Rycal is S107 having the formula III:



(III)

In certain embodiments, the Rycal is selected from the group of compounds of the general Formula IV:



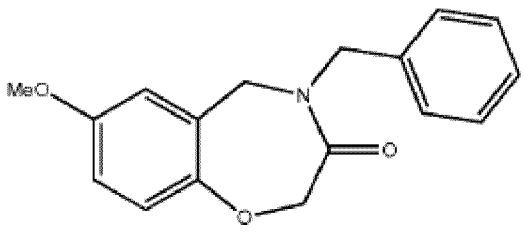
(IV)

wherein

- n is 0, 1, 2, 3, or 4;
- X is O, $-NR_5$ or $-C(R_5)_2$;
- each R is independently selected from the group consisting of Z, R_5, $-OR_5$, $-SR_5$, $-N(R_5)_2$, $-NR_5C(=O)OR_5$, $-C(=O)N(R_5)_2$, $-C(=O)OR_5$, $-C(=O)R_5$, $-OC(=O)R_5$, NO_2, CN, $-CZ_3$, OCZ_3, $-N_3$, and $-P(=O)R_8R_9$;
- R_1 and R_3 are each independently selected from the group consisting of oxo, R_5, $-CH_2OR_5$, $-CH_2OC(=O)R_6$, $-C(=O)OR_5$, $-C(=O)NHR_5$, $-C(=O)R_5$, and $-OC(=O)R_5$;
- R_2 is selected from the group consisting of R_5, $-(C=O)R_6$, $-(C=S)R_6$, and $-(CH_2)_mR_{10}$, wherein m is 1, 2, 3, 4, 5, or 6; or
- R1 and R_2 together with the carbon and nitrogen to which they are respectively attached, form an unsubstituted or substituted heterocycle; or
- R_2 and R_3 together with the nitrogen and carbon to which they are respectively attached, form an unsubstituted or substituted heterocycle other than a piperazine; or
- R_3 and R_4 together with the carbon atoms to which they are respectively attached, form an unsubstituted or substituted cycloalkyl or heterocyclic ring; or
- R_4 is selected from the group consisting of R_5 and oxo;

- each R_5 is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, cycloalkylalkyl, heterocyclylalkyl, alkylaryl, and alkylheteroaryl;
- R_6 is selected from the group consisting of R5, $-(CH_2)_bNR_{13}R_{14}$, $-NR_5OR_5$, $-OR_5$, $-C(O)OR_5$, $-C(=O)NR_{13}R_{14}$, $-(CH_2)_CY$, and $-C(=O)R_5$, wherein b is 0, 1, 2, 3, 4, 5, or 6 and c is 1, 2, 3, 4 or 5;
- R_{10} is selected from the group consisting of R_5, $-OR_5$, $-SO_2R_{11}$, $-C(=O)R_{12}$, $-NH(C=O)R_{12}$, $-O(C=O)R_{12}$, and $-P(=O)R_8R_9$;
- R_8, R_9, R_{11} and R_{12} are independently selected from the group consisting of R_5, $-OR_5$, and $-N(R_5)_2$;
- Y is selected from the group consisting of Z, $-CO_2R_5$, $-C(=O)NR_{13}R_{14}$, and $-OR_5$;
- Z is a halogen selected from F, Cl, Br and I;
- R_{13} and R_{14} are independently selected from the group consisting of R_5, or R_{13} and R_{14} together with the N to which they are bonded may form an unsubstituted or substituted heterocycle; and
- wherein each alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, cycloalkylalkyl, heterocyclylalkyl, alkylaryl, and alkylheteroaryl may be substituted or unsubstituted;
- wherein the nitrogen in the benzoxazepine ring may optionally be a quaternary nitrogen;
- and all enantiomers, diastereomers, tautomers, pharmaceutically acceptable salts, hydrates, solvates, complexes, polymorphs, metabolites, and prodrugs thereof;

In some embodiments, the Rycal of Formula IV is selected from the group consisting of ARM136, ARM137, ARM138, ARM139, ARM140, ARM146, ARM147, ARM148, ARM149, ARM150, ARM151, ARM152, ARM153, ARM156, ARM157, ARM159, ARM160, ARM161, ARM166, ARM167, ARM182, ARM186, ARM187, ARM189, ARM 200, ARM203, ARM 205, ARM217, ARM251, ARM252, ARM258, ARM277, ARM279, ARM282, ARM291, ARM293, ARM296, ARM301, ARM302, ARM306, ARM311, ARM312, ARM313, ARM318, ARM322, ARM324, ARM326, ARM331, ARM335, ARM337, ARM351, ARM352, ARM353, ARM354, ARM397, ARM398, ARM399, ARM423, ARM454, ARM463, ARM466, ARM470, ARM473 and ARM477. The structures of these agents are provided in the Detailed Description of the International Patent Application WO2008/144483 and as follows:



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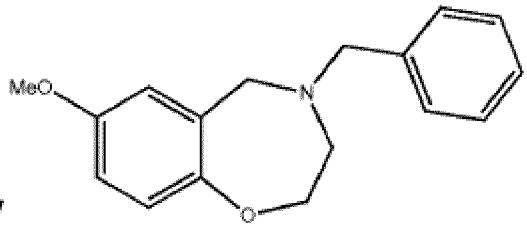
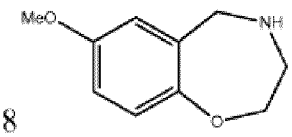


ARM137

ARM138



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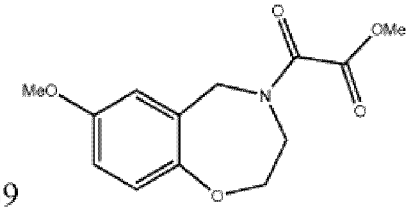

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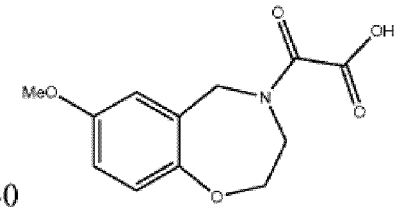

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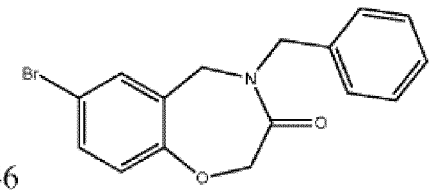

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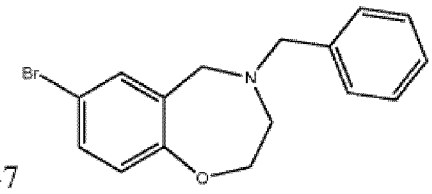

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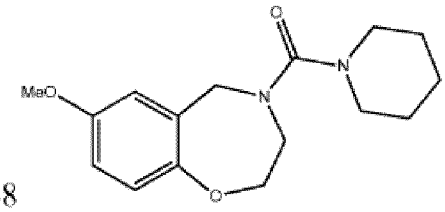

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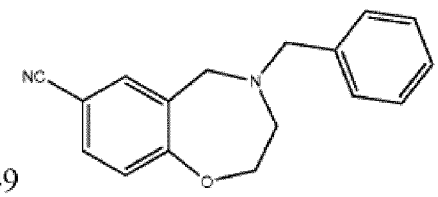

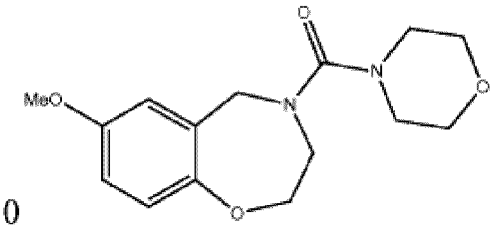

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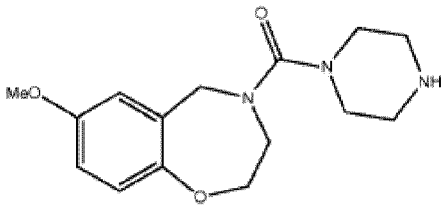

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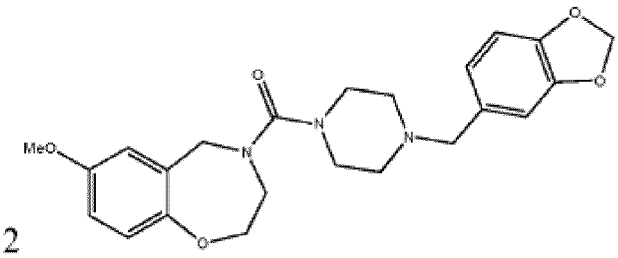

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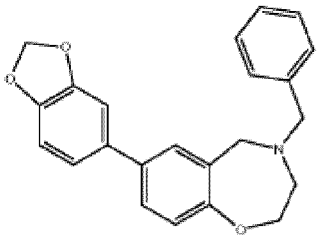

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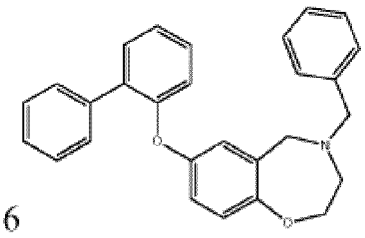

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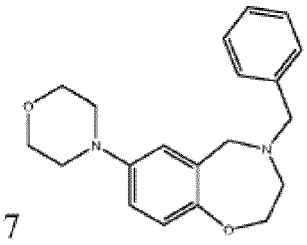

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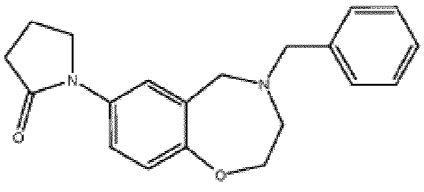

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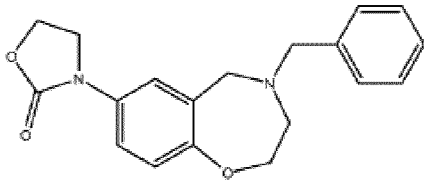

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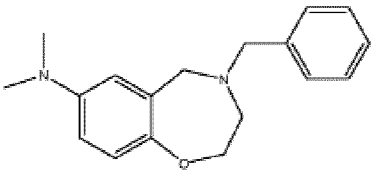

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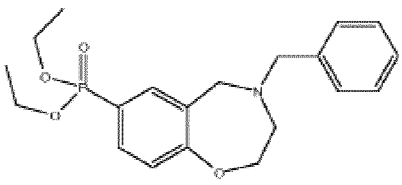

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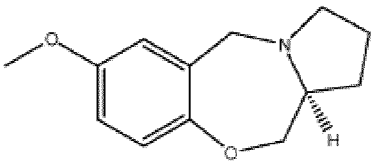

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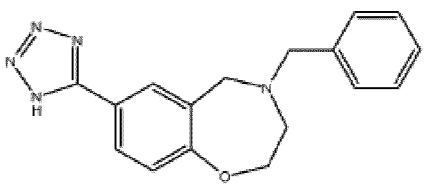

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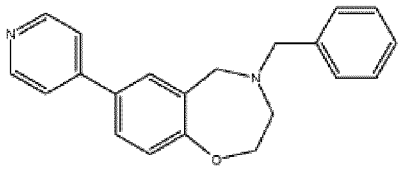

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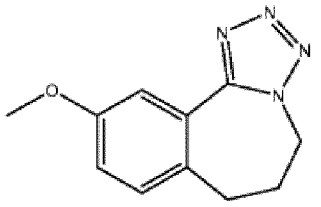

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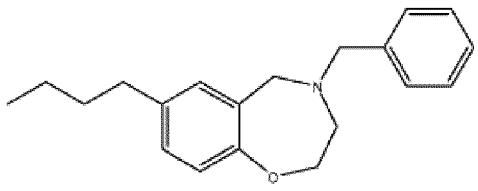

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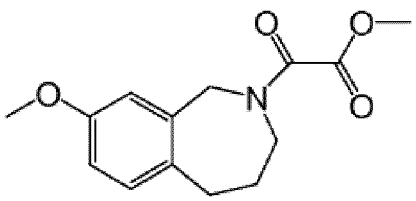

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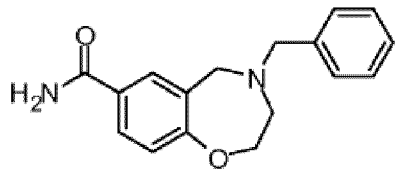

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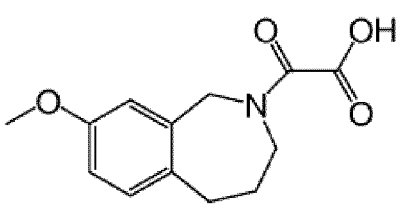

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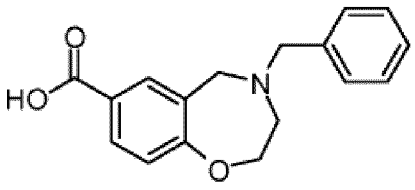

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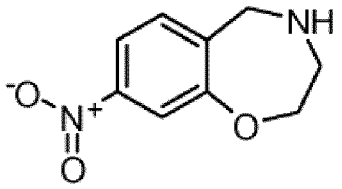

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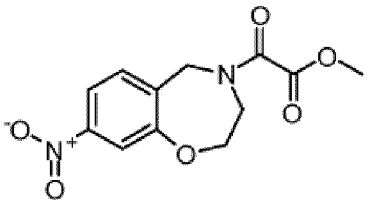

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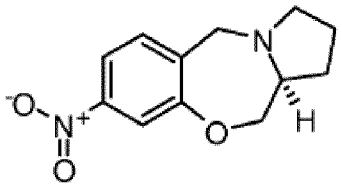

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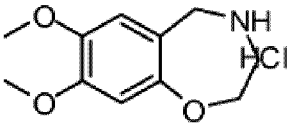

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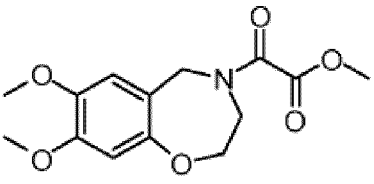

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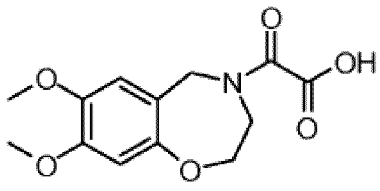

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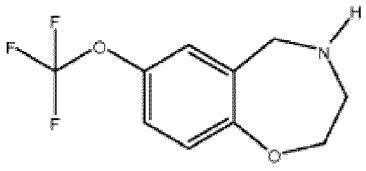

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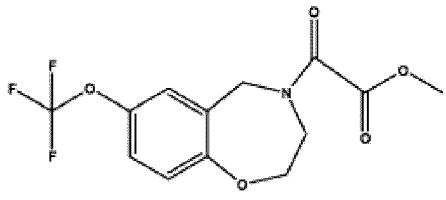

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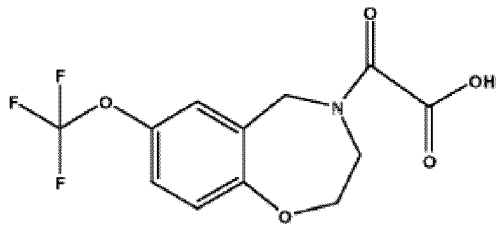

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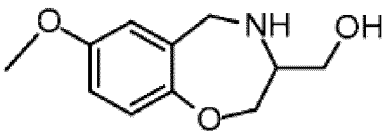

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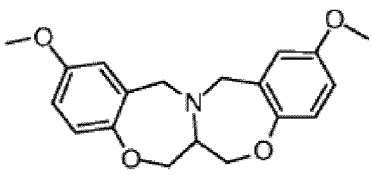

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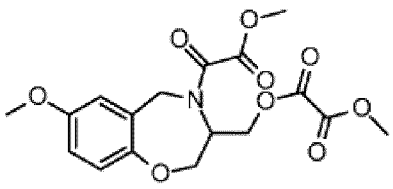

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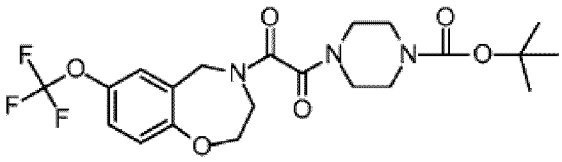

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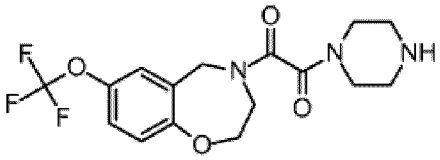

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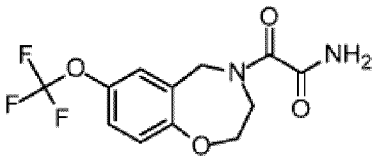

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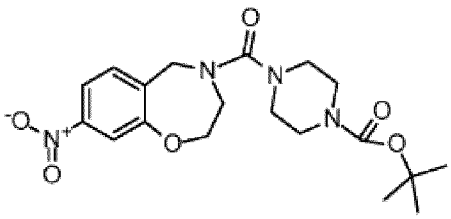

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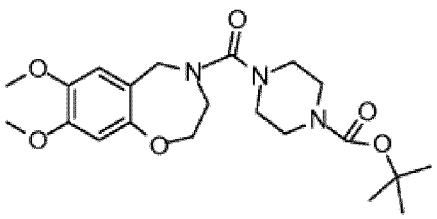

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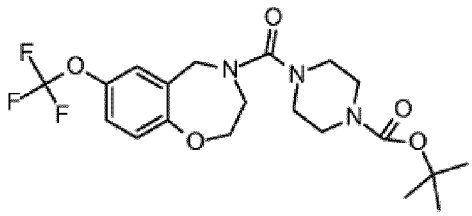

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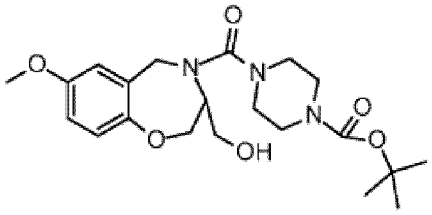

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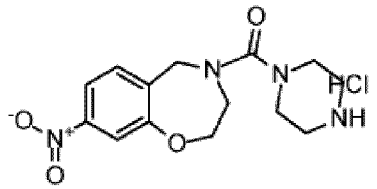

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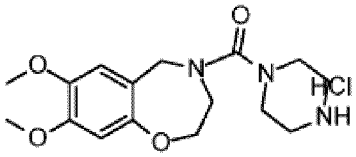

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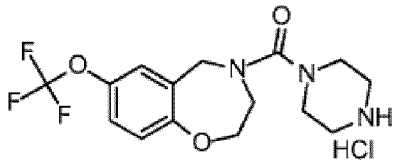

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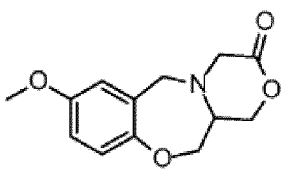

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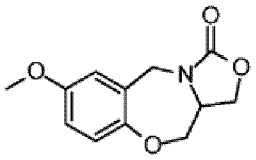

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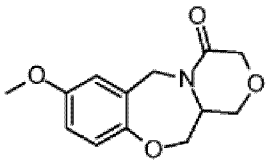

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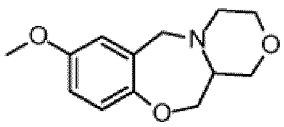

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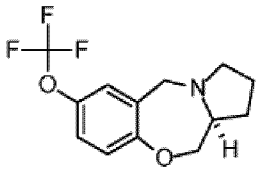

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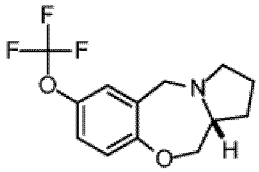

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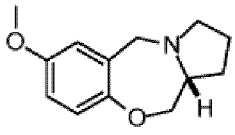

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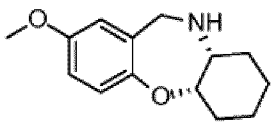

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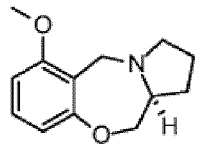

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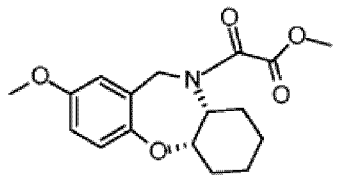

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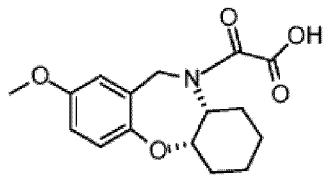

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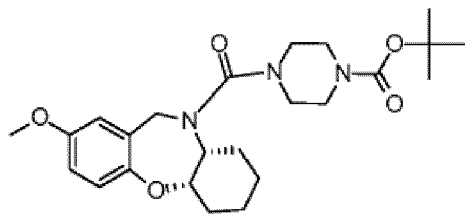

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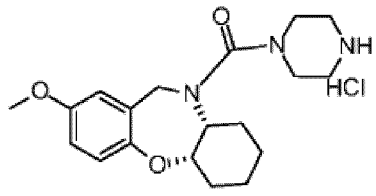

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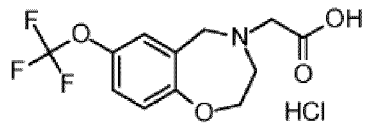

ARM477

Typically, the agent according to the invention is administered to the subject in a therapeutically effective amount. As used herein, the terms "effective amount" or "therapeutically effective amount" or "pharmaceutically effective amount" refer to a quantity sufficient to achieve a desired therapeutic (including prophylactic effect), e.g., an amount which results in the prevention of, or a decrease in, ventilator-induced diaphragmatic dysfunction, or symptoms associated therewith. In the context of therapeutic or prophylactic applications, the amount of a composition administered to the subject will depend on the type and severity of the disease and on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. It will also depend on the degree, severity and type of disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. The compositions can also be administered in combination with one or more additional therapeutic compounds. A suitable amount of the agents of the invention effective in the subject ranges from about 0.01 mg/kg/day to about 20 mg/kg/day, and/or is an amount sufficient to achieve plasma levels ranging from about 300 ng/ml to about 1000 ng/ml. Alternatively, the amount of compounds from the invention ranges from about 10 mg/kg/day to about 20 mg/kg/day. Also included are amounts of from about 0.01 mg/kg/day or 0.05 mg/kg/day to about 5 mg/kg/day or about 10 mg/kg/day which can be administered.

The agents of the invention are formulated into pharmaceutical compositions for administration to human subjects in a biologically compatible form suitable for administration in vivo.

Accordingly, a further aspect of the invention relates a pharmaceutical composition comprising at least one agent of the invention in admixture with a pharmaceutically acceptable diluent and/or carrier.

The pharmaceutically-acceptable carrier must be "acceptable" in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof. The pharmaceutically-acceptable carrier employed herein is selected from various organic or inorganic materials that are used as materials for pharmaceutical compositions and which are incorporated as analgesic agents, buffers, binders, disintegrants, diluents, emulsifiers, excipients, extenders, gellants, glidants, skin-penetration enhancers, solubilizers, stabilizers, suspending agents, tonicity agents, vehicles and viscosity-increasing agents. If

necessary, pharmaceutical additives, such as antioxidants, aromatics, colorants, flavor-improving agents, preservatives, and sweeteners, are also added. Examples of acceptable pharmaceutical carriers include carboxymethyl cellulose, crystalline cellulose, glycerin, gum arabic, lactose, magnesium stearate, methyl cellulose, powders, saline, sodium alginate, sucrose, starch, talc and water, among others.

The pharmaceutical compositions of the present invention are prepared by methods well-known in the pharmaceutical arts. For example, the agents of the invention are brought into association with a carrier and/or diluent, as a suspension or solution. Optionally, one or more accessory ingredients (e.g., buffers, flavoring agents, surface active agents, and the like) also are added. The choice of carrier is determined by the solubility and chemical nature of the agent, chosen route of administration and standard pharmaceutical practice.

Additionally, the compounds of the present invention are administered to the subject by known procedures including, without limitation, oral administration, sublingual or buccal administration, parenteral administration, transdermal administration, via inhalation or intranasally, vaginally, rectally, and intramuscularly. The agents of the invention are administered parenterally, by epifascial, intracapsular, intracranial, intracutaneous, intrathecal, intramuscular, intraorbital, intraperitoneal, intraspinal, intrasternal, intravascular, intravenous, parenchymatous, subcutaneous or sublingual injection, or by way of catheter.

For oral administration, a formulation of the agents of the invention may be presented as capsules, tablets, powders, granules, or as a suspension or solution. The formulation has conventional additives, such as lactose, mannitol, cornstarch or potato starch. The formulation also is presented with binders, such as crystalline cellulose, cellulose derivatives, acacia, cornstarch or gelatins. Additionally, the formulation is presented with disintegrators, such as cornstarch, potato starch or sodium carboxymethylcellulose. The formulation also is presented with dibasic calcium phosphate anhydrous or sodium starch glycolate. Finally, the formulation is presented with lubricants, such as talc or magnesium stearate.

For parenteral administration (i.e., administration by injection through a route other than the alimentary canal), the agents of the invention are combined with a sterile aqueous solution that is isotonic with the blood of the subject. Such a formulation is prepared by dissolving a solid active ingredient in water containing physiologically-compatible

substances, such as sodium chloride, glycine and the like, and having a buffered pH compatible with physiological conditions, so as to produce an aqueous solution, then rendering said solution sterile. The formulation is presented in unit or multi-dose containers, such as sealed ampoules or vials. The formulation is delivered by any mode of injection, including, without limitation, epifascial, intracapsular, intracranial, intracutaneous, intrathecal, intramuscular, intraorbital, intraperitoneal, intraspinal, intrasternal, intravascular, intravenous, parenchymatous, subcutaneous, or sublingual or by way of catheter into the subject's heart. For transdermal administration, the agents of the invention are combined with skin penetration enhancers, such as propylene glycol, polyethylene glycol, isopropanol, ethanol, oleic acid, JV-methylpyrrolidone and the like, which increase the permeability of the skin to the agents of the invention and permit the compounds to penetrate through the skin and into the bloodstream. The compound/enhancer compositions also may be further combined with a polymeric substance, such as ethylcellulose, hydroxypropyl cellulose, ethylene/vinylacetate, polyvinyl pyrrolidone, and the like, to provide the composition in gel form, which are dissolved in a solvent, such as methylene chloride, evaporated to the desired viscosity and then applied to backing material to provide a patch.

The composition may be provided in unit dose form such as a tablet, capsule or single-dose vial. Suitable unit doses, i.e., therapeutically effective amounts, can be determined during clinical trials designed appropriately for each of the conditions for which administration of a chosen compound is indicated and will, of course, vary depending on the desired clinical endpoint. The present invention also provides articles of manufacture for treating and preventing disorders, such as cardiac disorders, in a subject. The articles of manufacture comprise a pharmaceutical composition of one or more of the agents of the invention.

A further aspect of the invention relates to a method for screening a plurality of test substances useful for treatment of ventilator-induced diaphragmatic dysfunction comprising the steps consisting of (a) testing each of the test substances for its ability stabilize the RyR1-calstabin1 interaction and/or to reduce SR Ca^{2+} leak via RyR1 channel and (b) and positively selecting the test substances capable of restoring said integrated stress response.

The screening method of the invention is implemented by any assay well known in the art. Examples of suitable assays and screening methods that may be used to identify new

compounds that possess these properties such that they may be useful for the treatment of VIDD are described in the International Patent Publication WO 2008/064264 ("In vivo methods for identifying and screening compounds that modulate calstabin binding to a ryanodine receptor"), WO 2008/140592 ("Radioactively labeled 1,4-benzothiazepines and methods of screening for compounds that bind ryanodine receptors") and WO 2004/080283 ("Type 1 ryanodine receptor-based methods"), the entire contents of each of which are hereby incorporated by reference. The selected test substances indirectly decrease the open probability of RyR1 when examined under conditions that simulate diastole, by inhibiting the depletion of the stabilizing subunit calstabin1 from the RyR1 complex and thereby stabilizing the closed state of the channel, particularly PKA-phosphorylated, and/or nityrosylated, and/or oxidized RyR1, and thereby decrease the Ca^{2+} current through such channels under resting conditions when muscles are relaxed. The selected test substance may exert their effects, at least in part, by increasing the affinity with which calstabin1 proteins bind to RyR1, and/or by inhibiting a decrease in binding of calstabin1 to RyR1, and/or by inhibiting dissociation of calstabin1 from RyR1, particularly PKA phosphorylated RyR1.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

DETAILED DESCRIPTION OF THE FIGURES:

Figures 1A to 1C. VIDD is associated with defective RyR1 in human diaphragm muscle. Representative immunoblots (**A**) of immunoprecipitated RyR1 of human diaphragm samples collected from short term (control) and long term (MV) mechanically ventilated patients (see table 5A). (Each blot corresponds to adjacent wells of the same gel). Bar graphs (**B**) show quantification of immunoblots, relative to total RyR1 immunoprecipitated (mean ± SEM, n = 10 and 9 in control and MV respectively, * $p<0.05$, MV vs control). CysNO, thio-nitrosylation; DNP, 2,4-dinitrophenylhydrazone; P*RyR1, phosphorylated RyR1 (at serine 2844). **C**) Single channel traces of single RyR1 incorporated in planar lipid bilayers, corresponding to representative experiments performed with human diaphragm biopsies from control and MV groups. VIDD increases RyR1 open probability (P_o), Mean Po was 0.00062 ± 0.00035 in control (ranging from 0 to 0.00635, n=18). After MV, the channels had a higher Po value of 0.01745 ± 0.01259 (ranging from 0 to 0.28482, n=23) but not significantly

different from the control (see Fig. 5A). The lack of significance was due to a large variability in MV sample. 78% of the channels had a Po in the same range as the control channels and 22% had a Po significantly higher (0.07982 ± 0.05259).

Figures 2A to 2D. VIDD is associated with defective RyR1 in porcine diaphragm muscle.

A) Diaphragmatic contractile function was assessed by *in vivo* measurements of transdiaphragmatic pressure (Pdi) at t=0 (baseline) and t=72 (72 hours of anesthesia) in control (Control_0 and control_72) and mechanically ventilated pigs (MV_0 and MV_72), produced by supramaximal stimulation and expressed as a function of stimulation frequency. (*, $p<0.05$, MV_72 vs. MV_0). **B**) Single channel traces of single RyR1 incorporated in planar lipid bilayers, corresponding to representative experiments performed with porcine diaphragm biopsies from control and MV groups after 72 hours of anesthesia. As for human samples, VIDD increases RyR1 open probability (P_o) in pigs. Mean Po was 0.00115±0.00069 (n=12) in control (ranging from 0.00001 to 0.00827). After MV, the channels had a higher Po value of 0.00839±0.00582 (n=13) (ranging from 0.00001 to 0.06789) but not significantly different from the control (see Fig 5A). The lack of significance was due to a large variability in MV sample. 16% had a Po significantly higher (0.05394 ± 0.01396). This was never observed in control condition and reflected RyR leaky behavior induced by MV in pigs VIDD as in human. **C**) Representative immunoblots of immunoprecipitated RyR1 of pig diaphragm samples collected from control and mechanically ventilated (MV) groups after 72 hours of anesthesia (each blot corresponds to adjacent wells of the same gel). Bar graphs (**D**) show quantification of immunoblots relative to total RyR1 immunoprecipitated (mean ± SEM, n = 5 in both groups, * $p<0.05$, MV vs. control). CysNO, thio-nitrosylation ; DNP, 2,4-dinitrophenylhydrazone; P*RyR1, phosphorylated RyR1 (at serine 2844). In panels B, C and D Control and MV refer to samples collected at t=72. Data are expressed as mean ± SEM.

Figures 3A to 3F. VIDD is associated with early impairment of RyR1 in murine diaphragm muscle.

A) Representative records of specific diaphragmatic force production measured *ex vivo* at 1, 30 and 100Hz in muscle bundles under isometric conditions in control and mechanically ventilated (MV) mice. **B**) Averaged force – frequency relationships recorded in control (n=8), MV (n=10) and MV treated with trolox (n=8) groups. Representative immunoblots (each blot corresponds to adjacent wells of the same gel) of immunoprecipitated

RyR1 (**C**) and bar graphs (**D**) showing the quantification of immunoblots relative to total RyR1 immunoprecipitated of murine diaphragm samples collected from control, MV, and MV-trolox groups. CysNO, thio-nitrosylation ; DNP, 2,4-dinitrophenylhydrazone; P*RyR1, phosphorylated RyR1 (at serine 2844). Spontaneous SR Ca^{2+} release events were recorded in fluo-4-loaded permeabilized diaphragm fibers by laser scanning confocal microscopy. **E**) Representative ΔF/F line- scan images (1.54 ms per line) recorded in control, after MV and after MV with trolox treatment. **F**) Mean Ca^{2+} sparks frequency is used as an index of resting SR Ca^{2+} leak. Results are expressed as mean ± SEM (*, $p<0.05$, MV vs. control; #, $p<0.05$, MV-trolox vs. MV).

Figures 4A to 4F. RyR1 stabilization prevents VIDD.

A) Representative records of specific diaphragmatic force production measured *ex vivo* at 1, 30 and 100Hz in muscle under isometric conditions in wild-type mice and **B**) Averaged specific force–frequency relationships recorded in wild-type MV (MV, n=10) and wild-type MV treated with S107 (MV_S107, n=5). Spontaneous SR Ca^{2+} release events (Ca^{2+} sparks) were recorded in fluo-4-loaded permeabilized diaphragm fibers by laser scanning confocal microscopy. **C**) Comparison of mean Ca^{2+} sparks frequency between MV and MV_107, used as an index of resting SR Ca^{2+} leak. Results are expressed as mean ± SEM (*, $p<0.05$, vs. MV and MV_S107). **D**) Representative records of specific diaphragmatic force production in calstabin1 knockout (Cal1-/-) mice and **E)** Averaged specific force–frequency relationships recorded in calstabin1 knockout MV ($Cal1^{-/-}$_MV, n=6) and $Cal1^{-/-}$_MV treated with S107 ($Cal1^{-/-}$_MV-S107, n=6). While S107 prevented VIDD in wild type mice, no effect was observed in $Cal1^{-/-}$ mice (19.9±4.5 and 18.9±3 $N.cm^{-2}$ in $cal1^{-/-}$ and $cal1^{-/-}$ -MV respectively). **F**) Comparison of mean Ca^{2+} sparks frequency between $Cal1^{-/-}$_MV and $Cal1^{-/-}$_MV-S107. Results are expressed as mean ± SEM.

Figure 5A: RyR1 open probability in human and pig diaphragm muscles in control condition and after MV.

In control and MV human samples the Po was 0.00062± 0.00035 (n=18) and 0.01745±0.01259 (n=23). In pigs, similar results were obtained, with Po of 0.00115±0.00069 (n=12) and 0.00839±0.00582 (n=13) in control and MV respectively. Results are expressed as mean ± SEM.

Figure 5B: Ventilation in Continuous Positive Airway Pressure (Cpap) mode does not affect RyR1 remodeling.

Bar graphs represent the quantification of immunoblots relative to total RyR1 immunoprecipitated. CysNO, thio-nitrosylation; DNP, 2,4-dinitrophenylhydrazone; P*RyR1, phosphorylated RyR1 (at serine 2844). Results are expressed as mean ± SEM, n = 10 and 5 in control and CPAP respectively (*, $p<0.05$ CPAP vs. control).

Figure 5C: Ventilation in Continuous Positive Airway Pressure (Cpap) mode does not affect RyR1 function.

Spontaneous SR Ca^{2+} release events were recorded in fluo-4-loaded permeabilized diaphragm fibers by laser scanning confocal microscopy in control and CPAP condition. Mean Ca^{2+} sparks frequency used, as an index of resting SR Ca^{2+} leak was not significantly different between CPAP and control mice. (Results are expressed as mean ± SEM).

Figure 5D: Force frequency relationships of control and cal1-/- diaphragm muscle in resting condition.

Averaged force – frequency relationships recorded in control (n=8) and calstabin deficiency mice (n=4) in basal condition (i.e. non ventilated, -NV). The force production in basal condition is not affected by the lack of calstabin1 at the age of 10-12 weeks. Results are expressed as mean ± SEM.

EXAMPLES

EXAMPLE 1: MATERIAL AND METHODS

Human model of VIDD

The study in humans was conducted in accordance with the World Medical Association Guidelines for research in humans, and approved by the institutional ethics board of the Montpellier University Hospital (protocol # NCT00786526). All subjects or their surrogates provided written informed consent to participate in the study. All animal experiments in our study followed the guidelines for animal experiments established by the recommendations of the institutional care committee and the recommendations of the Helsinki Declaration. The protocol has been approved by the Animal Care and Use Committee Languedoc-Roussillon and recorded under the reference #CEEA-LR-12078.

The study design, as previously used included 2 groups of subjects: 1) - patients with brain death destined for organ donation, who had received MV for at least 24 hours prior to organ harvest (MV, n=9); and 2) patients anesthetized and supported with MV for 2-3 hours during thoracic surgery for localized lung nodules (Control, n=10). All subjects were required to have undergone MV via an endotracheal tube in fully controlled mode, i.e., without significant spontaneous breathing efforts during the MV period, with a tidal volume (VT) in the average range of 7-8.5 ml/kg of the body weight, Respiratory Rate (RR) from 12 to 24 min, and positive end-expiratory pressure (PEEP) level at 2-5 cm H_2O .. Diaphragm biopsies (approximately 1 cm^3) were obtained from the zone of apposition of the costal diaphragm at the mid-axillary line. In the MV group, the biopsies were obtained before circulatory arrest and removal of other organs. Each biopsy was partitioned and quick frozen in liquid nitrogen and kept at -80°C. Secondarily tissue blocks were prepared as required for RyR biochemical and RyR1 functional analysis with single channel recordings in lipid bilayers as detailed in the last methodology paragraph.

Porcine model of VIDD

As previously described in our laboratory, ten piglets (15–20 kg) separated into 2 groups of animals who both were intubated and ventilated for 72 consecutive hours: 1) a group of 5 piglets mechanically ventilated in a totally controlled mode (MV), with a tidal volume (VT) at 10 –12 ml/kg of the body weight, RR from 15 to 30 min, and positive end-expiratory pressure level at 5 cm H_2O. The absence of spontaneous breathing was verified on the ventilator trend graphs, and electromyographic activity of the diaphragm was measured to ensure that no electrical activity was present in the diaphragm; 2) a group of 5 piglets ventilated in a totally spontaneous pediatric mode (Control), setting in phase with ideal body weight of the piglet, inspiratory flow trigger at 0.3 l/min, percentage of mechanical ventilation between 100 and 150%, positive end-expiratory pressure level at 5 cm H_2O, and expiratory trigger at 25% of peak inspiratory flow. Electromyographic activity of the diaphragm was similarly assessed to verify that active piglets were able to breath spontaneously.

In both groups, oxygenation was maintained with FIO_2 from 25 to 35% and minute ventilation to assess normocapnia. The two groups received the same care, except for the ventilator mode. In brief, piglets were anesthetized with intravenous pentobarbital sodium (5–6 mg/kg), intubated with a cuffed endotracheal tube, and mechanically ventilated (Galileo®; Hamilton Medical AG, Rhazuns, Switzerland). Anaesthesia was maintained with continuous

intravenous propofol (15–20 mg/kg/h), midazo-lam (0.1– 0.3 mg/h), and ketamine (3–4 mg /kg/ h). The level of sedation was monitored with bispectral index (BIS®; Aspect, Norwood, MA). Heating pads were used as needed to maintain a normal body temperature of 38.5°–39.5°C. A carotidal arterial catheter (PiCCO®; Pulsion, Munich, Germany) was inserted for the monitoring of heart rate, arterial blood pressure, and cardiac output. Arterial pressure of carbon dioxide levels was checked by using a capnograph (DELTATRAC®; Datex-Ohmeda, Helsinki, Finland) and then verified by arterial blood gases (iSTAT®; Abbott, Abbott Park,IL). Parenteral nutrition was given from the first day (10% glucose solution and 20% amino acids solution, and HYPERAMINE 20%®; Braun, Boulogne Billancourt, France) providing 30–35 kcal/kg/day. All procedures were performed aseptically. The animals received prophylactic intravenous antibiotics three times daily (amoxicillin– clavulanate, 100 mg/kg/day).

In vivo Diaphragm Contractile function in porcine model of VIDD

Diaphragm contractile function was assessed in vivo by measuring transdiaphragmatic Pressure (Pdi).). In brief, double air-filled balloon-tipped catheters were placed transorally in the stomach and the distal third of the esophagus. Bipolar transvenous pacing catheters were introduced via each internal jugular vein and adjusted to achieve stimulation of the phrenic nerve and subsequent contraction of the diaphragm by supramaximal stimulation at frequencies ranging from 20 to 120 Hz with trains of stimulation of 2 s and 150 ms pulse duration.

Murine model of VIDD

The experimental design has been described in recent study .. In brief, 35 adult male mice (10 to 12 weeks old, 25 to 30g) C57/BL6 mice were separated into five groups. Three groups were intubated with a 22-gauge angiocatheter and mechanically ventilated for 6 consecutive hours using a volume-driven small-animal ventilator (MINIVENT®, Harvard Apparatus, Saint-Laurent, Canada). Tidal volume was established at 10µl/mg body weight with a respiratory rate of 150 breaths/min, a positive end-expiratory pressure (PEEP) level from 2 to 4 cm H_2O and a fraction of inspired oxygen of 0.21. Non-spontaneous ventilation was defined as a lack of diaphragm contractile activity attested by repetitive stereotypical deflections observed in the airway pressure curve.

The mice were divided into five groups. The first group (MV-Trolox) received a priming dose of trolox (0.125 ml of saline containing 5g/l trolox, corresponding to ~20

mg/kg) intravenously (IV) infused over a 5-minute period, 20 minutes before start of MV. During MV, a constant IV infusion of trolox at a rate of 4 mg/kg per hours (~0.025 ml/h) was maintained. The second group of mice (MV-S107) was treated for 7–days before the start of MV, with S107 in their drinking water (final concentration, 0.25 $mg.ml^{-1}$) as previously reported and received same volume of IV saline 20 minutes before starting and during MV. The mice drank about 3 ml per day (water consumption was variable, and we recorded water bottle and body weight to monitor consumption) for a daily dose of ~0.75 mg (~37.5 mg/kg/day). The third group (MV) received the same volume of IV saline 20 minutes before starting and during MV. The fourth group (CPAP) (n=7) was intubated and treated in an identical manner to the first three groups but breathed spontaneously with a constant positive airway pressure (CPAP) of 3-4 cm H20. For this purpose, the angio-catheter was connected to an air compressor to deliver a high inspiratory flow rate (1 l/min room air) while the expiratory port was placed under a water seal to obtain PEEP. The fifth group (Control, n=7 mice) did not receive any treatment and served as control.

To address the specificity of S107 on RyR1 dysfunction in VIDD, we similarly ventilated 12 calstabin1 deficient mice (10-12 weeks-old), separated into two groups. The first group ($Cal1^{-/-}$-MV-S017) received S107 treatment similarly to the C57BL6 mice, 5-7 days before the start of MV. The second group ($Cal1^{-/-}$-MV) received the same amount of water volume. All mice had similar body weights (27 ±0.9 g).

All groups mechanically ventilated or under CPAP received the same general care. Mice were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and orally intubated with a 22-gauge angiocatheter. General care applied during the experiments also included continuous reheating using a homeothermic blanket (Homeothermic Blanket Control unit, Harvard Apparatus, Saint-Laurent, Canada, set at 35 °C), and hourly intraperitoneal injection of 0.05 ml of Ringers Lactate solution to maintain hemodynamic stability and compensate insensible losses, as well as bladder expression, eye lubrication and passive limb movements.

In vitro Contractile function in murine muscle samples

At the end of the protocol of MV, the entire diaphragm was surgically excised and mice were euthanized, by exsanguination. Isometric contractile properties were assessed as described previously in detail. The excised diaphragm strip was mounted into jacketed tissue

bath chambers filled with equilibrated and oxygenated Krebs solution. The muscles were supra-maximally stimulated using square wave pulses (Model S48; Grass Instruments, West Warwick, RI). The force–frequency relationship was determined by sequentially stimulating the muscles for 600 ms at 10, 20, 30, 50, 60, 80, 100 and 120Hz with 1 minute between each stimulation train. After measurement of contractile properties, muscles were measured at Lo (the length at which the muscle produced maximal isometric tension), dried and weighted. For comparative purposes, diaphragmatic force production was normalized for total muscle strip cross-sectional area and expressed in $N.cm^{-2}$. The total muscle strip cross-sectional area was determined by dividing muscle weight by its length and tissue density (1.056 g/cm^3).

The rest of the diaphragm was partitioned, one part was quick frozen in liquid nitrogen and secondarily used for biochemical analysis, and the other part was used freshly for Ca^{2+} spark measurements.

RyR1 biochemical analysis

Muscle biopsies were homogenized in 150 µl of buffer containing 5% SDS, 5% beta-mercaptoethanol, 10% glycerol, 10 mM EDTA and 50 mM Tris/HCl buffer (pH= 8.0). Each sample was immediately denatured at 90°C for 4 min. After centrifugation (5000 rpm) at 4°C, supernatant protein concentrations were measured in duplicate using the BCA protein assay, equilibrated at the same concentration by dilution with loading buffer and aliquoted at 2 µg/µl. RyR1 was immunoprecipitated from 250 µg of homogenate using an anti-RyR antibody (4 µg RyR1-1327) in 0.5 ml of a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na_3VO_4, 1% Triton-X100, and protease inhibitors) for 1 hr at 4°C. The immune complexes were incubated with protein A Sepharose beads (Amersham Pharmacia) at 4°C for 1 hr and the beads were washed three times with buffer. Proteins were separated on SDS-PAGE gels (4-20% gradient) and transferred onto nitrocellulose membranes for 2 hr at 200 mA (SemiDry transfer blot, Bio-Rad). To prevent non-specific antibody binding, the membranes were incubated with blocking solution (LICOR Biosciences) and washed with Tris-buffered saline with 0.1% Tween-20.

Blots were respectively incubated with primary antibody to RyR1 (RyR1-1327, an affinity-purified rabbit polyclonal antibody raised against a KLH-conjugated peptide, corresponding to residues 1327–1339 of mouse skeletal RyR1, with an additional cysteine residue added to the amino terminus), and affinity purified with the unconjugated peptide. We

also used antibody to calstabin1 (1: 2500 in blocking buffer, LICOR Biosciences); phospho-epitope–specific antibody to human RyR2 phosphorylated on Ser-2808 (1:5,000), which detects PKA-phosphorylated mouse RyR1 (on Ser-2844) and RyR2 (on Ser-2808); antibody to S-nitrosylated cysteine residues (1:1000, Sigma). To determine RyR1 oxidation, the immunoprecipitate was treated with 2, 4-dinitrophenyl hydrazine, and the derivatized carbonyls were detected using an OxyBlot protein oxidation detection Kit (catalog S7150, Chemicon International Inc.). After three washes, membranes were incubated with infrared-labeled secondary antibodies. Control samples were analyzed on each gel for normalization and total levels of RyR1 were not different between groups.

Calcium sparks measurements.

Diaphragm muscles samples were dissected and stored in a HEPES buffered physiological medium (in mM: 119 NaCl, 5 KCl, 1.25 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 1.1 mannitol, 10 HEPES, pH 7.4). Muscles were then rapidly placed in a dissecting chamber and the solution exchanged with a relaxing solution (in mM: 140 K-glutamate, 10 HEPES, 10 $MgCl_2$, 0.1 EGTA, pH 7.0). Bundles of 5 to 10 EDL fibers were manually dissected, mounted as described previously and permeabilized in a relaxing solution containing 0.01% saponin for 30 s. After washing with saponin free solution, the solution was changed to an internal medium for imaging: (in mM) 140 K-glutamate, 5 Na_2ATP, 10 glucose, 10 HEPES, 4.4 $MgCl_2$, 1.1 EGTA, 0.3 $CaCl_2$, Fluo-3 0.05 pentapotassium salt (Invitrogen), pH 7.0, for sparks acquisition as previously reported. Potential sparks were empirically identified using an autodetection algorithm. The mean fluorescence (F0) value for the image was calculated by summing and averaging the temporal F at each spatial location, while ignoring potential spark areas. This F0 value was then used to create a smoothing routine, potential spark locations were visualized and analyzed for spatiotemporal properties as described previously 9. Image analysis was performed using IDL (v5.5, Research System, Inc.). Statistical comparisons were performed using an ANOVA test with a significance level set at $P<0.05$ (Sigmastat v3.5).

Sarcoplasmic reticulum vesicle preparation

Diaphragms were homogenized on ice in 300 mM sucrose, 20 mM PIPES (pH 7.0) in the presence of protease inhibitors (Roche) and centrifuged at 8000 rpm (5900 g) for 20 min at 4°C. The following supernatant was ultracentrifuged at 32 000 rpm (100 000 g) for 1 h at 4°C. The final pellet containing microsomal fractions enriched in SR vesicles was

resuspended and aliquoted in 300 mM sucrose, 5 mM PIPES (pH 7.0) containing protease inhibitors. Samples were frozen in liquid nitrogen and stored at -80°C.

Single channel data from planar lipid bilayer measurements

Planar lipid bilayers were formed from a 3:1 mixture of phosphatidylethanolamine and phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) suspended (30 mg/ml) in decane by painting the lipid/decane solution across a 200-μm aperture in a side of a polysulfonate cup (Warner Instruments) separating two chambers. The *trans* chamber (1 ml) representing the intra-SR (luminal) compartment was connected to the headstage input of a bilayer voltage clamp amplifier (BC-525D, Warner Instruments) and the *cis* chamber (1 ml), representing the cytoplasmic compartment, was held at virtual ground. Solutions in both chambers were as follows: 1 mM EGTA, 250/125 mM HEPES/Tris, 50 mM KCl, 0.64 mM $CaCl_2$, pH 7.35 as *cis* solution and 53 mM $Ca(OH)_2$, 50 mM KCL, 250 mM HEPES, pH 7.35 as trans solution. The concentration of free Ca^{2+} in the *cis* chamber was calculated with WinMaxC program (version 2.50; www.stanford.edu/~cpatton/maxc.html). SR vesicles were added to the *cis* side and fusion with the lipid bilayer was induced by making the cis side hyperosmotic by the addition of 400-500 mM KCl. After the appearance of potassium and chloride channels, the *cis* compartment was perfused with the *cis* solution. Single-channel currents were recorded at 0 mV using a Bilayer Clamp BC-535 amplifier (Warner Instruments), filtered at 1 kHz and digitized at 4 kHz. All experiments were performed at room temperature. Data acquisition was performed by using Digidata 1440A and Axoscope 10.2 software and the recordings were analysed by using Clampfit 10.2 (Molecular Devices). Open probability was identified by 50% threshold analysis using a least 2 min of continuous record. At the conclusion of each experiment, ryanodine (5μM) was added to the *cis* chamber to confirm channels as RyRs.

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EXAMPLE 2: LEAKY RYANODINE RECEPTORS CONTRIBUTE TO DIAPHRAGMATIC MUSCLE WEAKNESS DURING MECHANICAL VENTILATION

Results:

Leaky RyR1 in diaphragm fibers correlate with VIDD:

To evaluate the remodeling and functional abnormalities of RyR1 in the diaphragms of human patients subjected to MV, we obtained diaphragm biopsies from brain-dead organ donor patients who had undergone long term MV (98±65 hours, referred to as MV) before organ harvest; we then compared these specimens with control diaphragm biopsies obtained on short term MV (2.3±0.4 hours, referred to as control) patients during thoracic surgery for removal of solitary lung nodules (see Table 5A for patients description).

Supplementary Table 1: Clinical description of patients.

	Control **Short-Term MV**	**MV** **Long-Term MV**
Duration of MV	2.3±0.4	98±65 **
Number of patients	**10**	**9**
Age (Years)	53±9	39±19 *
Sex (M/F)	9/1	3/6
Weight (kg)	72±15	74±14
Height (cm)	170±10	170±11
BMI (kg/cm^2)	25±5	25±3
Reason for surgery/ cause of brain death	Stage 1A adenocarcinoma of the lung	Stroke (7), Motor vehicle accident (1), Cardiac arrest (1)
MV = mechanical ventilation. BMI = body mass index (defined as the weight in kilograms divided by the square of the height in meters). * $p < 0.05$; ** $p < 0.01$		

SR fractions were purified to analyze the biochemical properties of RyR1 macromolecular complex (Figs. 1A-1B). RyR1 immunoprecipitation after mechanical ventilation revealed a significant increase in S-nitrosylation, oxidation and phosphorylation

on Ser-2844 together with calstabin1 dissociation from RyR1. These biochemical abnormalities were associated with an increased RyR1 open probability (Po) measured in a subgroup of channels incorporated within planar lipid bilayers (Fig. 1C and 5A), indicative of increased Ca^{2+} leak.

Because one limitation of human patient samples is the potential influence of co-morbidities and confounding factors associated with critical illness, we next evaluated RyR1 biochemistry and function in diaphragm from healthy pigs anesthetized and mechanically ventilated for 72 hours in comparison to identically anesthetized control pigs. As previously described a significant reduction in diaphragmatic contractile function was noted after MV (Fig. 2A). SR fractions incorporated into planar lipid bilayers demonstrated an RyR1 leak similar to that observed in human MV diaphragm samples (Fig. 2B and 5A). This RyR1 leak was also associated with remodeling of the RyR1 macromolecular complex, characterized again by a significant increase in S-nitrosylation, oxidation and phosphorylation on Ser-2844 and loss of calstabin1 from the RyR1 complex (Figs. 2C-2D).

Defective RyR1 function: an early pathophysiological event in VIDD:

The experiments described above clearly demonstrate that MV may contribute to structural and functional remodeling of RyR1. However, in these two models, histological damage to muscle fibers is present which could conceivably account for both the reduction in diaphragmatic force production and RyR1 remodeling. Therefore, to examine very early proximal events in the course of VIDD, we took advantage of a mouse model that exhibits a major significant loss of diaphragmatic force-generating capacity after only 6 hours of MV (Fig. 3A-B). We were thereby able to evaluate RyR1 remodeling prior to the onset of histological alterations associated with the later stages of VIDD.

MV-induced diaphragm muscle weakness in mice was associated with significant RyR1 remodeling consisting once again of S-nitrosylation, oxidation, Ser-2844 phosphorylation, and calstabin1 dissociation from RyR1 (Figs. 3C-3D). RyR1 functional properties were next evaluated *in situ* by measuring spontaneous SR Ca^{2+} release events (i.e. Ca^{2+} sparks). Note that a significant increase in spontaneous Ca^{2+} sparks frequency reflects an exaggerated level of RyR1 leak. After 6 hours of MV, Ca^{2+} spark frequency was significantly increased in diaphragm fibers (Figs. 3E-3F). It is important to note that this RyR1 impairment is due to MV only, since animals that were anesthetized and immobilized for 6 hours and

maintained only on a continuous positive airway pressure (CPAP) mode of respiration, and this did not induce VIDD. Furthermore, there was no biochemical remodeling of RyR1 (Fig. 5B) and no functional alteration of the channel complex as indicated by similar Ca^{2+} spark frequency of experimental mice as the control mice (Fig. 5C).

Since MV induces oxidative stress in the diaphragm, and antioxidant treatment has been reported to prevent VIDD, a group of mice was continuously injected with trolox, a permeable analog of vitamin E used as an antioxidant scavenger. As previously reported in rats mechanically ventilated for 12 hours, trolox treatment in mice ventilated for 6 hours prevented MV-induced diaphragm muscle weakness (Figs. 3A-3B) along with MV-induced RyR1 biochemical remodeling (Figs. 3C-3D) and the associated increase in Ca^{2+} spark frequency (Figs. 3E-3F).

RyR1 as a novel therapeutic target in VIDD:

As described above, a pharmacological approach aimed at reducing oxidative stress appears to prevent MV-induced RyR1 dysfunction. However, to directly target RyR1 and thus prove its role as a major pathophysiological target in VIDD, we treated mechanically ventilated mice with the rycal S107. Rycals are small orally available agents known to prevent depletion of calstabin1 from the RyR1 complex in spite of its PKA phosphorylation, S-nitrosylated and/or oxidation. In wild-type mice, S107 prevented the loss of muscle weakness induced by MV (Fig. 4A-B) as well as the previously observed increase in Ca^{2+} spark frequency (Fig. 4C).

To address the specificity of S107, experiments were further conducted in calstabin1 knockout ($Cal1^{-/-}$) mice. At baseline, wild type and $Cal1^{-/-}$ mice exhibited similar diaphragmatic muscle specific force production (Fig. 5D). After 6 hours of MV, $Cal1^{-/-}$ mice exhibited diaphragmatic weakness (Figs. 4D-4E) as well as an increase in Ca^{2+} spark frequency (Fig. 4F) that was comparable to that recorded in wild-type mice. However, in $Cal1^{-/-}$ mice subjected to MV, S107 treatment failed to prevent both the decrease in force production and the increase in Ca^{2+} spark frequency, thus confirming that the beneficial effects of the rycal S107 in preventing these manifestations of VIDD were dependent upon the RyR1 stabilizer, calstabin1.

Explanation of the significance of the invention:

The present work provides the first evidence, consistently found across the three different models of VIDD (humans, pigs, and mice) employed in this study, of impaired regulation and function of diaphragmatic intracellular Ca^{2+} release channels, i.e., ryanodine receptors (RyR). This RyR1 dysfunction depends on the MV-induced oxidative stress that has been extensively described. RyRs are highly sensitive to oxidative/nitrosative stress in skeletal muscle as well as in the heart. This occurs in many pathological situations including heart failure, diabetes, and Duchenne muscular dystrophy (DMD)[29,38]. Postranslational modification of RyR1 also progresses with aging and partially accounts for age-dependent muscle weakness. Therefore, as previously reported in skeletal muscle during heart failure and aging, an overly leaky RyR1 similar to that observed in the present study may account for a reduction in Ca^{2+} transient and force production, i.e. impaired excitation-contraction coupling, without the need for invoking other muscle pathology such as atrophy or injury. Evidence is provided herein that postranslational modifications of RyR1 are associated with a leaky channel. This is observed in the mouse models by evaluating the frequency of spontaneous Ca^{2+} release events (i.e. Ca^{2+} sparks). The term, Ca^{2+} sparks, refers to a local Ca^{2+} release events arising from a single Ca^{2+} release unit (CRU).). Each CRU corresponds to a cluster of RyRs. Depending on the type of muscle and species, a CRU may contain a variable number of RyRs, remaining approximate (from a few tens up to a few hundred). Therefore, by measuring Ca^{2+} sparks in a muscle fiber, we have a direct measurement *in situ* of the gating behavior of RyRs belonging to a single CRU. In a CRU, RyRs are functionally coupled. The most common mechanism involved in triggering a rapid opening of RyR in a CRU is a calcium-induced calcium release mechanisms. However, a cooperative mechanism involving calstabine has also been proposed to explain coupled RyR gating in a CRU. Thus, posttranslational modifications of RyR may not only affect individual RyR properties but also CRU behavior. To date the threshold of modified channels (i.e. depleted from calstabin1, oxidized, nitrosylated and or phosphorylated) to affect the overall properties of a single CRU has not been characterized. This nevertheless explains the heterogeneity of the leaky behavior measured in RyR incorporated into lipid planar bilayer was heterogeneous. After MV only about 20% of the channels had a Po higher than 0.01 while 80% had a Po lower than 0.001, with a range comparable with control RyR. This leaky behavior was never observed in control condition, which had more homogeneous Po.

Taken together, these findings suggest that excessive SR Ca^{2+} leak due to redox-dependent modifications of the RyR1 is a key factor in VIDD pathogenesis that occurs at a

very early stage. Trolox was previously reported to prevent VIDD in a rat model. It was also reported that oxidative stress is required for MV-induced activation of calpain and caspase-3 in the diaphragm. Our data demonstrate that trolox also prevents RyR dysfunction induced by MV.

These data suggest that impaired RyR1 function is an early proximal event, which precedes histological damage. Furthermore, this early RyR1-dependent defect in intracellular Ca^{2+} homeostasis may be an important mediator of later histological remodeling in VIDD as previously reported in DMD. Indeed, impaired Ca^{2+} signaling may in part be responsible for the activation of Ca^{2+}-dependent proteolytic enzymes (caspases and calpains) as well as Ca^{2+}-dependent gene expression changes involved in deleterious muscle injury and wasting processes. One would expect these effects to be exacerbated with an increased duration of MV.

The present study also demonstrates that preventing RyR1 leak with S107, a small molecule known to stabilize the RyR1-calstabin1 interaction, can prevent muscle weakness induced by MV in mice. This concept is further proven by the lack of effect of S107 in calstabin1 deficient mice as already reported in other forms of skeletal muscle and cardiac pathophysiology. As mentioned earlier, the RyR complex is a converging target in many pathophysiological situations, and all of them have certainly not yet been reported. This can be explained in part by the ubiquitous function of Ca^{2+} in cellular processes but also in the complexity and fragility of the RyR macromolecular complex. Therefore, the fact that RyR1 is a potential mediator of muscle weakness in VIDD suggests that patients with co-morbidities and/or confounding factors that may affect RyR function such as heart failure or aging, might have a greater vulnerability to VIDD.

In conclusion, this study demonstrates the pathophysiological role of RyR1 in VIDD and strongly supports the hypothesis that preventing the RyR1-mediated SR Ca^{2+} leak induced by MV may provide a new therapeutic approach to prevent diaphragm muscle dysfunction in patients who require artificial respiratory support.

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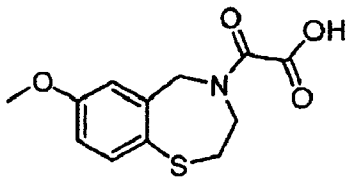
All publications, references, patents and patent applications cited herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art, from a reading of the disclosure, that various changes in form and detail can be made without departing from the true scope of the invention in the appended claims.

CLAIMS:

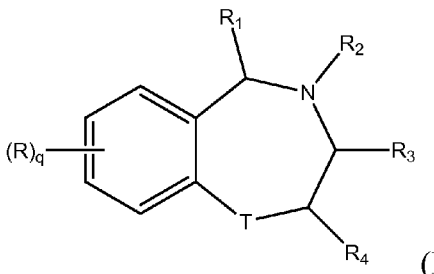
1. A method of treating ventilator-induced diaphragmatic dysfunction in a subject in need thereof comprising administering the subject with a therapeutically effective amount of an agent capable of stabilizing RyR1-calstabin1 interaction.

2. The method of claim 1 wherein the agent has the formula I:



(I)

3. The method of claim 1 wherein the agent is selected from the group consisting of compounds of the general Formula II:



(II)

wherein,

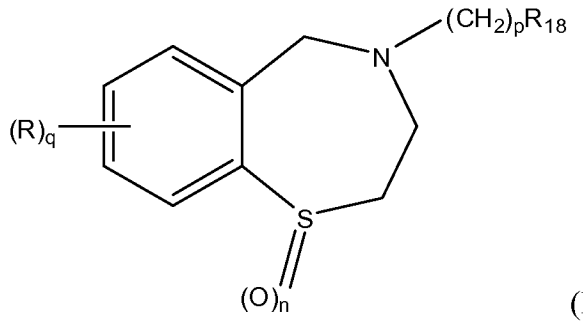
- T is O, CH2, NH, or $S=(O_2)_n$;
- n is 0, 1, or 2;
- q is 0, 1, 2, 3, or 4;
- each R is independently selected from the group consisting of H, halogen, -OH, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-SO_3H$, $-S(=O)_2$alkyl, -S(=O)alkyl, $-OS(=O)_2CF_3$, acyl, -O-acyl, alkyl, alkoxyl, alkylamino, alkylarylamino, alkylthio, cycloalkyl, alkylaryl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio, and (hetero-)arylamino; wherein each acyl, -O-acyl, alkyl, alkoxyl, alkylamino, alkylarylamino, alkylthio, cycloalkyl, alkylaryl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio, and (hetero-)arylamino may be optionally substituted;

- R_1 is selected from the group consisting of H, oxo, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted;
- R_2 is selected from the group consisting of H, $-C(=O)R_5$, $-C(=S)R_6$, $-SO_2R_7$, $-P(=O)R_8R_9$, $-(CH_2)_m-R_{10}$, alkyl, aryl, alkylaryl, heteroaryl, cycloalkyl, cycloalkylalkyl, and heterocyclyl; wherein each alkyl, aryl, alkylaryl, heteroaryl, cycloalkyl, cycloalkylalkyl, and heterocyclyl may be optionally substituted and wherein m is 0, 1, 2, 3, or 4;
- R_3 is selected from the group consisting of H, $-CO_2Y$, $-C(=O)NHY$, acyl, -O-acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted; and wherein Y is selected from the group consisting of H, alkyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl, and wherein each alkyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted;
- R_4 is selected from the group consisting of H, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl; wherein each alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, heteroaryl, and heterocyclyl may be optionally substituted;
- R_5 is selected from the group consisting of $-NR_{15}R_{16}$, $-(CH_2)zNR_{15}R_{16}$, $-NHNR_{15}R_{16}$, -NHOH, $-OR_{15}$, $-C(=O)NHNR_{15}R_{16}$, $-CO_2R_{15}$, $-C(=O)NR_{15}NR_{16}$, $-CH_2X$, acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkyl, alkenyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted, and wherein z is 1, 2, 3, 4, 5, or 6;
- R_6 is selected from the group consisting of $-OR_{15}$, $-NHNR_{15}R_{16}$, -NHOH, $-NR_{15}R_{16}$, $-CH_2X$, acyl, alkenyl, alkyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted;
- R_7 is selected from the group consisting of $-OR_{15}$, $-NR_{15}R_{16}$, $-NHNR_{15}R_{16}$, -NHOH, alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each alkyl, alkenyl, alkynyl, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted;

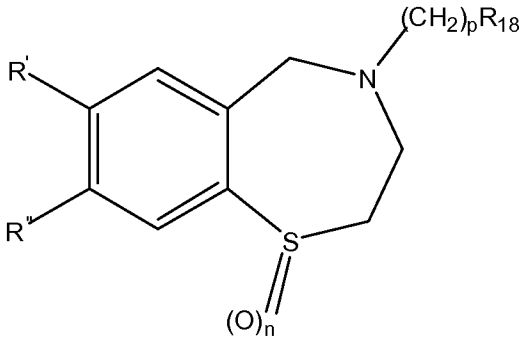
- R_8 and R_9 independently are selected from the group consisting of OH, acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted;
- R_{10} is selected from the group consisting of $-NR_{15}R_{16}$, OH, $-SO_2R_{11}$, $-NHSO_2R_{11}$, $C(=O)(R_{12})$, $NHC=O(R_{12})$, $-OC=O(R_{12})$, and $-P(=O)R_{13}R_{14}$;
- R_{11} , R_{12}, R_{13}, and R_{14} independently are selected from the group consisting of H, OH, NH_2, $-NHNH_2$, -NHOH, acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted; X is selected from the group consisting of halogen, -CN, $-CO_2R_{15}$, $-C(=O)NR_{15}R_{16}$, $-NR_{15}R_{16}$, $-OR_{15}$, $-SO_2R_7$ and $–P(=O)R_8R_9$ and
- R_{15} and R_{16} independently are selected from the group consisting of H, acyl, alkenyl, alkoxyl, OH, NH_2, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl; wherein each acyl, alkenyl, alkoxyl, alkyl, alkylamino, aryl, alkylaryl, cycloalkyl, cycloalkylalkyl, heteroaryl, heterocyclyl, and heterocyclylalkyl may be optionally substituted; and optionally R_{15} and R_{16} together with the N to which they are bonded may form a heterocycle which may be substituted; the nitrogen in the benzothiazepine ring may optionally be a quaternary nitrogen;

and enantiomers, diastereomers, tautomers, pharmaceutically acceptable salts, hydrates, solvates, complexes, and prodrugs thereof, or any combination thereof.

4. The method of claim 3 wherein the agent is selected from the group consisting of compounds of formula II-k or II-k-1:



(II-k)



(II-k-1)

- wherein:
- - R, R' and R" are independently selected from the group consisting of H, halogen, -OH, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-SO_3H$, $-S(=O)_2$alkyl, -S(=O)alkyl, $-OS(=O)_2CF_3$, acyl, alkyl, alkoxyl, alkylamino, alkylthio, cycloalkyl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio, and (hetero-)arylamino; and wherein each acyl, alkyl, alkoxyl, alkylamino, cycloalkyl, aryl, heterocyclyl, heterocyclylalkyl, alkenyl, alkynyl, (hetero-)aryl, (hetero-)arylthio may be substituted or unsubstituted;
- - R_{18} is selected from the group consisting of H, $-NR_{15}R_{16}$, $-C(=O)NR_{15}R_{16}$, $-(C=O)OR_{15}$, $-OR_{15}$, alkyl, aryl, cycloalkyl, heterocyclyl, and at one labeling group; wherein each alkyl, aryl, cycloalkyl, and heterocyclyl may be substituted or unsubstituted;
- and further wherein:
- - q is 0, 1, 2, 3, or 4;
- - p is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10; and
- n is 0, 1, or 2;

and enantiomers, diastereomers, tautomers, pharmaceutically acceptable salts, hydrates, solvates, complexes and prodrugs thereof.

5. The method of claim 4 wherein the agent is selected from the group consisting of:
- compounds of formula II-k wherein each R is independently selected from the group

consisting of H, halogen, -OH, OMe, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-S(=O)_2C_1$-C_4alkyl, $-S(=O)C_1$-C_4alkyl, $-S$-C_1-C_4alkyl, $-OS(=O)_2CF_3$, Ph, $-NHCH_2Ph$, -C(=O)Me, -OC(=O)Me, morpholinyl and propenyl; and n is 0, 1 or 2;

- compounds of formula II-k wherein R is H or is OMe at position 7 of the benzothiazepine ring;

- compounds of formula II-k-1 wherein R' and R" are independently selected from the group consisting of H, halogen, -OH, OMe, $-NH_2$, $-NO_2$, -CN, $-CF_3$, $-OCF_3$, $-N_3$, $-S(=O)_2C_1$-C_4alkyl, $-S(=O)C_1$-C_4alkyl, $-S$-C_1-C_4alkyl, $-OS(=O)_2CF_3$, Ph, $-NHCH_2Ph$, -C(=O)Me, -OC(=O)Me, morpholinyl and propenyl, and n is 0, 1 or 2;

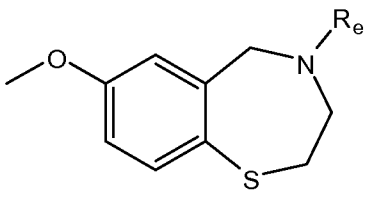
- compounds of formula II-k-1 wherein R' is H or OMe, and R" is H;

- compounds of formula II-k or II-k-1, wherein R_{18} is selected from the group consisting of $-NR_{15}R_{16}$, $-(C=O)OR_{15}$, $-OR_{15}$, alkyl, aryl, and at one labeling group; and wherein each alkyl and aryl may be substituted or unsubstituted;

- compounds of formula II-k or II-k-1 wherein, m is 1, and R_{18} is Ph, C(=O)OMe, C(=O)OH, aminoalkyl, NH_2, NHOH, or NHCbz; those compounds of formula II-k or II-k-1 wherein m is 0, and R_{18} is C_1-C_4 alkyl, such as Me, Et, propyl, and butyl;

- compounds of formula II-k or II-k-1 wherein m is 2, and R_{18} is pyrrolidine, piperidine, piperazine, or morpholine;

- compounds of Formula II-o:

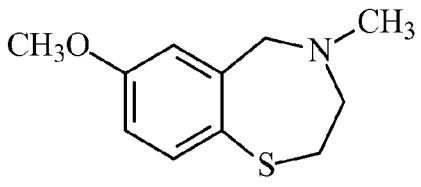


(II-o)

wherein:

- R_e is $-(C_1$-C_6 alkyl)-phenyl, $-(C_1$-C_6 alkyl)-$C(O)R_b$, or substituted or unsubstituted $-C_1$-C_6 alkyl; and
- R_b is -OH or $-O-(C_1$-C_6 alkyl), and

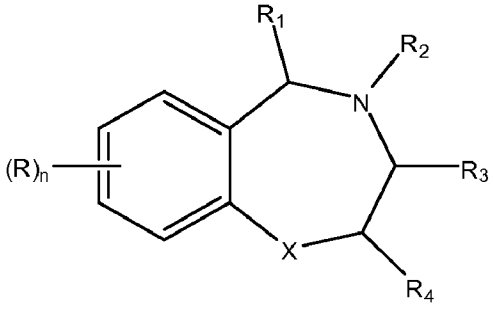
- wherein the phenyl or substituted alkyl is substituted with one or more of halogen, hydroxyl, -C_1-C_6 alkyl, -O-(C_1-C_6 alkyl), -NH_2, -NH(C_1-C_6 alkyl), -N(C_1-C_6 alkyl)$_2$, cyano, or dioxolane; and
- S107 having the formula III:



(III).

6. The method of claim 3 wherein the agent is selected from the group consisting of S1, S2, S3, S4, S5, S6, S7, S9, S11, S12, S13, S14, S19, S20, S22, S23, S24, S25, S26, S27, S36, S37, S38, S40, S43, S44, S45, S46, S47, S48, S49, S50, S51, S52, S53, S54, S55, S56, S57, S58, S59, S60, S61, S62, S63, S64, S66, S67, S68, S69, S70, S71, S72, S73, S74, S75, S76, S77, S78, S79, S80, S81, S82, S83, S84, S85, S86, S87, S88, S89, S90, S91, S92, S93, S94, S95, S96, S97, S98, S99, S100, S101, S102, S103, S104, S107, S108, S109, S110, S111, S112, S113, S114, S115, S116, S117, S118, S119, S120, S121, S122, S123, S136, S137, S138, S139, S140, S146, S147, S148, S149, S150, S151, S152, S153, S156, S157, S159, S160, S161, S166, S167, S182, S186, S189, S203, S217, S251, S252, S258, S277, S279, S282, S291, S293, S296, S301, S302, S306, S311, S312, S313, S318, S322, S324, S326, S331, S335, S337, S351, S352, S353, S354, S397, S398, S399, S423, S454, S463, S466, S470, S473, S477 and salts thereof.

7. The method of claim 1 wherein the agent is selected from the group of compounds of the general Formula IV:



(IV)

wherein

- n is 0, 1, 2, 3, or 4;
- X is O, -NR_5 or -$C(R_5)_2$;

- each R is independently selected from the group consisting of Z, R_5, $-OR_5$, $-SR_5$, $-N(R_5)_2$, $-NR_5C(=O)OR_5$, $-C(=O)N(R_5)_2$, $-C(=O)OR_5$, $-C(=O)R_5$, $-OC(=O)R_5$, NO_2, CN, $-CZ_3$, OCZ_3, $-N_3$, and $-P(=O)R_8R_9$;
- R_1 and R_3 are each independently selected from the group consisting of oxo, R_5, $-CH_2OR_5$, $-CH_2OC(=O)R_6$, $-C(=O)OR_5$, $-C(=O)NHR_5$, $-C(=O)R_5$, and $-OC(=O)R_5$;
- R_2 is selected from the group consisting of R_5, $-(C=O)R_6$, $-(C=S)R_6$, and $-(CH_2)_mR_{10}$, wherein m is 1, 2, 3, 4, 5, or 6; or
- R1 and R_2 together with the carbon and nitrogen to which they are respectively attached, form an unsubstituted or substituted heterocycle; or
- R_2 and R_3 together with the nitrogen and carbon to which they are respectively attached, form an unsubstituted or substituted heterocycle other than a piperazine; or
- R_3 and R_4 together with the carbon atoms to which they are respectively attached, form an unsubstituted or substituted cycloalkyl or heterocyclic ring; or
- R_4 is selected from the group consisting of R_5 and oxo;
- each R_5 is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, cycloalkylalkyl, heterocyclylalkyl, alkylaryl, and alkylheteroaryl;
- R_6 is selected from the group consisting of R5, $-(CH_2)_bNR_{13}R_{14}$, $-NR_5OR_5$, $-OR_5$, $-C(O)OR_5$, $-C(=O)NR_{13}R_{14}$, $-(CH_2)_cY$, and $-C(=O)R_5$, wherein b is 0, 1, 2, 3, 4, 5, or 6 and c is 1, 2, 3, 4 or 5;
- R_{10} is selected from the group consisting of R_5, $-OR_5$, $-SO_2R_{11}$, $-C(=O)R_{12}$, $-NH(C=O)R_{13}$, $-O(C=O)R_{12}$, and $-P(=O)R_8R_9$;
- R_8, R_9, R_{11} and R_{12} are independently selected from the group consisting of R_5, OR_5, and $-N(R_5)_2$;
- Y is selected from the group consisting of Z, $-CO_2R_5$, $-C(=O)NR_{13}R_{14}$, and $-OR_5$;
- Z is a halogen selected from F, Cl, Br and I;
- R_{13} and R_{14} are independently selected from the group consisting of R_5, or R_{13} and R_{14} together with the N to which they are bonded may form an unsubstituted or substituted heterocycle; and
- wherein each alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, cycloalkylalkyl, heterocyclylalkyl, alkylaryl, and alkylheteroaryl may be substituted or unsubstituted;

- wherein the nitrogen in the benzoxazepine ring may optionally be a quaternary nitrogen;

8. The method of claim 7 wherein the agent is selected from the group consisting of ARM136, ARM137, ARM138, ARM139, ARM140, ARM146, ARM147, ARM148, ARM149, ARM150, ARM151, ARM152, ARM153, ARM156, ARM157, ARM159, ARM160, ARM161, ARM166, ARM167, ARM182, ARM186, ARM187, ARM189, ARM 200, ARM203, ARM 205, ARM217, ARM251, ARM252, ARM258, ARM277, ARM279, ARM282, ARM291, ARM293, ARM296, ARM301, ARM302, ARM306, ARM311, ARM312, ARM313, ARM318, ARM322, ARM324, ARM326, ARM331, ARM335, ARM337, ARM351, ARM352, ARM353, ARM354, ARM397, ARM398, ARM399, ARM423, ARM454, ARM463, ARM466, ARM470, ARM473 and ARM477.

9. The method of claim 1 wherein the subject suffers from respiratory failure and/or heart failure which can be aggravated by sepsis, metabolic disorder, neuromuscular diseases, or surgery along with post-surgical recovery.

10. The method of claim 1 wherein the subject suffers from a disease selected from the group consisting of Chronic Obstructive Pulmonary Disease (COPD), pneumonia, sepsis, Acute Respiratory Distress Syndrome (ARDS), Severe Acute Respiratory Syndrome (SARS) and cystic fibrosis (CF).

11. The method of claim 1 wherein the subject suffers from a trauma.

12. The method of claim 1 wherein ventilator-induced diaphragmatic dysfunction results from prolonged controlled mechanical ventilation (MV) greater than 12 hours.

13. The method of claim 1 wherein the agent is administered before MV, immediately after MV initiation, during MV, and/or immediately after MV.

14. A method for screening a plurality of test substances useful for treatment of ventilator-induced diaphragmatic dysfunction comprising the steps consisting of (a) testing each of the test substances for its ability stabilize the RyR1-calstabin1 interaction and/or to reduce SR Ca2+ leak via RyR1 channel and (b) and positively selecting the test substances capable of restoring said integrated stress response.

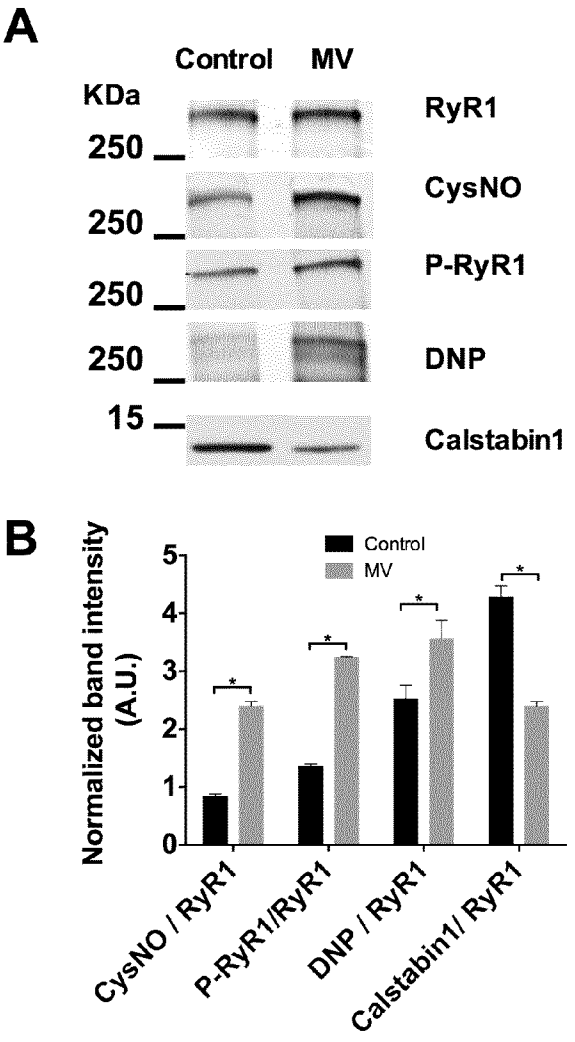

A
Control
MV
KDa
250
250
250
250
15
RyR1
CysNO
P-RyR1
DNP
Calstabin1
B
Normalized band intensity (A.U.)
Control
MV
0
1
2
3
4
5
*
*
*
*
CysNO / RyR1
P-RyR1/RyR1
DNP / RyR1
Calstabin1/ RyR1
C
Control
P(o)= 0.00043
4 pA
1 s
MV
P(o)= 0.28482

Figure 1

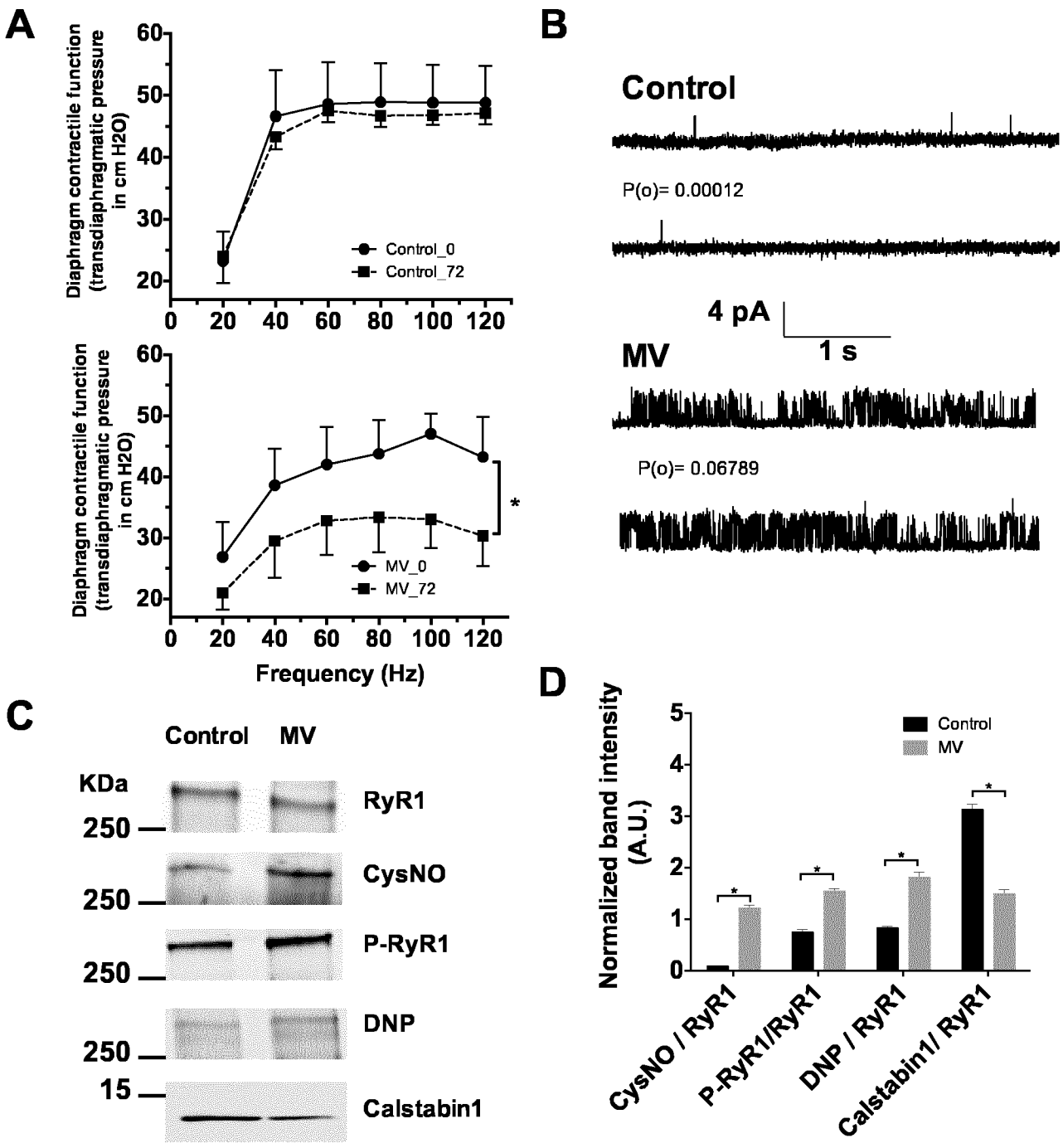

A
Diaphragm contractile function (transdiaphragmatic pressure in cm H2O)
Control_0
Control_72
MV_0
MV_72
Frequency (Hz)
*
B
Control
P(o)= 0.00012
4 pA
1 s
MV
P(o)= 0.06789
C
Control
MV
KDa
250
250
250
250
15
RyR1
CysNO
P-RyR1
DNP
Calstabin1
D
Normalized band intensity (A.U.)
Control
MV
CysNO / RyR1
P-RyR1/RyR1
DNP / RyR1
Calstabin1/ RyR1

Figure 2

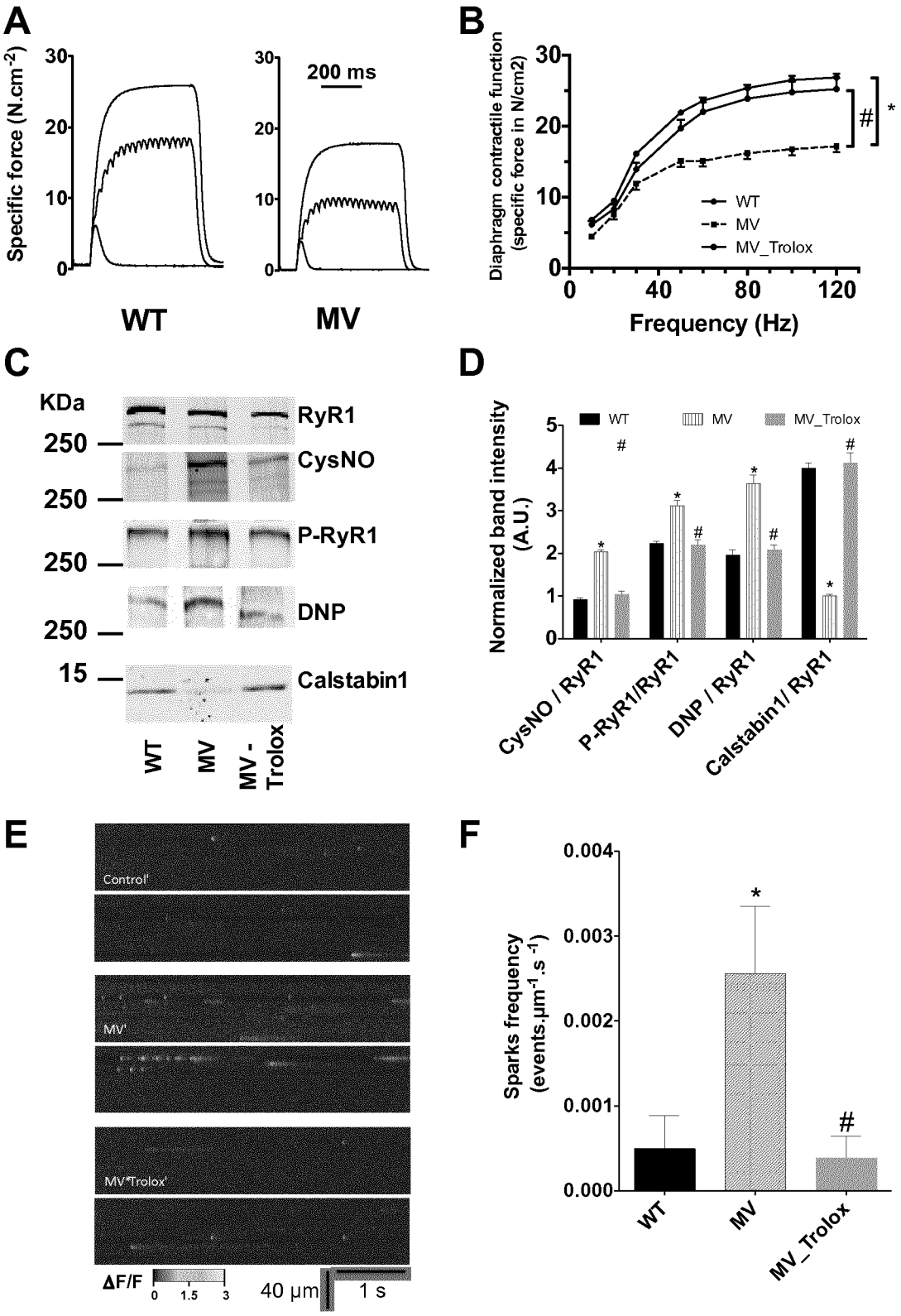

A
Specific force (N.cm-2)
30
20
10
0
200 ms
WT
MV
B
Diaphragm contractile function (specific force in N/cm2)
WT
MV
MV_Trolox
Frequency (Hz)
0
40
80
120
#
*
C
KDa
250
250
250
250
15
RyR1
CysNO
P-RyR1
DNP
Calstabin1
WT
MV
MV - Trolox
D
Normalized band intensity (A.U.)
WT
MV
MV_Trolox
CysNO / RyR1
P-RyR1/RyR1
DNP / RyR1
Calstabin1/ RyR1
E
Control
MV
MV+Trolox
ΔF/F
0
1.5
3
40 µm
1 s
F
Sparks frequency (events.µm-1.s-1)
0.004
0.003
0.002
0.001
0.000
WT
MV
MV_Trolox

Figure 3

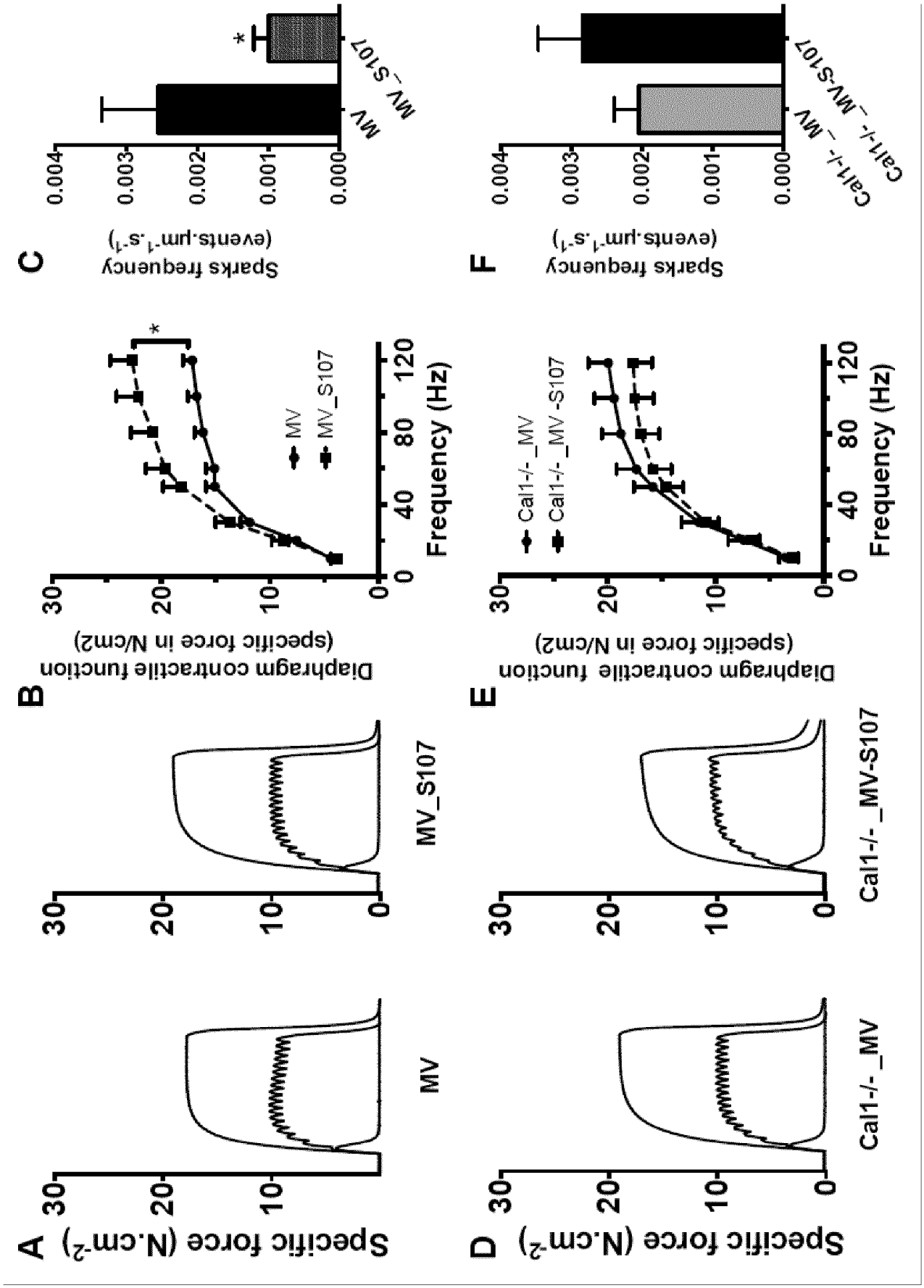

A
Specific force (N.cm-2)
MV
MV_S107
B
Diaphragm contractile function
(specific force in N/cm2)
MV
MV_S107
Frequency (Hz)
C
Sparks frequency
(events.µm-1.s-1)
MV
MV_S107
D
Specific force (N.cm-2)
Cal1-/-_MV
Cal1-/-_MV-S107
E
Diaphragm contractile function
(specific force in N/cm2)
Cal1-/-_MV
Cal1-/-_MV-S107
Frequency (Hz)
F
Sparks frequency
(events.µm-1.s-1)
Cal1-/-_MV
Cal1-/-_MV-S107

Figure 4

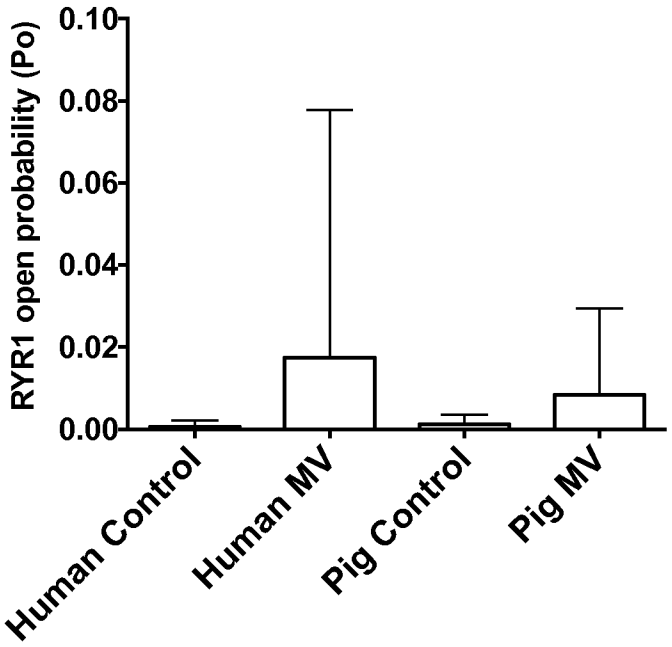


Figure 5A

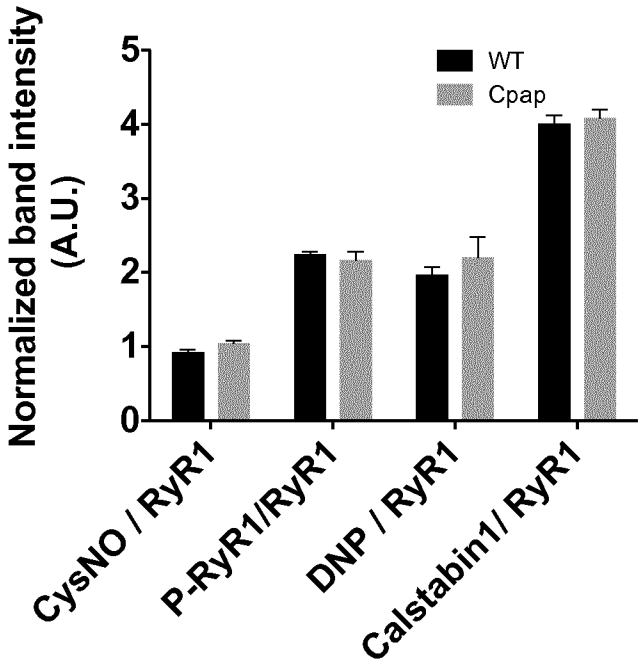


Figure 5B

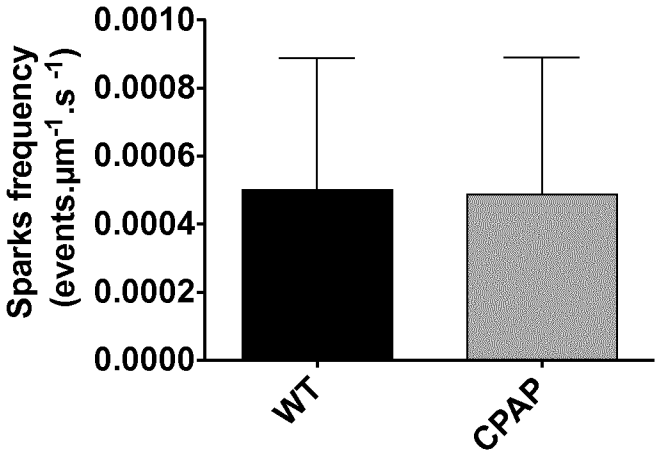


Figure 5C

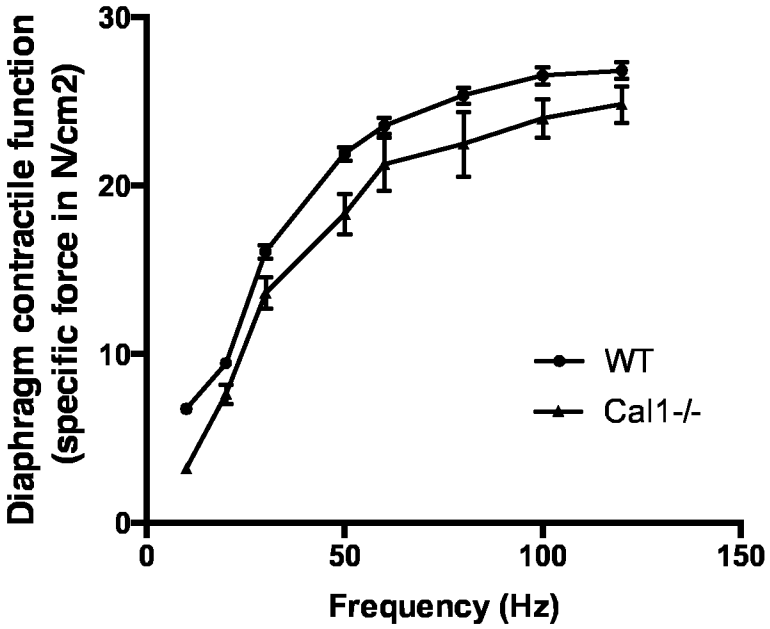


Figure 5D

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/065705

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/5513 A61K31/553 A61K31/554 A61P11/00 G01N33/6872 G01N33/68
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JENNA L. BETTERS ET AL: "Trolox Attenuates Mechanical Ventilation-induced Diaphragmatic Dysfunction and Proteolysis", AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE, vol. 170, no. 11, 1 December 2004 (2004-12-01), pages 1179-1184, XP055088885, ISSN: 1073-449X, DOI: 10.1164/rccm.200407-939OC cited in the application the whole document ----- -/--	1

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 September 2014	01/10/2014

Name and mailing address of the ISA/	Authorized officer
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/194767 A1 (MARKS ANDREW R [US] ET AL MARKS ANDREW ROBERT [US] ET AL) 31 August 2006 (2006-08-31) page 33, paragraphs 219, 220 claim 117	14
X	WO 2012/019076 A1 (UNIV COLUMBIA [US]; UNIV MONTPELLIER [FR]; MARKS ANDREW R [US]; LACAMP) 9 February 2012 (2012-02-09) page 21	14
A	SAMIR JABER ET AL: "Clinical review: Ventilator-induced diaphragmatic dysfunction - human studies confirm animal model findings!", CRITICAL CARE, vol. 15, no. 2, 1 January 2011 (2011-01-01), page 206, XP055088830, ISSN: 1364-8535, DOI: 10.1183/09031936.00091108 page 4, left-hand column - page 6, left-hand column, paragraph 1	1-14

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/065705

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2006194767 A1	31-08-2006	NONE	
WO 2012019076 A1	09-02-2012	NONE	