Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 2836511 C 2021/10/19

(11)(21) 2 836 511

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2012/05/28
(87) Date publication PCT/PCT Publication Date: 2012/12/06
(45) Date de délivrance/Issue Date: 2021/10/19
(85) Entrée phase nationale/National Entry: 2013/11/18
(86) N° demande PCT/PCT Application No.: CA 2012/050349
(87) N° publication PCT/PCT Publication No.: 2012/162828
(30) Priorité/Priority: 2011/06/02 (US61/492,556)

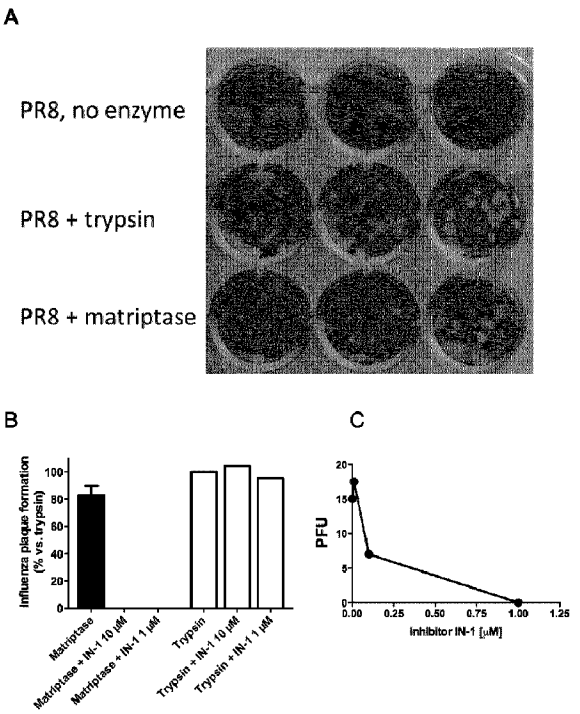
(51) Cl.Int./Int.Cl. *C07K 5/11* (2006.01), *A61K 31/713* (2006.01), *A61K 38/07* (2006.01), *A61K 39/42* (2006.01), *A61K 9/12* (2006.01), *A61K 9/72* (2006.01), *A61P 31/14* (2006.01), *A61P 31/16* (2006.01), *A61P 35/00* (2006.01), *C07K 5/10* (2006.01), *C12N 9/64* (2006.01)

(72) Inventeurs/Inventors:
RICHTER, MARTIN, CA;
LEDUC, RICHARD, CA;
COLOMBO, ELOIC, CA;
MARSAULT, ERIC, CA

(73) Propriétaire/Owner:

(54) Titre : INHIBITEURS DE MATRIPTASE ET UTILISATIONS DE CEUX-CI CONTRE LES INFECTIONS A ORTHOMYXOVIRIDAE

(54) Title: MATRIPTASE INHIBITORS AND USES THEREOF AGAINST ORTHOMYXOVIRIDAE INFECTIONS



(57) Abrégé/Abstract:
The present invention provides matriptase inhibitors and compositions for treating and preventing orthomyxovirus infections such as flu infections. The present invention also provides novel compounds, compositions, methods of use, uses and kits thereof for

CA 2836511 C 2021/10/19
(11)(21) 2 836 511
(13) C

(73) Propriétaires(suite)/Owners(continued):SOCPRA SCIENCES SANTE ET HUMAINES S.E.C., CA

(74) Agent: ROBIC

(57) Abrégé(suite)/Abstract(continued):
inhibiting matriptase. Such compounds are useful for treating and preventing orthomyxovirus infections, such as flu infections, and for inhibiting tumor growth, progression and/or metastasis.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 December 2012 (06.12.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/162828 A1

(51) International Patent Classification:
C07K 5/11 (2006.01) *A61P 31/14* (2006.01)
A61K 31/713 (2006.01) *A61P 31/16* (2006.01)
A61K 38/07 (2006.01) *A61P 35/00* (2006.01)
A61K 39/42 (2006.01) *C07K 5/10* (2006.01)
A61K 9/12 (2006.01) *C12N 9/64* (2006.01)
A61K 9/72 (2006.01)

(21) International Application Number:
PCT/CA2012/050349

(22) International Filing Date:
28 May 2012 (28.05.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/492,556 2 June 2011 (02.06.2011) US

(71) Applicant *(for all designated States except US)*: **SOCPRA SCIENCES SANTÉ ET HUMAINES S.E.C.** [CA/CA]; 35, rue Radisson, Bureau 100, Sherbrooke, Québec J1L 1E2 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **RICHTER, Martin** [CA/CA]; 1120, rue Langevin, Sherbrooke, Québec J1E 1N1 (CA). **LEDUC, Richard** [CA/CA]; 1125, rue Pascal, Sherbrooke, Québec J1J 4B8 (CA). **COLOMBO, Éloïc** [FR/CA]; 54, rue Kennedy Nord, Sherbrooke, Québec J1E 2B3 (CA). **MARSAULT, Éric** [CA/CA]; 540, rue Meilleur, Sherbrooke, Québec J1J 2P6 (CA).

(74) Agent: **GOUDREAU GAGE DUBUC**; 2000 McGill College, Suite 2200, Montréal, Québec H3A 3H3 (CA).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

[Continued on next page]

(54) Title: MATRIPTASE INHIBITORS AND USES THEREOF AGAINST ORTHOMYXOVIRIDAE INFECTIONS

(57) Abstract: The present invention provides matriptase inhibitors and compositions for treating and preventing orthomyxovirus infections such as flu infections. The present invention also provides novel compounds, compositions, methods of use, uses and kits thereof for inhibiting matriptase. Such compounds are useful for treating and preventing orthomyxovirus infections, such as flu infections, and for inhibiting tumor growth, progression and/or metastasis.

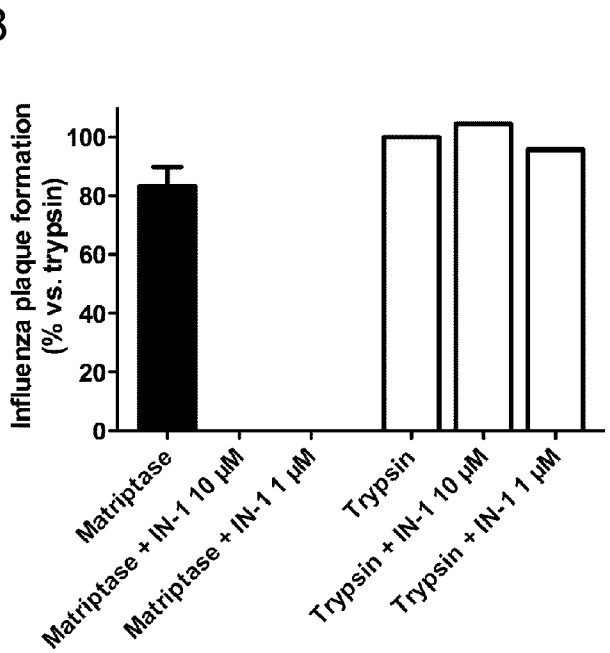


Figure 7

WO 2012/162828 A1

WO 2012/162828 A1

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

TITLE OF THE INVENTION

MATRIPTASE INHIBITORS AND USES THEREOF AGAINST ORTHOMYXOVIRIDAE INFECTIONS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] Blank.

FIELD OF THE INVENTION

[0002] The present invention relates to the prevention and/or treatment of orthomyxoviridae infections (*e.g.*, influenza infection).

REFERENCE TO SEQUENCE LISTING

[0003] Pursuant to 37 C.F.R. 1.821(c), a sequence listing is submitted herewith as an ASCII compliant text file named 14692_25 - Seq Listing_ST25.txt, created on May 23, 2012 and having a size of 22 kilobytes.

BACKGROUND OF THE INVENTION

Influenza - introduction

[0004] Influenza is a highly contagious respiratory disease caused by the influenza virus. Influenza respiratory infections, and particularly influenza A because of its continuous antigenic evolution, have the potential to lead to deadly pandemics (*e.g.*, 1918, 1957 and 1968). In recent history, events such as those of Hong Kong in 1997, SARS in 2003, as well as the transmission of H5N1 avian influenza directly to humans, underscore the scope and severity of the consequences associated to such infections. In fact, according to the WHO there are 3 to 5 million severe cases of influenza each year and 250 000 to 500 000 deaths from the virus.

[0005] The influenza virus, which belongs to the orthomyxoviridae family, is enveloped and contains 8 single-stranded RNA segments coding for 10 to12 proteins. While there are three types of influenza viruses (A, B and C), only types A and B cause significant illnesses in humans, type A viruses being the most problematic. Type A influenza viruses are responsible for most of the seasonal epidemics and are further subtyped according to surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). The influenza virus evades the host immune system by undergoing a continuous antigenic evolution through the processes called antigenic « drift » and « shift». Antigenic drift is the evolution of viral strains through frequent mutations among antibody binding sites of surface antigens leading to the emergence of new variants not adequately recognized by the host immune system. Antigenic drift is the reason why, every season, it is necessary to identify and predict the most probable strains that will circulate in order to produce the most appropriate vaccines for annual vaccinations. Antigenic shift results from the reassortment

of the genetic material of co-circulating strains, leading to the replacement of surface glycoproteins HA, and less frequently NA, which in turn leads to the emergence of severe epidemics and even pandemics (1). Such a scenario is possible for the H5N1 avian virus which has a 50-60% death rate and the H5 component of which has never circulated in the human population. Because new viruses generated through antigenic shift also undergo antigenic drift, this means that in the case of the H5N1 virus, this could lead to efficient transmission from human to human and thus to a pandemic.

[0006] Unlike surface antigens, the internal proteins of the virus do not sustain the same mutational pressures and remain more conserved between strains. During the host adaptive cellular immune response, CD8 T cells eliminate infected epithelial cells through a process that involves perforin, granzymes, and cytokines such as TNF-α and IFNγ. Although it does not provide sterilizing immunity, as does the humoral response, the cellular response significantly reduces lung viral titers. In addition, as opposed to the humoral response which is ineffective against viruses bearing mutated surface antigens, the cellular response recognizes internal epitopes which tend to be conserved between viral strains. It has been shown both experimentally and clinically that the influenza virus easily evades the host humoral response to its surface antigens thanks to its particular mutational characteristics.

[0007] Two classes of anti-influenza drugs are currently available: inhibitors of the viral M2 channel (*e.g.*, amantadine and rimantadine) and inhibitors of the viral neuraminidase (*e.g.*, zanamivir and oseltamivir) (2). Inhibitors of the viral M2 channel interact directly with the viral M2 ionic channel which participates in the acidification and the decapsulation of the virus in cellular endosomes, and the viral neuraminidase allow the detachment of nascent virions. Targeting viral proteins has proven to be an effective strategy. However, because of the mutational characteristics of the influenza virus and the widespread use of antiviral drugs, resistance has become an important problem (2, 3). As a result, inhibitors of the M2 ion channel are no longer recommended for the prophylactic treatment of influenza. Furthermore, according to the Center for Disease Control, almost all currently circulating strains of H3N2 influenza A are resistant to amantadine and almost all circulating H1N1 influenza A seasonal strains are resistant to oseltamivir (Tamiflu™). Fortunately, most H1N1 influenza A strains stemming from the 2009 pandemic are susceptible to the drug, although several resistant strains of this virus have been isolated. Therefore, targeting host cellular mechanisms that are crucial for influenza viral entry, protein synthesis, maturation, and replication, provides an exciting alternative strategy potentially obviating the resistance problem.

[0008] Hemagglutinin (HA) protein plays an essential role in binding to and entering into host cells during the virus infection process. Hemagglutinin (HA) binds to monosaccharide sialic acids that are present on the surface of its target host cells. The cell membrane then engulfs the virus through endocytosis and forms endosomes. The binding affinity of a type of influenza virus to sialic acids on epithelial cells of the respiratory system, typically in the nose, pharynx, trachea, bronchi, bronchioles, alveoli and lungs of mammals and intestines of birds, can affect the capability of the virus to infect the species and the capability to spread among different individuals.

[0009] Influenza HA is synthesized as a single protein precursor termed HA0 and since the virus does not

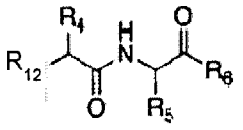
encode any protease, host cell proteases are required for the cleavage of HA0 into subunits HA1 and HA2. This cleavage is required for the protein to change conformation in the acidic conditions in the endosome (9, 10). This change in the protein's conformation exposes the hydrophobic fusion peptide located in the HA2 subunit (11, 12). This allows the virus to fuse with the host cell. The hemagglutinin proteins of pathogenic avian influenza viruses are characterized by multibasic cleavage sites containing furin-like recognition sequences RXXR (13, 14). Since some subtilisin-like proteases such as furin or other proprotein convertases are ubiquitous, the HA glycoprotein of avian viruses utilizes multiple tissues and sites for its activation and allows infection and replication of these viruses in many cell types (pantropicity) (14). One of the severe manifestations of avian flu virus is a life-threatening encephalitis. On the other hand, the HA glycoprotein of non-avian viruses does not have the polybasic furin-recognition site. These viruses have monobasic cleavage sites recognized by other proteases (*e.g.*, TTSPs) of the host (14).

[0010] Blank

SUMMARY OF THE INVENTION

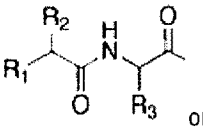
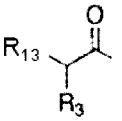
[0011] In the studies described herein, the inventors have shown that the type II transmembrane protease matriptase (also known as ST14 or TMPRSS14) is capable of cleaving and activating influenza HA. Matriptase mRNA was identified in several human respiratory system epithelial cells as well as in a RNA extract from healthy human lungs. Furthermore, the inventors have demonstrated that the matriptase protein is expressed in human epithelial cell lines of the respiratory system. Adding matriptase to Mandin-Darby Canine Kidney (MDCK) cells, (which do not allow multicycle influenza replication without an exogenous protease such as trypsin), promotes viral plaque formation, thereby indicating that matriptase is able to activate HA and promotes multicycle influenza replication in these cells. In addition, using RNA interference technology it was shown that siRNAs targeting matriptase significantly blocks influenza replication in a human epithelial cell line that can promote influenza replication without exogenous enzyme addition. Furthermore, the inventors have generated a class of selective potent matriptase inhibitors possessing a "serine trap" (*e.g.*, a ketobenzothiazole moiety) which block matriptase activity thereby inhibiting HA activation and influenza replication in human airway epithelial cells.

[0012] In accordance with a first aspect of the present invention, there is provided a compound of formula (1):



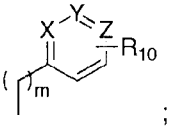
(1)

[0013] or a pharmaceutically acceptable salt, hydrate, solvate, or stereoisomer thereof, wherein

R_{12} is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl,  or  or one or more amino acids residues;

R_{13} is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl or SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl or one or more amino acids residues;

[0014] R_1 is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl or SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl or one or more amino acids residues;

[0015] R_2 and R_5 are independently -CH_2-R_8 or ;

wherein R_8 is (CH_2)n-NH(C=NH)NH- R_{14} or (CH_2)n-NH-R_{14}, wherein,

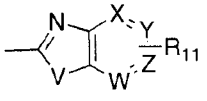
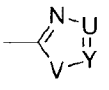
n is 1 to 4;

m is 0 to 3;

R_{10} is -C(=NH)-NH_2, NH_2 or NH alkyl; and

R_{14} is H, alkyl, NH_2, NO_2 or COalkyl;

[0016] R_3 and R_4 are independently an alkyl, an aryl, a substituted alkyl, a substituted aryl, an heteroaryl, a substituted heteroaryl, a side chain of an amino acid or a substituted side chain of an amino acid;

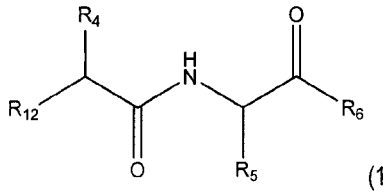
R_6 is , , CF_3, CO_2H, $CONH_2$, CONH-alkyl, CONH-aryl or CO-(amino acid residue)$_p$, wherein p is 1 to 3;

wherein W, X, Y and Z are independently N or CH;

V and U are independently O, NH, NCH_3 or S; and

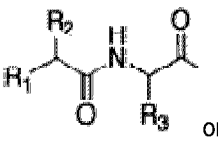
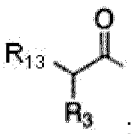
R_{11} is H, CO_2H, CONH-alkyl, CONH-aryl, aryl, heteroaryl, halide or CO-(amino acid residue)$_q$, wherein q is 1 or 2.

[0016a] In a specific embodiment, there is provided a compound of formula (1):



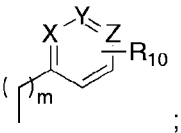
(1)

or a pharmaceutically acceptable salt, hydrate, solvate, or stereoisomer thereof, wherein

R_{12} is NHR_7; wherein R_7 is  or  ;

R_{13} is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl or one or more amino acids residues;

R_1 is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl or one or more amino acids residues;

R_2 and R_5 are independently -CH_2-R_8 or  ;

wherein R_8 is (CH_2)n-NH(C=NH)NH- R_{14} or (CH_2)n-NH-R_{14}, wherein,

n is 1 to 4;

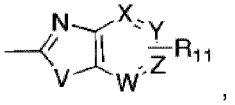
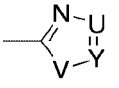
m is 0 to 3;

R_{10} is -C(=NH)-NH_2, NH_2 or NH alkyl; and

R_{14} is H, alkyl, NH_2, NO_2 or COalkyl;

R_3 is a linear alkyl; an alkyl substituted with $(CO)NH_2$, (CO)OH, NH_2, NHCO-alkyl, NHCO-aryl, $NHSO_2$-alkyl, $NHSO_2$-aryl or heteroaryl; an aryl, a substituted aryl, an heteroaryl or a substituted heteroaryl;

R_4 is an alkyl, an aryl, a substituted alkyl, a substituted aryl, an heteroaryl, a substituted heteroaryl, a side chain of an amino acid or a substituted side chain of an amino acid;

R_6 is  ,  , CF_3, CO_2H, $CONH_2$, CONH-alkyl, CONH-aryl or CO-(amino acid residue)$_p$, wherein p is 1 to 3;

wherein W, X, Y and Z are independently N or CH;

V and U are independently O, NH, NCH_3 or S; and

R_{11} is H, CO_2H, CONH-alkyl, CONH-aryl, aryl, heteroaryl, halide or CO-(amino acid residue)$_q$, wherein q is 1 or 2;

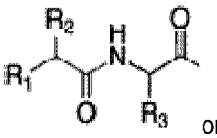
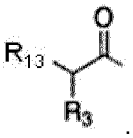
wherein unless indicated otherwise:

the substituted aryl, heteroaryl or alkyl is an aryl, heteroaryl or alkyl substituted with alkyl; alkenyl; alkynyl; hydroxy; alkoxy; amide; halide; heterocyclic; aryl; aryl substituted on any or all possible aryl positions by hydroxyl, amide, halides, nitrile, nitro, alkyl, amino, amides or sulfonamides; guanidine; amidine; heteroaryl; aryloxy; halogen; haloalkyl; cyano; nitro; amino (-NH_2); lower alkylamino; lower dialkylamino; NHCO-alkyl; NHCO-aryl; $NHSO_2$-alkyl; $NHSO_2$-aryl; amido (-(CO)NR_a); azido; acyl (—(CO)R_a); alkoxymethyl; mercapto (—S—R_a); sulfoxy (—SO—R_a); sulfonyl —$(SO)_2$—R_a); sulfonamide (—$S(O)_2N(R_a)_2$); carbonate (—O(CO)—O—R_a); oxyacyl (—O(CO)—R_a); carboxyl (—CO_2H); ester (—(CO)OR_6); carbamate (—O(CO)—$N(R_a)_2$); wherein R_a is H or lower alkyl, lower alkenyl,

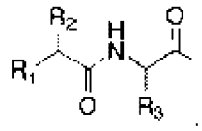
lower alkynyl, aryl, heteroaryl or heterocycle; a side chain of a natural amino acid or a substituted side chain of a natural amino acid; and

the substituted side chain of an amino acid is a side chain of an amino acid substituted with alkoxy, hydroxyl, halide, aminoalkyl, amide, sulfonamide, aryl, alkyl, heteroaryl, amidine.

[0017] In a specific embodiment,

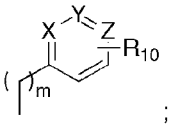
R_7 is  or .

[0018] In another specific embodiment,

R_7 is .

[0019] In another specific embodiment,

(i) R_1 is -H or -NH_2;

(ii) R_2 is ;

(iii) m is 1;

(iv) W is CH;

(v) X is CH;

(vi) Y is CH;

(vii) R_{10} is -C(=NH)NH_2; or

(viii) any combination of (i) to (vii).

[0020] In another specific embodiment,

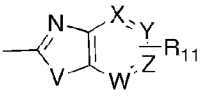
(i) R_1 is -NH_2;

(ii) R_2 is -CH_2-R_8;

(iii) R_3 is a substituted alkyl;

(iv) R_4 is an alkyl;

(v) R_5 is -CH_2-R_8;

(v) R_6 is ;

(vi) R_8 is -$(CH_2)_3$NH(C=NH)NH_2;

(vii) W is CH;

(viii) X is CH;

(ix) Y is CH;

(x) Z is CH;

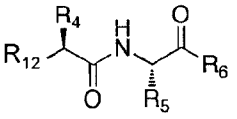
(xi) V is S;

(xii) R_{11} is H; or

(xiii) any combination of (i) to (xii).

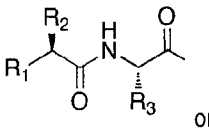
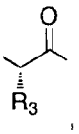
[0021] In another specific embodiment, R3 is a substituted alkyl, which is substituted with an alkyl, aryl, $(CO)NH_2$, (CO)OH, NH_2, NHCO-alkyl, NHCO-aryl, $NHSO_2$-alkyl, $NHSO_2$-aryl or heteroaryl. In another specific embodiment, R_3 is a substituted C_1 to C_6 alkyl. In another specific embodiment, R_3 is a substituted C_1 to C_3 alkyl. In another specific embodiment, R_3 is $-(CH_2)_2C(=O)NH_2$. In another specific embodiment, R_4 is a C_1 to C_6 alkyl. In another specific embodiment, R_4 is a C_1 to C_3 alkyl. In another specific embodiment, R_4 is $-CH_3$. In another specific embodiment, R_2 and R_5 have the (S) configuration.

[0022] In another specific embodiment, the compound is of formula (1.1):



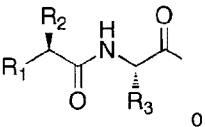
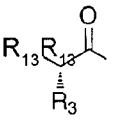
wherein

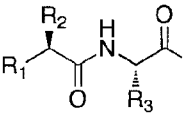
R_{12} is H, NH_2 or NHR_7, wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl,

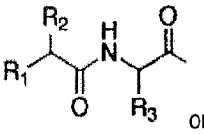
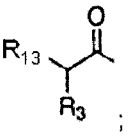

or

,

and

[0023] wherein R_1, R_2, R_3, R_4, R_5, R_6, R_8, R_{10}, R_{11} and R_{13} are as defined in herein.

[0024] In another specific embodiment, R_7 is  or .

[0025] In another specific embodiment, R_7 is .

[0026] In another specific embodiment, R_{12} is NH_2,  or ;

R_{13} is NH_2;

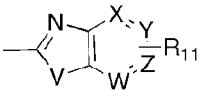
R_1 is NH_2;

R_2 is the side chain of arginine;

R_5 is the side chain of arginine or lysine;

R_3 is the side chain of glutamine;

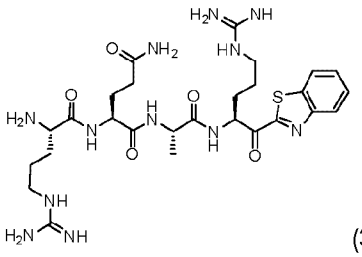
R_4 is the side chain of alanine;

R_6 is  ;

wherein W, X, Y and Z are CH; and V is S; and

R_{11} is H.

[0027] In another specific embodiment, the compound is of formula (3):



(3).

[0028] In accordance with another aspect of the present invention, there is provided a composition comprising the compound of the present invention, and

(a) a further therapeutic agent for treating or preventing an orthomyxovirus infection and/or associated symptoms;

(b) a pharmaceutically acceptable excipient; or

(c) a combination of (a) and (b).

[0029] In a specific embodiment, said orthomyxovirus infection is an influenza infection.

[0030] In another specific embodiment, said further therapeutic agent is a viral M2 ion channel inhibitor or a neuraminidase inhibitor.

[0031] In another specific embodiment, said further therapeutic agent is Tamiflu™ (oseltamivir), Relenza™ (zanamivir), laninamivir, peramivir, amantadine, rimantadine, ribavirin vitamin C, Cold Fx™, echinacea, ginseng or any combination thereof.

[0032] In another specific embodiment, the composition is formulated for direct administration into lungs.

[0033] In another specific embodiment, the composition is for administration by an inhaler or nebulizer.

[0034] In accordance with another aspect of the present invention, there is provided a method of preventing or treating an orthomyxovirus infection in a subject, said method comprising administering to said subject a therapeutically effective amount of a matriptase inhibitor, or a composition comprising said inhibitor, to said subject.

[0035] In a specific embodiment of the method, said matriptase inhibitor is a compound is a composition as defined herein.

[0036] In another specific embodiment, said composition is a composition as defined herein.

[0037] In another specific embodiment, said matriptase inhibitor is one or more of the compounds depicted in Tables 2 to 6.

[0038] In another specific embodiment, said matriptase inhibitor is an antibody specifically recognizing the catalytic domain of matriptase. In another specific embodiment, said antibody specifically recognizing the catalytic domain of matriptase comprises the sequence of the light and/or heavy chain CDR1, CDR2 and CDR3 defined in Table 1. In another specific embodiment, said antibody specifically recognizing the catalytic domain of matriptase comprises the sequence of the light and/or heavy chain defined in Table 1. In another specific embodiment, said matriptase inhibitor is an RNA interference agent that decreases the expression of matriptase. In another specific embodiment, wherein said RNA interference agent is an siRNA.

[0039] In another specific embodiment, the orthomyxovirus infection is an influenza infection. In another specific embodiment, the influenza infection is an influenza type A infection.

[0040] In accordance with another aspect of the present invention, there is provided a method of inhibiting matriptase activity in a cell, comprising contacting the cell with an effective amount of: (i) a compound as defined herein; or (ii) a composition as defined herein; whereby said matriptase activity is inhibited. In a specific embodiment, said cell is an epithelial cell of the respiratory system.

[0041] In accordance with another aspect of the present invention, there is provided a use of a matriptase inhibitor, or a composition comprising said inhibitor, for treating or preventing an orthomyxovirus infection in a subject.

[0042] In accordance with another aspect of the present invention, there is provided a use of a matriptase inhibitor, or a composition comprising said inhibitor, for the manufacture of a medicament for treating or preventing an orthomyxovirus infection in a subject.

[0043] In a specific embodiment, said matriptase inhibitor is a compound as defined herein. In another

specific embodiment, said composition or medicament is a composition as defined herein. In another specific embodiment, said matriptase inhibitor is one or more of the compounds depicted in Tables 2 to 6. In another specific embodiment, said matriptase inhibitor is an antibody specifically recognizing the catalytic domain of matriptase.In another specific embodiment, said antibody specifically recognizing the catalytic domain of matriptase comprises the sequence of the light and/or heavy chain CDR1, CDR2 and CDR3 defined in Table 1. In another specific embodiment, said antibody specifically recognizing the catalytic domain of matriptase comprises the sequence of the light and/or heavy chain defined in Table 1. In another specific embodiment, said matriptase inhibitor is an RNA interference agent that decreases the expression of matriptase. In another specific embodiment, said RNA interference agent is an siRNA. In another specific embodiment, the orthomyxovirus infection is an influenza infection. In another specific embodiment, the influenza infection is an influenza type A infection.

[0044] In accordance with another aspect of the present invention, there is provided a use of (i) a compound as defined herein; or (ii) a composition as defined herein, for inhibiting matriptase activity in a cell.

[0045] In accordance with another aspect of the present invention, there is provided a use of (i) a compound as defined herein; or (ii) a composition as defined herein, for the manufacture of a medicament for inhibiting matriptase activity in a cell. In a specific embodiment, said cell is an epithelial cell of the respiratory system.

[0046] In accordance with another aspect of the present invention, there is provided a matriptase inhibitor, or a composition comprising said inhibitor, for treating or preventing an orthomyxovirus infection in a cell.

[0047] In accordance with another aspect of the present invention, there is provided a matriptase inhibitor, or a composition comprising said inhibitor, for the manufacture of a medicament for treating or preventing an orthomyxovirus infection in a cell. In a specific embodiment, said matriptase inhibitor is a compound as defined herein. In another specific embodiment, said composition or medicament is a composition as defined herein. In another specific embodiment, said matriptase inhibitor is one or more of the compounds depicted in Tables 2 to 6. In another specific embodiment, said matriptase inhibitor is an antibody specifically recognizing the catalytic domain of matriptase. In another specific embodiment, said antibody specifically recognizing the catalytic domain of matriptase comprises the sequence of the light and/or heavy chain CDR1, CDR2 and CDR3 defined in Table 1. In another specific embodiment, said antibody specifically recognizing the catalytic domain of matriptase comprises the sequence of the light and/or heavy chain defined in Table 1. In another specific embodiment, said matriptase inhibitor is an RNA interference agent that decreases the expression of matriptase. In another specific embodiment, said RNA interference agent is an siRNA. In another specific embodiment, the orthomyxovirus infection is an influenza infection. In another specific embodiment, the influenza infection is an influenza type A infection.

[0048] In accordance with another aspect of the present invention, there is provided a compound as defined herein, or the composition as defined herein, for inhibiting matriptase activity in a cell.

[0049] In accordance with another aspect of the present invention, there is provided a compound as defined

herein, or a composition as defined herein, for the manufacture of a medicament for inhibiting matriptase activity in a cell. In a specific embodiment, said cell is an epithelial cell of the respiratory system.

[0050] In accordance with another aspect of the present invention, there is provided a kit comprising a matriptase inhibitor, or a composition comprising said inhibitor, and instructions to use same for treating or preventing an orthomyxovirus infection in a subject. In another specific embodiment, said matriptase inhibitor or composition is the matriptase inhibitor or composition as defined herein. In another specific embodiment, the orthomyxovirus infection is an influenza infection. In another specific embodiment, the influenza infection is an influenza type A infection.

[0051] In accordance with another aspect of the present invention, there is provided a method for inhibiting tumor growth, progression and/or metastasis in a subject in need thereof, the method comprising administering an effective amount of a compound as defined herein, or a composition a compound as defined herein, to said subject.

[0052] In accordance with another aspect of the present invention, there is provided a use of a compound as defined herein, or a composition as defined herein, for inhibiting tumor growth, progression and/or metastasis in a subject.

[0053] In accordance with another aspect of the present invention, there is provided a use of the compound as defined herein, or the composition as defined herein, for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject

[0054] In accordance with another aspect of the present invention, there is provided a compound as defined herein, or a composition as defined herein, for inhibiting tumor growth, progression and/or metastasis in a subject.

[0055] In accordance with another aspect of the present invention, there is provided a compound as defined herein, or a composition as defined herein, for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.

[0056] In accordance with another aspect of the present invention, there is provided a composition comprising the compound of the present invention, and (a) a further therapeutic agent for treating or preventing flu infections and/or associated symptoms; (b) a pharmaceutically acceptable excipient; or (c) a combination of (a) and (b).

[0057] In a specific embodiment of the composition of the present invention, said further therapeutic agent is a viral M2 ion channel inhibitor or a neuraminidase inhibitor. In another specific embodiment of the composition of the present invention, said further therapeutic agent is Tamiflu™ (oseltamivir), Relenza™ (zanamivir), laninamivir, peramivir, amantadine, rimantadine, ribavirin, vitamin C, Cold Fx™, echinacea, ginseng or any combination thereof.

[0058] In another specific embodiment of the composition of the present invention, the composition is

formulated for direct administration into lungs. In another specific embodiment of the composition of the present invention, the composition is formulated for administration by an inhaler or nebulizer.

[0059] In accordance with a further aspect of the present invention, there is provided a method of preventing or treating an orthomyxovirus infection in a subject, said method comprising administering to said subject a therapeutically effective amount of: (i) the compound of the present invention; (ii) the composition of the present invention; or (iii) an siRNA that decreases the expression of matriptase, whereby said flu infection is prevented or treated.

[0060] In a specific embodiment of the method of the present invention, the orthomyxovirus infection is an influenza infection. In another specific embodiment of the method of the present invention, the influenza infection is an influenza type A infection.

[0061] In accordance with a further aspect of the present invention, there is provided a method of inhibiting matriptase activity in a cell, comprising contacting the cell with an effective amount of: (i) the compound of the present invention; (ii) the composition of the present invention; or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase, whereby said matriptase activity is inhibited.

[0062] In a specific embodiment of the method of the present invention, said cell is an epithelial cell of the respiratory system.

[0063] In accordance with a further aspect of the present invention, there is provided a use of: (i) the compound of the present invention; (ii) the composition of the present invention; or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase, for (a) treating or preventing an orthomyxovirus infection; or (b) inhibiting matriptase activity in a cell.

[0064] In accordance with a further aspect of the present invention, there is provided a use of: (i) the compound of the present invention; (ii) the composition of the present invention; or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase, in the preparation of a medicament for (a) treating or preventing an orthomyxovirus infection; or (b) inhibiting matriptase activity in a cell.

[0065] In a specific embodiment of the use of the present invention, the orthomyxovirus infection is an influenza infection. In another specific embodiment of the use of the present invention, the influenza infection is an influenza type A infection.

[0066] In another specific embodiment of the use of the present invention, said cell is an epithelial cell of the respiratory system.

[0067] In accordance with a further aspect of the present invention, there is provided a compound of the present invention or a composition of the present invention or a short interfering RNA molecule (siRNA) that

decreases the expression of matriptase, for (a) treating or preventing an orthomyxovirus infection; or (b) inhibiting matriptase activity in a cell.

[0068] In a specific embodiment of the compound, composition or siRNA of the present invention, the orthomyxovirus infection is an influenza infection. In another specific embodiment of the compound, composition or siRNA of the present invention, the influenza infection is an influenza type A infection. In another specific embodiment of the compound, composition or siRNA of the present invention, said cell is an epithelial cell of the respiratory system.

[0069] In accordance with a further aspect of the present invention, there is provided a kit comprising: (i) the compound of the present invention; (ii) the composition of the present invention; or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase, and instructions to use same for (a) treating or preventing an orthomyxovirus infection; or (b) inhibiting matriptase activity in a cell.

[0070] In a specific embodiment of the kit of the present invention, the orthomyxovirus infection is an influenza infection. In another specific embodiment of the kit of the present invention, the influenza infection is an influenza type A infection. In another specific embodiment of the kit of the present invention, said cell is an epithelial cell of the respiratory system.

[0071] In a specific embodiment of the method, the use, the compound for use, composition for use or siRNA for use, or kit of the present invention, the influenza infection is cause by a type A virus. In a specific embodiment of the method, the use, the compound for use, composition for use or siRNA for use, or kit of the present invention, the influenza infection is cause by a type B virus. In a specific embodiment of the method, the use, the compound for use, composition for use or siRNA for use, or kit of the present invention, the influenza infection is cause by a type C virus.

[0072] In another aspect, the present invention relates to a composition having formula (1), (1.1) or (1.2) for the prevention or treatment of influenza infection. In an embodiment, the composition further comprises a pharmaceutically acceptable excipient. In an embodiment, the composition inhibits hemagglutinin activation. In an embodiment, the composition inhibits sustained viral replication and propagation. In an embodiment the composition reduces viral load/titre in an infected subject's blood, cells or in a nasopharingeal swab of that subject. In an embodiment, the cells are epithelial cells of the respiratory system (*e.g.*, nasal, of the pharynx, tracheal, bronchial, bronchiolar, alveolar and lung epithelial cell).

[0073] In a further aspect, the present invention concerns a method of treating or preventing an orthomyxovirus infection in a subject in need thereof comprising administering an effective amount of i) a compound or composition of the present invention comprising a matriptase inhibitor of the present invention or ii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase. In an embodiment, the inhibitor is that of formula (1). In a further embodiment, the inhibitor is that of formula (1.1). In a further embodiment, the

inhibitor is that of formula (1.2).

[0074] Accordingly, the composition of the present invention may comprise additional products for preventing or treating any symptoms of an orthomyxovirus infection (*e.g.*, influenza) (*e.g.*, antitussif, expectorant, anti-inflammatory analgesic, decongestant) or may be used in combination with any additional flu medicine. Non-limiting examples of additional flu medicine include viral M2 ion channel inhibitors and neuraminidase inhibitors. Known therapeutic agents for the prevention and treatment of flu infections includes Tamiflu™ (oseltamivir), Relenza™ (zanamivir), laninamivir, peramivir, amantadine, rimantadine, ribavirin, vitamin C, Cold Fx™, echinacea, ginseng, etc. In another particular embodiment, the composition of the present invention comprises as sole medicinal ingredient for the prevention or treatment of flu infection a composition comprising the compound of formula (1), (1.1), (1.2), (2), (2.1) or (3).

[0075] The present invention also provides a method of inhibiting matriptase activity in a cell comprising contacting the cell with an effective amount of (i) a compound of the present invention; (ii) a composition comprising a compound of the present invention or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase, whereby matriptase activity is inhibited. In an embodiment, the inhibitor is that of formula (1). In another embodiment, the inhibitor is that of formula (1.1). In another embodiment, the inhibitor is that of formula (1.2). In another embodiment, the inhibitor is that of formula (2). In another embodiment, the inhibitor is that of formula (2.1). In another embodiment, the inhibitor is that of formula (3).

[0076] In another aspect, the present invention is concerned with a method of inhibiting influenza infection in a cell comprising contacting the cell with an effective amount of (i) a compound of the present invention; (ii) a composition comprising a compound of the present invention; or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase, whereby influenza infection is inhibited. In an embodiment, the inhibitor is that of formula (1). In another embodiment, the inhibitor is that of formula (1.1). In another embodiment, the inhibitor is that of formula (1.2). In another embodiment, the inhibitor is that of formula (2). In another embodiment, the inhibitor is that of formula (2.1). In another embodiment, the inhibitor is that of formula (3).

[0077] The present invention also relates to the use of (i) a compound of the present invention; (ii) a composition comprising a compound of the present invention; or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase, for (a) treating or preventing an orthomyxovirus infection; or (b) inhibiting matriptase activity in a cell.

[0078] The present invention also relates to the use of (i) a compound of the present invention; (ii) a composition comprising a compound of the present invention; or (iii) a short interfering RNA molecule (siRNA) that decreases the expression of matriptase for the manufacture of a medicament for the prevention or treatment of flu infections.

[0079] In another aspect, the present invention relates to the use of the compound or compositions of the

present invention for research assays (*e.g.*, biochemical, enzymatic assays, *in vitro* or *in vivo* models).

[0080] The compositions of the present invention comprising an inhibitor or RNAi or antibody of the present invention may be formulated in various ways according to the route of administration. The compositions or RNAis of the present invention may be administered in any suitable way including for example orally, nasally or topically. Administration is carried out in a customary manner, preferably directly to the lung of the subject (*e.g.*, via a nebulizer or an inhaler).

[0080a] In another aspect, the present invention relates to :

- Use of the compound as defined herein, or the composition as defined herein, for treating or preventing an influenza type A, B or C infection in a subject.
- Use of the compound as defined herein, or the composition as defined herein, for the manufacture of a medicament for treating or preventing an influenza type A, B or C infection in a subject.
- Use of (i) the compound as defined herein; or (ii) the composition as defined herein, for inhibiting matriptase activity in a cell.
- Use of (i) the compound as defined herein; or (ii) the composition as defined herein, for the manufacture of a medicament for inhibiting matriptase activity in a cell.
- Use of the compound as defined herein, or the composition as defined herein, for inhibiting tumor growth, progression and/or metastasis in a subject.
- Use of the compound as defined herein, or the composition as defined herein, for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.
- The compound as defined herein, or the composition as defined herein, for inhibiting tumor growth, progression and/or metastasis in a subject.
- The compound as defined herein, or the composition as defined herein, for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.
- A kit comprising a compound as defined herein, or a composition as defined as defined herein, and instructions to use same for treating or preventing an influenza type A, B or C infection in a subject.
- Use of the compound as defined herein, or the composition the compound as defined herein, for inhibiting tumor growth, progression and/or metastasis in a subject.
- Use of the compound the compound as defined herein, or the composition the compound as defined herein,

Date Reçue/Date Received 2020-08-06

for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.

– The compound the compound as defined herein, or the composition the compound as defined herein, for inhibiting tumor growth, progression and/or metastasis in a subject.

– The compound the compound as defined herein, or the composition the compound as defined herein, for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.

[0081] Other and features of the present invention will become more apparent upon reading of the following non-restrictive description of specific embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

[0082] Figures 1A and 1B show the matriptase nucleotide sequence (SEQ ID NO:1); and protein sequence (SEQ ID NO:2), respectively.

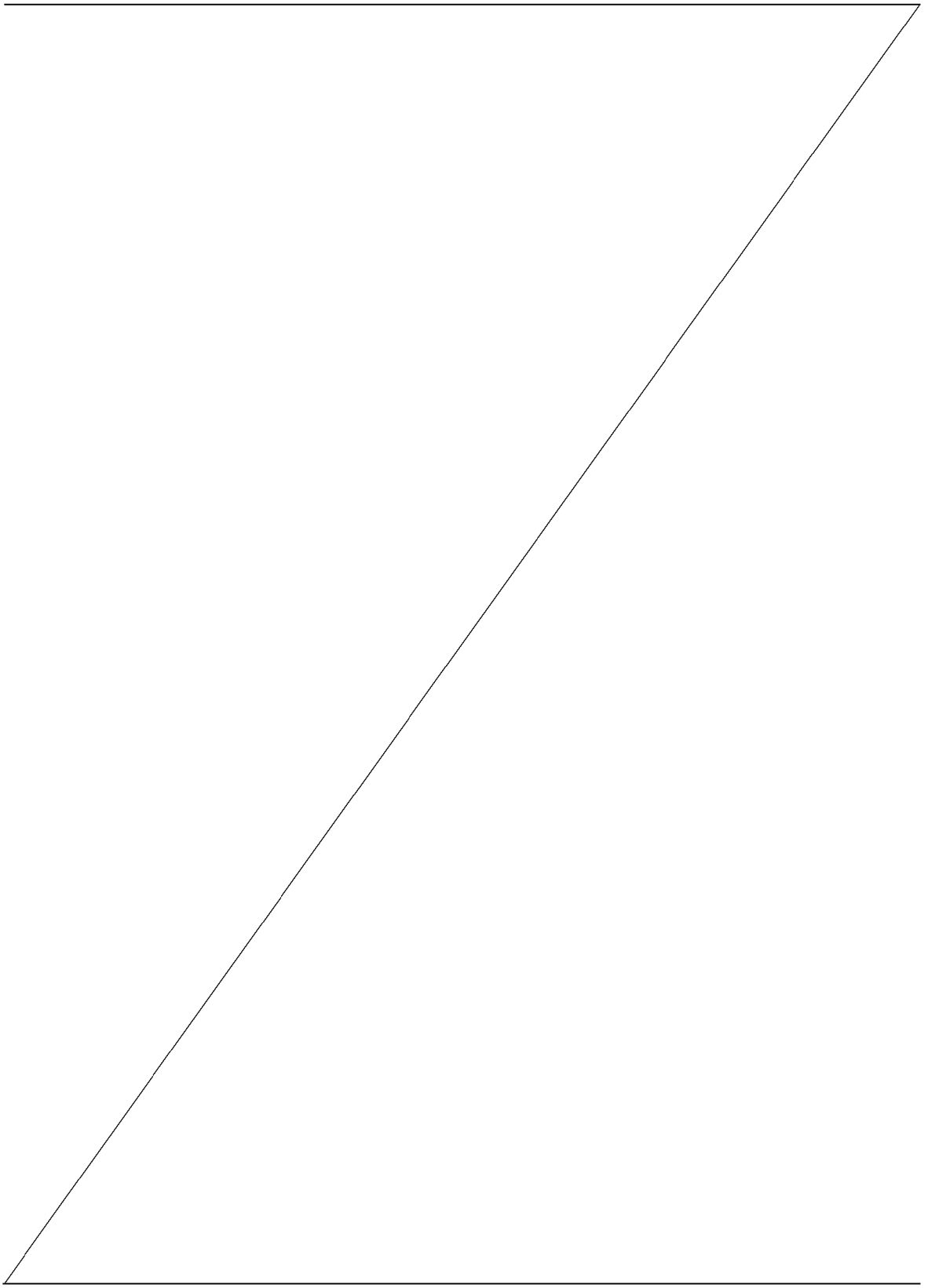
[0083] Figure 2 shows matriptase mRNA expression in human respiratory system epithelial cell lines (Calu-3, H292, A549, Beas-2B) and in an RNA extract from normal human lung (Lung; Ambion).

[0084] Figure 3 shows Western blot analyses of matriptase protein expression in human respiratory system epithelial cell lines (upper panel: A549, Calu-3, Beas-2 and H292; lower panel: Calu-3, H292, NHBE and 16-HBE). Whole cell protein extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Using a matriptase-specific rabbit anti-human/mouse antibody and anti-rabbit-HRP secondary antibody, bands were revealed using ECL reagent. In the lower panel, Calu-3, NCI-H292 and NHBE cell extracts were analyzed on the same gel and 16-HBE cell extracts were analyzed on a separate gel using smaller wells. Results presented are reconstituted from the two gels.

[0085] Figure 4 shows that the matriptase protein is expressed by airway epithelial cells (Calu-3 cells) and that it is localized at least in the plasma membranes of these cells. Upper panel: Immunofluorescence of matriptase in Calu-3 cells. Calu-3 cells were grown on coverslips and stained with DAPI (staining of the nucleus), DiI (staining of membranes) and rabbit anti-human matriptase antibody and anti-rabbit Alexa-488. Fluorescence was detected using an epifluorescence microscope and appropriate filters. Lower panel: Calu-3 cells were grown in transwell chambers to confluence (tight junctions; elevated electrical resistance). Transwell membranes were removed and placed in paraffin blocks. Five-micron sections were cut and stained with DAPI (staining of the nucleus) and rabbit anti-human matriptase antibody and anti-rabbit Alexa-647. Fluorescence was detected using confocal microscopy. The straight line under the cells represents non-specific staining of the transwell membrane on which cells were

Date Reçue/Date Received 2020-08-06

grown.



Date Reçue/Date Received 2020-08-06

[0086] Figure 5 shows the cleavage of HA sequence internally quenched fluorescent octapeptides by Matriptase. Internally quenched ortho-Aminobenzoic acid (Abz) fluorescent peptide substrates (IQFPs) were designed based on consensus sequences of influenza HAs most relevant to humans (H1 and H3) with the general structure Abz-XXXR↓GLFG-Tyr(3-NO_2) (SEQ ID NO: 6). H1 sequence was Abz-IQSR↓GLFG-Tyr(3-NO_2) (SEQ ID NO: 7) and H3 sequence was Abz-KQTR↓GLFG-Tyr(3-NO_2) (SEQ ID NO: 8). Downward pointing arrow represents cleavage site between enzyme recognition site (P4-P1) and first four amino acids of the influenza fusion peptide (P1'-P4'; conserved among all influenza strains). IQFPs containing HA sequences of H1 or H3 at fixed concentration were incubated with recombinant Matriptase as previously described (Beliveau, F., A. Desilets, and R. Leduc. 2009. Probing the substrate specificities of matriptase, matriptase-2, hepsin and DESC1 with internally quenched fluorescent peptides. *Febs J* **276**:2213-2226). These data indicate that Matriptase has the potential to cleave H1 and H3 sequences.

[0087] Figure 6 shows Western blot analyses of influenza HA cleavage by matriptase. A/Puerto Rico/8/34 influenza virus (PR8) HA0 viruses (viruses with uncleaved HA) were generated as described in Klenk *et al.* 1984. HA0 viruses were incubated with matriptase 500nM (or trypsin 200nM as positive control) for 2h at 37°C and viral particles were disrupted using triton X-100 at a final concentration of 1%. Viral protein extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Using a H1 (H1N1/Pan)-specific rabbit anti-viral protein antibody and anti-rabbit-HRP secondary antibody, bands were revealed using ECL reagent. HA0 indicates uncleaved HA and HA1 represents the HA1 fragment of cleaved HA.

[0088] Figure 7 shows that matriptase promotes multicycle influenza replication in MDCK cells infected with 25 PFU of A/Puerto Rico/8/34 influenza virus (PR8). Panel A: Recombinant matriptase (43nM) was added to MDCK cells and cells were infected with 25 PFU of A/Puerto Rico/8/34 influenza virus (PR8). Viral plaques are observed in the presence of matriptase (or trypsin the standard protease used in this assay) thereby indicating viral activation by the enzyme. Compared to trypsin, matriptase was approx. 80% as effective in viral plaque initiation. Panel B: Compilation of results and efficacy of IN-1 (Compound **1**) at inhibiting matriptase in this assay (no effect on trypsin indicating selectivity). Panel C: Concentration-response curve of inhibition of PR8 plaque formation in the presence of IN-1 in MDCK cells supplemented with Matriptase (43nM). Viral plaques are observed in the presence of matriptase (or trypsin) thereby indicating viral activation by the enzyme.

[0089] Figure 8 shows that siRNAs targeting matriptase mRNA significantly block matriptase protein expression. Calu-3 cells were transfected with two different siRNAs (50nmol) targeted toward matriptase mRNA using various concentrations of Lipofectamine RNAiMAX™ (0.5 to 1.5µl). Scrambled siRNAs were used as controls. Panel A: Western blot of total protein extracts from Calu-3 cells. Matriptase was identified using a rabbit anti-human ST14 (matriptase) antibody. S: transfected scrambled siRNA control; 1: transfected siRNA 1 targeted to matriptase; 2: transfected siRNA 2 targeted to matriptase; Ctrl untransfected cells. Densitometric analysis of siRNA knock-down of matriptase protein expression in Calu-3 cells. Panel B: Densitometric analysis of blots performed in (A) revealed that matriptase-targeted siRNAs significantly inhibited matriptase protein expression in Calu-3 cells 48h after

transfection.

[0090] Figure 9 shows that matriptase promotes influenza HA activation and viral replication in airway epithelial cells. Calu-3 cells were transfected (Lipofectamine RNAiMAX™ 1.5µl) with siRNAs targeted to matriptase 48h before infection (scrambled siRNAs were used as controls). Following transfection, cells were washed and infected with influenza A/Puerto Rico/8/34 (1000 PFU) for 1h to allow viral attachment. Cells were then washed and incubated for 48h. Supernatants were collected and assayed for infectious viral particles using the MDCK/trypsin system.

[0091] Figure 10 shows that a ketobenzothiazole tetrapeptide representative of the compounds of the invention inhibits matriptase. Recombinant purified human matriptase (amino acids 596-855) (1nM) was incubated with vehicle (1% DMSO) or 1µM of inhibitors (IN-1 and IN-2) for 10 minutes at room temperature. Proteolytic activity was monitored using Boc-Gln-Ala-Arg-AMC as a fluorogenic peptide (50µM). Results are presented as relative activity compared to the control reaction (vehicle = 1).

[0092] Figures 11A and 11B show the dissociation of enzyme:inhibitor (EI) complex using dilution experiments. Figure 11A reports the dissociation curves for ketobenzothiazole inhibitor IN-1 and Figure 11B reports the dissociation curves for irreversible inhibitor Glu-Gly-Arg chloromethyl ketone (EGR-CMK). Matriptase and increasing concentrations of (A) RQAR-ketobenzothiazole (IN-1, compound **1** in Table 7) or (B) EGR-CMK were pre-incubated for 20 minutes at room temperature and diluted 2000 times in a reaction buffer containing 400 µM Boc-QAR-AMC. Final concentration of matriptase was 0.25 nM and was varied as indicated for inhibitors. Proteolytic activity in the reaction buffer was measured as described below.

[0093] Figure 12 shows that the ketobenzothiazole inhibitor IN-1, inhibits matriptase *in cellulo*. Results show the enzymatic activity measured (fluorescence units per minutes per µl of media) under each condition.

[0094] Figures 13A, 13B and 13C show the selectivity of IN-1 for matriptase. Figure 13A shows the inhibition of the enzymatic activity of various proteases (1nM) by compound IN-1 (1µM). Data is presented as relative activity compared to the control reaction (vehicle = 1) (white columns). Figures 13B and 13C show the IC_{50} determination of IN-1 for matriptase (B) and hepsin (C), respectively. Relative activities (Vi/Vo) were plotted as a function of the log transformation of inhibitor concentration (log I). Data were fitted by non-linear regression analysis using GraphPad™ Prism 5 software. IC_{50} corresponds to the concentration of inhibitor that results in a response half way between the maximal response and the maximal inhibitory response.

[0095] Figure 14 shows that compound IN-1 completely abrogates influenza replication in MDCK cells with matriptase as the activating serine protease. MDCK cells were cultured to confluence and infected with 25 PFU of influenza A/Puerto Rico/8/34 virus for 1h (PR8) to allow viral attachment, in incomplete medium containing BSA. Cells were then washed and a 1.8% Avicel microcrystalline cellulose solution in MEM containing 43nM of matriptase was added to allow viral plaque formation. Matriptase inhibitor IN-1 was added to the assay at 1h post-viral

attachment.

[0096] Figure 15 is a graphic representation of a compilation of results presented in Figures 7 and 14, and additional experiments using trypsin as the activating protease. Viral plaques were counted in each replicate and results were compared.

[0097] Figure 16 shows that the IN-1 compound blocks influenza replication in airway epithelial cells. Upper panel: Calu-3 cells were cultured to confluence, washed and infected with influenza A/Puerto Rico/8/34 (1000 PFU) for 1h to allow viral attachment. Cells were then washed and incubated for 72h in the presence or absence of 1μM IN-1 (or DMSO as the vehicle control 1:1000 final concentration). Supernatants were collected and assayed for infectious viral particles using the MDCK/trypsin viral plaque assay system. Lower panel: Inhibitory concentration-response curve of IN-1 in Calu-3 cells infected with influenza PR8 (H1N1). The procedure is identical to that described above except that IN-1 was added at increasing concentrations. Data was plotted using GraphPad™ Prism software and IC_{50} was determined.

[0098] Figure 17 shows inhibitory concentration-response curve of IN-1 in Calu-3 cells infected with influenza viruses A/Hong Kong/X-31 or a strain of the 2009 pandemic H1N1 virus isolated in Quebec during the pandemic (A/California-like/04/09). Calu-3 cells were grown to confluence and infected with 1000 PFU of influenza virus for 1h. Cells were washed and IN-1 was added at increasing concentrations. Cell supernatants containing newly formed viruses were harvested and titrated using MDCK cells. Data was plotted using GraphPad™ Prism software.

[0099] Figure 18 shows a molecular modeling of the docking of IN-1 in the active site of matriptase. The image was generated using Pymol (The PyMOL Molecular Graphics System, Version 1.2r1pre, Schrödinger, LLC.26). Numbers refer to matriptase numbering.

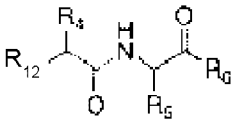
DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[00100] The results presented herein show for the first time that matriptase, a type II transmembrane serine protease, is expressed in epithelial cells of the respiratory system both at the mRNA and protein levels. Moreover, the inventors have discovered that matriptase is capable of promoting multicycle influenza replication both in a system where an exogenous protease is required for influenza hemagglutinin activation and in a system where the cells possess all of the required enzymes to activate the influenza virus. Further, results confirmed that matriptase is involved in influenza replication in airway epithelial cells through inhibition assays using new inhibitors as well as in a siRNA knockdown system targeting matriptase. Finally, the results presented herein show that matriptase inhibitors are effective at blocking influenza replication *in vitro* and that these show selectivity for matriptase.

[00101] The matriptase inhibitors described target the host cell (a host protein) and not the virus directly. It is hypothesized that this may reduce viral adaptation since the viral proteins are not targeted directly, thus obviating continuous viral mutations. Second, since both influenza A and B types require proteolytic cleavage of hemagglutinin

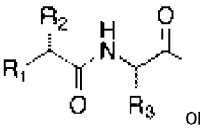
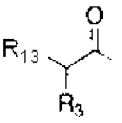
for their activation, it is hypothesized that the matriptase inhibitors described herein has a broad spectrum of activity. As the current anti-influenza antivirals (M2 ion channel blockers and neuraminidase inhibitors) exhibit more and more resistance, inhibition of matriptase may be used as first line therapy. Inhibition of matriptase may also be used in combination with other antivirals (*e.g.*, M2 ion channel blockers and neuraminidase inhibitors) in attempts to minimize the generation of escape mutants that lead to antiviral resistance.

[00102] The present invention relates to a compound of formula (1):



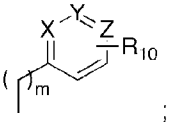
(1)

[00103] or a pharmaceutically acceptable salt, hydrate, solvate, or stereoisomer thereof, wherein

[00104] R_{12} is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl,  or  or one or more amino acid residues, in an embodiment 1 to 4 (1, 2, 3 or 4);

[00105] R_{13} is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl, or one or more amino acid residues, in an embodiment 1 to 3 (1, 2 or 3);

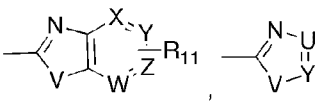
[00106] R_1 is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl, or one or more amino acid residues, in an embodiment 1 to 3 (1, 2 or 3);

[00107] R_2 and R_5 are independently -CH_2-R_8 or ;

[00108] wherein R_8 is (CH_2)n-NH(C=NH)NH-R_{14} or (CH_2)n-NH- R_{14}, wherein n is 1 to 6, in an embodiment 1 to 4 (1, 2, 3 or 4), in an embodiment 1 to 3 (1, 2 or 3); m is 0 to 6, in an embodiment 0 to 3 (0, 1, 2 or 3); and R_{10} is -C(=NH)-NH_2, NH_2 or NH alkyl; and R_{14} is H, alkyl, NH_2, NO_2, COalkyl;

[00109] R_3 and R_4 are independently an alkyl, an aryl, a substituted alkyl or a substituted aryl, a heteroaryl, a substituted heteroaryl, a side chain of an amino acid or a substituted side chain of an amino acid. In an embodiment R4 is the side chain of alanine, valine, isoleucine, leucine, glutamine, alanine, aspartic acid, asparagine, glutamic

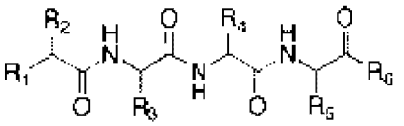
acid, serine, homoserine, alpha-amino adipic acid, phenylalanine, tyrosine, tryptophan, histidine, pyridylalanine, thienylalanine, thiazolylalanine, furylalanine or naphthylalanine, the side chain being substituted or not; and

[00110] R_6 is a serine trap, in an embodiments R_6 is  , CF_3, CO_2H, $CONH_2$, CONH-alkyl, CONH-aryl or CO-(amino acid residue)$_p$, wherein p is 1 or more, in a specific embodiment 1 to 6, in a more specific embodiment 1 to 3;

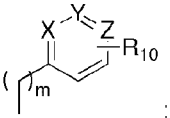
[00111] wherein W, X, Y and Z are independently N or CH; V and U are independently O, NH, NCH_3 or S;

[00112] R_{11} is H, CO_2H, CONH-alkyl, CONH-aryl, aryl, heteroaryl, halide or CO-(amino acid residue)$_q$, wherein q is 1 or more, in a specific embodiment 1 to 4, in a more specific embodiment 1 to 2.

[00113] The present invention also relates to a compound of formula (2):

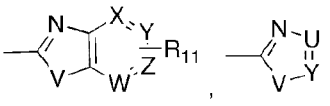
 (2)

[00114] or a pharmaceutically acceptable salt, hydrate, solvate, or stereoisomer thereof, wherein R_1 is H, NH_2, NHR_7 or one or more amino acid residues; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl or SO_2aryl;

[00115] R_2 and R_5 are independently $-CH_2-R_8$ or  ;

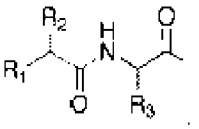
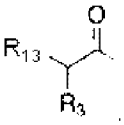
[00116] wherein R_8 is (CH_2)n-NH(C=NH)NH.R_{14} or (CH_2)n-NH_2; n is 1 to 3; m is 0 to 3; and R_{10} is -C(=NH)-NH_2, NH_2 or NH alkyl; R_{14} is H, alkyl, NH_2, NO_2, COalkyl;

[00117] R_3 and R_4 are independently an alkyl, an aryl, a substituted alkyl or a substituted aryl, a heteroaryl, a substituted heteroaryl, a side chain of an amino acid or a substituted side chain of an amino acid. In an embodiment R4 is the side chain of alanine, valine, isoleucine, leucine, glutamine, alanine, aspartic acid, asparagine, glutamic acid, serine, homoserine, alpha-amino adipic acid, phenylalanine, tyrosine, tryptophan, histidine, pyridylalanine, thienylalanine, thiazolylalanine, furylalanine or naphthylalanine, the side chain being substituted or not; and

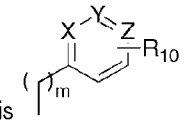
[00118] R_6 is  , CF_3, CO_2H, CONH-alkyl, CONH-aryl or CO-(amino acid residue)$_p$, wherein p is 1 or more, in a specific embodiment 1 to 6, in a more specific embodiment 1 to 3; wherein W, X, Y and Z are independently N or CH; V and U are independently O, NH, NCH_3 or S; and

[00119] R_{11} is H, CO_2H, CONHalkyl, CONHaryl, aryl, heteroaryl, halide or CO-(amino acid residue)$_q$, wherein q is 1 or more, in an embodiment 1 to 4, in a more specific embodiment 1 to 2.

[00120] In an embodiment, R_{12} is H. In another embodiment, R_{12} is NH_2. In another embodiment, R_{12} is NHR_7.

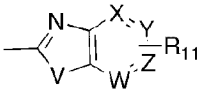
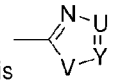
[00121] In an embodiment, R_7 is an alkyl (substituted or not). In another embodiment, R_7 is an aryl. In another embodiment, R_7 is (C=O)-alkyl. In another embodiment, R_7 is (C=O)-aryl. In another embodiment, R_7 is SO_2-alkyl. In another embodiment, R_7 is SO_2-aryl. In another embodiment, R_7 is . In another embodiment, R_7 is .

[00122] In an embodiment, R_1 and/or R_{13} is an alkyl. In another embodiment, R_1 and/or R_{13} is an aryl. In another embodiment, R_1 and/or R_{13} is (C=O)-alkyl. In another embodiment, R_1 and/or R_{13} is (C=O)-aryl. In another embodiment, R_1 and/or R_{13} is SO_2-alkyl. In another embodiment, R_1 and/or R_{13} is SO_2-aryl.

[00123] In an embodiment, R_2 and/or R_5 is $-CH_2-R_8$. In another embodiment, R_2 and/or R_5 is .

[00124] In an embodiment, R_{10} is $-C(=NH)-NH_2$. In another embodiment, R_{10} is NH_2. In another embodiment, R_{10} is NH-alkyl.

[00125] In an embodiment, R_3 and/or R_4 is an alkyl. In another embodiment, R_3 and/or R_4 is an aryl. In another embodiment, R_3 and/or R_4 is a substituted alkyl. In another embodiment, R_3 and/or R_4 is a substituted aryl.

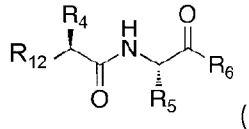
[00126] In an embodiment, R_6 is . In another embodiment, R_6 is . In another embodiment, R_6 is CF_3. In another embodiment, R_6 is CO_2H. In another embodiment, R_6 is $CONH_2$. In another embodiment, R_6 is CONH-alkyl. In another embodiment, R_6 is CONH-aryl. In another embodiment, R_6 is CO-(amino acid residue)$_p$, wherein p is 1 or more, in a specific embodiment 1 to 6, in a more specific embodiment 1 to 3.

[00127] In an embodiment W, X, Y and/or Z is N. In another embodiment, W, X, Y and/or Z is CH.

[00128] In an embodiment, V and/or U is O. In another embodiment, V and/or U is NH. In another embodiment, V and/or U is NCH_3. In another embodiment, V and/or U is S.

[00129] In an embodiment, R_{11} is H. In another embodiment, R_{11} is CO_2H. In another embodiment, R_{11} is CONH-alkyl. In another embodiment, R_{11} is CONH-aryl. In another embodiment, R_{11} is aryl. In another embodiment, R_{11} is heteroaryl. In another embodiment, R_{11} is halide.

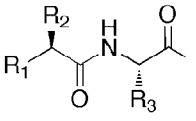
[00130] In an embodiment, the compound is of formula (1.1):



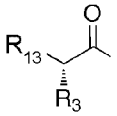
(1.1)

wherein

R_{12} is H, NH_2 or NHR_7, wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl,



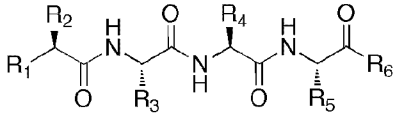
,



or one or 2 amino acid residue(s); and

[00131] wherein R_1, R_2, R_3, R_4, R_5, R_6, R_8, R_{10}, R_{11} and R_{13} are as defined above.

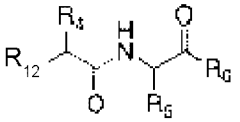
[00132] In a specific embodiment, the compound is of formula (2.1):



(2.1)

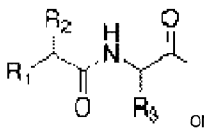
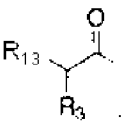
[00133] wherein R_1, R_2, R_3, R_4, R_5 and R_6 are as defined above.

[00134] In a specific embodiment, the compound is of formula (1.1)



(1)

[00135] or a pharmaceutically acceptable salt, hydrate, solvate, or stereoisomer thereof, wherein

[00136] R_{12} is NH_2,  or ;

[00137] R_{13} is NH_2;

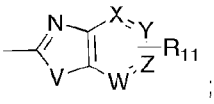
[00138] R_1 is as defined above, or in a specific embodiment, R_1 is NH_2;

[00139] R_2 is the side chain of arginine, substituted or not, in an embodiment, not substituted;

[00140] R_5 is the side chain of arginine or lysine, substituted or not, in an embodiment, not substituted;

[00141] R_3 is the side chain of glutamine, substituted or not, in an embodiment, not substituted;

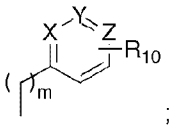
[00142] R_4 is the side chain of alanine, substituted or not, in an embodiment, not substituted;

[00143] R_6 is ;

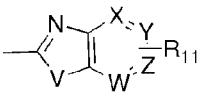
[00144] wherein W, X, Y and Z are CH; V is S; and

[00145] R_{11} is as defined above, in a specific embodiment, R_{11} is H. In a specific embodiment, of the formula 1 as defined above, all amino acid residues are in L-configuration. In a specific embodiment, on amino acid residue is in D configuration. In a specific embodiment, the compound of formula 1 is RQAR-R6, RQAK-R6, QAR-R6 or AR-R6, wherein all amino acids are in L-configuration. In a specific embodiment, the compound of formula 1 is RQAR-R6, wherein A is in D configuration and R, R and R are in L configuration.

[00146] In another specific embodiment of the compound of the present invention, (i) R_1 is -H or $-NH_2$;

[00147] (ii) R2 is ; (iii) m is 1; (iv) W is CH; (v) X is CH; (vi) Y is CH; (vii) R_{10} is $-C(=NH)NH_2$; or (viii) any combination of (i) to (vii).

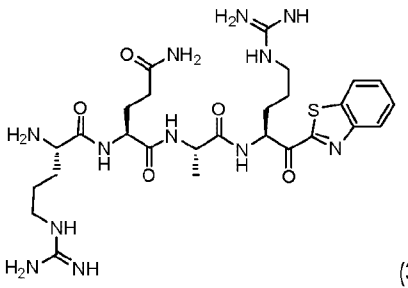
[00148] In another specific embodiment of the compound of the present invention, (i) R_1 is $-NH_2$; (ii) R_2 is $-CH_2-R_8$; (iii) R_3 is a substituted alkyl; (iv) R_4 is an alkyl; (v) R_5 is $-CH_2-R_8$;

[00149] (v) R_6 is ; (vi) R_8 is $-(CH_2)_3NH(C=NH)NH_2$ or $(CH_2)n-NH_2$, , in an embodiment, R_8 is $-(CH_2)_3NH(C=NH)NH_2$; (vii) W is CH; (viii) X is CH; (ix) Y is CH; (x) Z is CH; (xi) V is S; (xii) R_{11} is H; or (xiii) any combination of (i) to (xii).

[00150] In another specific embodiment of the compound of the present invention, R_3 is substituted alkyl, which is substituted with an alkyl, aryl, $(CO)NH_2$, (CO)OH, NH_2, NHCO-alkyl, NHCO-aryl, $NHSO_2$-alkyl, $NHSO_2$-aryl or heteroaryl. In another specific embodiment of the compound of the present invention, R_3 is $-(CH_2)_2(C=O)NH_2$. In another specific embodiment of the compound of the present invention, R_4 is -CH3. In another specific embodiment

of the compound of the present invention, R_2 and R_5 have the (S) configuration.

[00151] In another specific embodiment of the compound of the present invention, the compound is of formula (3):



(3)

DEFINITIONS

[00152] The articles "a," "an" and "the" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article.

[00153] The use of the word "a" "an" and "the" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one" but it is also consistent with the meaning of "one or more", "at least one", and "one or more than one".

[00154] As used in this specification and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, un-recited elements or method steps and are used interchangeably with, the phrases "including but not limited to" and "comprising but not limited to".

Compound

[00155] As used herein, the terms "molecule", "compound" and "agent" are used interchangeably and broadly to refer to natural, synthetic or semi-synthetic molecules or compounds. The term "compound" therefore denotes, for example, chemicals, macromolecules, cell or tissue extracts (from plants or animals) and the like. Non-limiting examples of compounds include peptides, antibodies, carbohydrates, nucleic acid molecules and pharmaceutical agents. The compound can be selected and screened by a variety of means including random screening, rational selection and by rational design using, for example, ligand modeling methods such as computer modeling. As will be understood by the person of ordinary skill, molecules having non-naturally occurring modifications are also within the scope of the term "compound". For example, the compounds of the present invention can be modified to enhance their activity, stability, and/or bioavailability, and also to lower its toxicity. The compounds or molecules identified in accordance with the teachings of the present invention have a therapeutic value in diseases or conditions related to

microbial infections, including Orthomyxoviridae infections such as influenza infection.

[00156] Compounds of the present invention may include alkyl, aryls, substituted alkyl and substituted aryls.

[00157] As used herein the term "alkyl" refers to substituted or unsubstituted, cyclic or acyclic, linear or branched alkyl (C_1 to C_{12}). In an embodiment, the alkyl is a C_1 to C_{10} alkyl, and in a more specific embodiment C_1-C_6 or C_1 to C_3. Whenever it appears herein, a numerical range, such as "1 to 12" or "C_1-C_{12}", refers to each integer in the given range; *e.g.*, "C_1-C_{12} alkyl" means that an alkyl group may comprise only 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc., up to and including 12 carbon atoms, although the term "alkyl" also includes instances where no numerical range of carbon atoms is designated. "Substituted alkyl" refers to alkyl moieties bearing substituents typically selected from alkyl, alkenyl, alkynyl, hydroxy, alkoxy, amide, halide, heterocyclic, aryl, substituted aryl (with substituents on any or all possible aryl positions including but not limited to hydroxyl, amide, halides, nitrile, nitro, alkyl, amino, amides, sulfonamides), guanidine, amidine, heteroaryl, aryloxy, halogen, haloalkyl, cyano, nitro, amino (-NH_2), lower alkylamino, lower dialkylamino, amido (-(CO)NR_6), azido, acyl (—(CO)R_6), alkoxymethyl, mercapto (—S—R_6), sulfoxy (—SO—R_6), sulfonyl —$(SO)_2$—R_6), sulfonamide (—$S(O)_2N(R_6)_2$),, carbonate (—O(CO)—O—R_6), oxyacyl (—O(CO)—R_6), carboxyl (—CO_2H), ester (—(CO)OR_6), carbamate (—O(CO)—$N(R_6)_2$), wherein R_6 is H or lower alkyl, lower alkenyl, lower alkynyl, aryl, heteroaryl, heterocycle, and the like. As used herein, "lower alkyl" refers to alkyl moieties having from 1 to about 6 carbon atoms, for example from 1 to 3 carbon atoms. More preferably, the substituent is an alkyl, aryl, -(CO)NH_2, -(CO)OH, -NH_2, NHCOalkyl, NHCOaryl, $NHSO_2$alkyl, $NHSO_2$aryl and heteroaryl, guanidine, amidine, halide, OH, OCH_3, NO_2, NH_2 or CO_2H. Alkyl, aryl, substituted alkyl and substituted aryl as used herein include the side chains of natural and non-natural amino acids. Hence, in specific embodiments, R_2, R_3, R_4 and/or R_5 are side chains of natural/standard or non-natural/non-standard amino acids. In an embodiment, R_2 and/or R_5 is a side chain of arginine, lysine, histidine or an analog/surrogate thereof such as ornithine or citrulline. In an embodiment, R_4 is a side chain of alanine, valine, isoleucine, leucine, or an analog/surrogate thereof. In an embodiment, R_4 is a side chain of glutamine, alanine, aspartic acid, asparagine, glutamic acid, serine, homoserine, alpha-amino adipic acid, phenylalanine, tyrosine, tryptophan, histidine, pyridylalanine, thienylalanine, thiazolylalanine, furylalanine, naphthylalanine, the side chain being substituted or not with Alkoxy, hydroxyl, halide, aminoalkyl, amide, sulfonamide, aryl, alkyl, heteroaryl or amidine. In an embodiment, at least one or more (*e.g.*, 2, 3 or 4) amino acid residue of the compound is in L configuration. In an embodiment, at least one or more (*e.g.*, 2, 3 or 4) amino acid residue of the compound is in D configuration. Unless indicated otherwise, as used herein in the terms "CONH-amino acid residue(s)" and "CO-amino-acid residue(s)" are used interchangeably: the NH of "CONH" is that of the amino acid residue lined to the CO.

[00158] As used herein, "alkenyl" refers to straight, branched chain or cyclic hydrocarbyl groups having at least one carbon-carbon double bond, and having in the range of about 2 up to about 100 carbon atoms, for example from 2 to 12 carbon atoms, and "substituted alkenyl" refers to alkenyl groups further bearing one or more substituents as set forth above. As used herein, "lower alkenyl" refers to alkenyl moieties having from 2 to about 6 carbon atoms, for example from 1 to 3 carbon atoms.

[00159] As used herein, "alkynyl" refers to straight or branched chain hydrocarbyl groups having at least one carbon-carbon triple bond, and having in the range of about 2 up to about 100 carbon atoms, for example from 2 to 12 carbon atoms and "substituted alkynyl" refers to alkynyl groups further bearing one or more substituents as set forth above. As used herein, "lower alkynyl" refers to alkynyl moieties having from 2 to about 6 carbon atoms, for example 2, 3 or 4 carbons.

[00160] As used herein the term "aryl" refers to substituted or unsubstituted aryl (C_5 to C_{14} (*e.g.*, C_5-C_6)). "Substituted aryl" refers to aryl groups further bearing one or more substituents as set forth above in the definition of "substituted alkyl" above. In a more specific embodiment, the substituent is an alkyl, a heterocycle, an halide, SH, OH, OCH_3, NO_2, NH_2, CO_2H, $CONH_2$ or SO_2NH_2.

[00161] As used herein the term "amino acid residue" refers to any natural/standard and non-natural/non-standard amino acid residue in (L) or (D) configuration, and includes alpha or alpha-disubstituted amino acids. It refers to isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, arginine, alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, proline, serine, tyrosine. It also includes beta-alanine, 3-amino-propionic acid, 2,3-diamino propionic acid, alpha-aminoisobutyric acid (Aib), 4-amino-butyric acid, *N*-methylglycine (sarcosine), hydroxyproline, ornithine (*e.g.*, L-ornithine), citrulline, *t*-butylalanine, *t*-butylglycine, *N*-methylisoleucine, phenylglycine, cyclohexylalanine, cyclopentylalanine, cyclobutylalanine, cyclopropylalanine, cyclohexylglycine, cyclopentylglycine, cyclobutylglycine, cyclopropylglycine, norleucine (Nle), norvaline, 2-napthylalanine, pyridylalanine, 3-benzothienyl alanine, 4-chlorophenylalanine, 2-fluorophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, penicillamine, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylix acid, beta-2-thienylalanine, methionine sulfoxide, L-homoarginine (hArg), N-acetyl lysine, 2-amino butyric acid, 2-amino butyric acid, 2,4,-diaminobutyric acid (D- or L-), *p*-aminophenylalanine, *N*-methylvaline, selenocysteine, homocysteine, homoserine (HoSer), cysteic acid, epsilon-amino hexanoic acid, delta-amino valeric acid, or 2,3-diaminobutyric acid (D- or L-), etc. These amino acids are well known in the art of biochemistry/peptide chemistry. Similarly, the terms "side chain of amino acid" refer to the side chain of any natural/standard and non-natural/non standard amino acid residue in (L) or (D) configuration as described above.

[00162] Compounds of the present invention which include peptides may comprise replacement of at least one of the peptide bonds with an isosteric modification. Compounds of the present invention which include peptides may be peptidomimetics. A peptidomimetic is typically characterised by retaining the polarity, three dimensional size and functionality (bioactivity) of its peptide equivalent, but wherein one or more of the peptide bonds/linkages have been replaced, often by proteolytically more stable linkages. Generally, the bond which replaces the amide bond (amide bond surrogate) conserves many or all of the properties of the amide bond, *e.g.* conformation, steric bulk, electrostatic character, potential for hydrogen bonding, etc. Typical peptide bond replacements include esters, polyamines and derivatives thereof as well as substituted alkanes and alkenes, such as aminomethyl and ketomethylene. For example, the peptide may have one or more peptide linkages replaced by linkages such as –CH_2NH–, –CH_2S–, –CH_2–CH_2–, –CH=CH– (*cis* or *trans*), –CH_2SO–, –$CH(OH)CH_2$–, or –$COCH_2$–, -N-NH-, -

CH_2NHNH-, or peptoid linkages in which the side chain is connected to the nitrogen atom instead of the carbon atom. Such peptidomimetics may have greater chemical stability, enhanced biological/pharmacological properties (*e.g.*, half-life, absorption, potency, efficiency, etc.) and/or reduced antigenicity relative its peptide equivalent.

Salts, esters, hydrates and solvates

[00163] The compounds of the present invention include pharmacologically acceptable salts and ester derivatives thereof as well as hydrates or solvates thereof and all stereoisomeric forms of the referenced compounds. The compounds and pharmacologically acceptable esters thereof of the present invention can form pharmacologically acceptable salts if necessary.

Salts

[00164] The terms "pharmacologically acceptable salt thereof" refer to a salt to which the compounds of the present invention can be converted. Preferred examples of such a salt include alkali metal salts such as a sodium salt, a potassium salt, a lithium salt, magnesium or calcium salts; alkaline earth metal salts such as a calcium salt and a magnesium salt; metal salts such as an aluminum salt, an iron salt, a zinc salt, a copper salt, a nickel salt and a cobalt salt; amine salts such as inorganic salts including an ammonium salt; organic salts or ammonium salts such as a t-octylamine salt, a dibenzylamine salt, a morpholine salt, a glucosamine salt, a phenylglycine alkyl ester salt, an ethylenediamine salt, an N-methylglucamine salt, a guanidine salt, a diethylamine salt, a triethylamine salt, a dicyclohexylamine salt, an N,N'-dibenzylethylenediamine salt, a chloroprocaine salt, a procaine salt, a diethanolamine salt, an N-benzyl-phenethylamine salt, a piperazine salt, a tetramethylammonium salt and a tris(hydroxymethyl)aminomethane salt; inorganic acid salts such as hydrohalic acid salts such as a hydrofluoride, a hydrochloride, a hydrobromide or a hydroiodide, a nitrate, a perchlorate, a sulfate or a phosphate; lower alkanesulfonates such as a methanesulfonate, trifluoromethanesulfonate or an ethanesulfonate; arylsulfonates such as a benzenesulfonate or a p-toluenesulfonate and the like, which are non-toxic to living organisms; organic acid salts such as an acetate, a malate, adipate, a fumarate, a succinate, a citrate, alginate, ascorbate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, cinnamate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, itaconate, lactate, maleate, mandelate, sulfonate, methanesulfonate, trifluoromethanesulfonates, ethanesulfonates 2-naphthalenesulfonate, nicotinate, nitrate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, tartrate, thiocyanate, tosylate, trifluoroacetic acid, undecanoate, a tartrate, an oxalate or a maleate; and amino acid salts such as a glycine salt, a lysine salt, an arginine salt, an ornithine salt, histidine, a glutamate or an aspartate salt. Additionally, basic nitrogen containing groups may be quaternized with such agents as lower alkyl halides such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates including dimethyl, diethyl, and dibutyl sulfate; and diamyl sulfates, long chain halides such as decyl, lauryl, myristyl and strearyl chlorides, bromides and iodides, aralkyl halides including benzyl and phenethyl bromides, and others. For further example, see S. M.

Berge, et al. "Pharmaceutical Salts," J. Pharm. Sci. 1977, 66, 1-19. Such salts can be formed quite readily by those skilled in the art using standard techniques.

[00165] Preferred examples of the salts formed with an acidic group present in the compounds of the present invention include metal salts such as alkali metal salts (*e.g.*, sodium salts, potassium salts and lithium salts), alkali earth metal salts (*e.g.*, calcium salts and magnesium salts), aluminum salts and iron salts; amine salts such as inorganic amine salts (*e.g.*, ammonium salts) and organic amine salts (*e.g.*, t-octylamine salts, dibenzylamine salts, morpholine salts, glucosamine salts, phenylglycinealkyl ester salts, ethylenediamine salts, N-methylglucamine salts, guanidine salts, diethylamine salts, triethylamine salts, dicyclohexylamine salts, N,N'-dibenzylethylenediamine salts, chloroprocaine salts, procaine salts, diethanolamine salts. N-benzylphenethylamine salts, piperazine salts, tetramethylammonium salts and tris(hydroxymethyl)aminomethane salts; and amino acid salts such as glycine salts, lysine salts, arginine salts, ornithine salts, glutamates and aspartates.

[00166] All salts are intended to be pharmaceutically acceptable salts within the scope of the invention and all salts are considered equivalent to the free forms of the corresponding compounds for purposes of the invention.

Esters

[00167] Physiologically/pharmaceutically acceptable esters are also useful as active medicaments. The term "pharmaceutically acceptable esters" embraces esters of the compounds of the present invention, in which hydroxy groups (*e.g.*, in carboxylic acid) have been converted to the corresponding esters and may act as a prodrug which, when absorbed into the bloodstream of a warm-blooded animal, may cleave in such a manner as to release the drug form and permit the drug to afford improved therapeutic efficacy. Such esters can be formed with inorganic or organic acids such as nitric acid, sulphuric acid, phosphoric acid, citric acid, formic acid, maleic acid, acetic acid, succinic acid, tartaric acid, methanesulphonic acid, p-toluenesulphonic acid and the like, which are non-toxic to living organisms. Further examples are the esters with aliphatic or aromatic acids such as acetic acid or with aliphatic alcohol (*e.g.*, alkyl esters, including methyl, ethyl, propyl, isopropyl, butyl, isobutyl or pentyl esters, and the like) or aromatic alcohols (*e.g.*, benzyl ester).

[00168] Esters can be prepared from their corresponding acids or salts by a variety of methods known to those skilled in the art, such as, for example, by first transforming the acid to the acid chloride and then reacting the acid chloride with a suitable alcohol. Other suitable methods for making esters are described in Kemp and Vellaccio, 1980.

[00169] Where esters of the invention have a basic group, such as an amino group, the compound can be converted to a salt by reacting it with an acid, and in the case where the esters have an acidic group, such as a sulfonamide group, the compound can be converted to a salt by reacting it with a base. The compounds of the present invention encompass such salts.

[00170] Salts and esters of the compounds of the present invention may be prepared by known method by employing appropriate starting materials or intermediate compounds that are readily available and/or are described herein.

[00171] Generally, a desired salt of a compound of this invention can be prepared *in situ* during the final isolation and purification of a compound by means well known in the art. For example, a desired salt can be prepared by separately reacting the purified compound in its free base or free acid form with a suitable organic or inorganic acid, or suitable organic or inorganic base, respectively, and isolating the salt thus formed. In the case of basic compounds, for example, the free base is treated with anhydrous HCl in a suitable solvent such as THF, and the salt isolated as a hydrochloride salt. In the case of acidic compounds, the salts may be obtained, for example, by treatment of the free acid with anhydrous ammonia in a suitable solvent such as ether and subsequent isolation of the ammonium salt. These methods are conventional and would be readily apparent to one skilled in the art.

[00172] The compounds of this invention may be esterified by a variety of conventional procedures including reacting the appropriate anhydride, carboxylic acid or acid chloride with the alcohol group of a compound of this invention. The appropriate anhydride is reacted with the alcohol in the presence of a base to facilitate acylation such as 1,8-bis[dimethylamino]naphthalene or N,N-dimethylaminopyridine. Or, an appropriate carboxylic acid can be reacted with the alcohol in the presence of a dehydrating agent such as dicyclohexylcarbodiimide, 1-[3-dimethylaminopropyl]-3-ethylcarbodiimide or other water soluble dehydrating agents which are used to drive the reaction by the removal of water, and, optionally, an acylation catalyst. Esterification can also be effected using the appropriate carboxylic acid in the presence of trifluoroacetic anhydride and, optionally, pyridine, or in the presence of N,N-carbonyldiimidazole with pyridine. Reaction of an acid chloride with the alcohol can be carried out with an acylation catalyst such as 4-DMAP or pyridine.

[00173] One skilled in the art would readily know how to successfully carry out these as well as other known methods of etherification of alcohols.

Prodrugs and solvates

[00174] Prodrugs and solvates of the compounds of the invention are also contemplated herein. A discussion of prodrugs is provided in T. Higuchi and V. Stella, Pro-drugs as Novel Delivery Systems (1987) 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, (1987) Edward B. Roche, ed., American Pharmaceutical Association and Pergamon Press. The term "prodrug" means a compound (*e.g.*, a drug precursor) that is transformed *in vivo* to yield a compound of the present invention or a pharmaceutically acceptable salt, hydrate or solvate of the compound. The transformation may occur by various mechanisms (*e.g.*, by metabolic or chemical processes), such as, for example, through hydrolysis in blood. A discussion of the use of prodrugs is provided by T. Higuchi and W. Stella, "Pro-drugs as Novel Delivery Systems," Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987.

[00175] For example, if a compound of the present invention or a pharmaceutically acceptable salt, hydrate or solvate of the compound contains a carboxylic acid functional group, a prodrug can comprise an ester formed by the replacement of the hydrogen atom of the acid group with a group such as, for example, (C1–C8)alkyl, (C2–C12)alkanoyloxymethyl, 1-(alkanoyloxy)ethyl having from 4 to 9 carbon atoms, 1-methyl-1-(alkanoyloxy)-ethyl having from 5 to 10 carbon atoms, alkoxycarbonyloxymethyl having from 3 to 6 carbon atoms, 1-(alkoxycarbonyloxy)ethyl having from 4 to 7 carbon atoms, 1-methyl-1-(alkoxycarbonyloxy)ethyl having from 5 to 8 carbon atoms, N-(alkoxycarbonyl)aminomethyl having from 3 to 9 carbon atoms, 1-(N-(alkoxycarbonyl)amino)ethyl having from 4 to 10 carbon atoms, 3-phthalidyl, 4-crotonolactonyl, gamma-butyrolacton-4-yl, di-N,N-(C1–C2)alkylamino(C2–C3)alkyl (such as β-dimethylaminoethyl), carbamoyl-(C_1–C_2)alkyl, N,N-di(C_1–C_2)alkylcarbamoyl-(C_1–C_2)alkyl and piperidino-, pyrrolidino- or morpholino(C_2–C_3)alkyl, and the like.

[00176] Similarly, if a compound of the present invention contains an alcohol functional group, a prodrug can be formed by the replacement of the hydrogen atom of the alcohol group with a group such as, for example, (C1–C6)alkanoyloxymethyl, 1-((C_1–C_6)alkanoyloxy)ethyl, 1-methyl-1-((C_1–C_6)alkanoyloxy)ethyl, (C_1–C_6)alkoxycarbonyloxymethyl, N-(C_1–C_6)alkoxycarbonylaminomethyl, succinoyl, (C_1–C_6)alkanoyl, α-amino(C_1–C_4)alkanyl, arylacyl and α-aminoacyl, or α-aminoacyl-α-aminoacyl, where each α-aminoacyl group is independently selected from the naturally occurring L-amino acids, $P(O)(OH)_2$, —$P(O)(O(C_1$–$C_6)alkyl)_2$ or glycosyl (the radical resulting from the removal of a hydroxyl group of the hemiacetal form of a carbohydrate), and the like.

[00177] If a compound of the present invention incorporates an amine functional group, a prodrug can be formed by the replacement of a hydrogen atom in the amine group with a group such as, for example, R-carbonyl, RO-carbonyl, NRR'-carbonyl where R and R' are each independently (C_1–C_{10})alkyl, (C_3–C_7)cycloalkyl, benzyl, or R-carbonyl is a natural α-aminoacyl or natural α-aminoacyl, —C(OH)C(O)OY1 wherein Y1 is H, (C_1–C_6)alkyl or benzyl, —$C(OY_2)Y_3$ wherein Y_2 is (C_1–C_4) alkyl and Y_3 is (C_1–C_6)alkyl, carboxy (C_1–C_6)alkyl, amino(C_1–C_4)alkyl or mono-N— or di-N,N-(C_1–C_6)alkylaminoalkyl, —C(Y4)Y5 wherein Y_4 is H or methyl and Y_5 is mono-N— or di-N,N-(C_1–C_6)alkylamino morpholino, piperidin-1-yl or pyrrolidin-1-yl, and the like.

[00178] One or more compounds of the invention may exist in unsolvated as well as solvated forms with pharmaceutically acceptable solvents such as water, ethanol, and the like, and it is intended that the invention embrace both solvated and unsolvated forms. “Solvate” means a physical association of a compound of this invention with one or more solvent molecules. This physical association involves varying degrees of ionic and covalent bonding, including hydrogen bonding. In certain instances the solvate will be capable of isolation, for example when one or more solvent molecules are incorporated in the crystal lattice of the crystalline solid. “Solvate” encompasses both solution-phase and isolatable solvates. Non-limiting examples of suitable solvates include ethanolates, methanolates, and the like. A “Hydrate” is a solvate wherein the solvent molecule is H_2O.

[00179] Preparation of solvates is generally known. Thus, for example, M. Caira et al, J. Pharmaceutical Sci., 93(3), 601–611 (2004) describe the preparation of the solvates of the antifungal fluconazole in ethyl acetate as well as from

water. Similar preparations of solvates, hemisolvate, hydrates and the like are described by E. C. van Tonder et al, AAPS Pharm Sci Tech., 5(1), article 12 (2004); and A. L. Bingham et al, Chem. Commun., 603–604 (2001). A typical, non-limiting, process involves dissolving the inventive compound in desired amounts of the desired solvent (organic or water or mixtures thereof) at a higher than ambient temperature, and cooling the solution at a rate sufficient to form crystals which are then isolated by standard methods. Analytical techniques such as, for example I. R. spectroscopy, show the presence of the solvent (or water) in the crystals as a solvate (or hydrate).

Hydrates

[00180] As used herein the terms, "pharmaceutically acceptable hydrate" refer to the compounds of the instant invention crystallized with one or more molecules of water to form a hydrated form.

Stereoisomers, diastereomers, enantiomers, racemates, tautomers

[00181] The compounds of the present invention have asymmetric carbon atoms and can exist in the form of diastereomers, optically pure enantiomers or as racemates. The term “compound” as used herein embraces all of these forms.

[00182] Diastereomers (sometimes called diastereoisomers) are stereoisomers that are not enantiomers. Diastereomerism occurs when two or more stereoisomers of a compound have different configurations at one or more (but not all) of the equivalent (related) stereocenters and are not mirror images of each other. When two diastereoisomers differ from each other at only one stereocenter they are epimers. Each stereocenter gives rise to two different configurations and thus to two different stereoisomers.

[00183] Diastereomers differ from enantiomers in that the latter are pairs of stereoisomers which differ in all stereocenters and are therefore mirror images of one another. Enantiomers of a compound with more than one stereocenter are also diastereomers of the other stereoisomers of that compound that are not their mirror image. Diastereomers have different physical properties and different reactivity, unlike enantiomers. Diastereomers of the present invention include tomatidine and 3 alpha-hydroxy-tomatidine for exemple.

[00184] For purposes of this Specification, "pharmaceutically acceptable tautomer" means any tautomeric form of any compound of the present invention.

[00185] The purification of enantiomers and the separation of isomeric mixtures of a compound of the present invention may be accomplished by standard techniques known in the art.

Inhibitors of matriptase for treatment of orthomyxoviridae infections

[00186] The present invention further concerns the use of any inhibitor of matriptase, i.e. any agent that decreases matriptase expression and/or activity, in target cells. Such inhibitors may be useful for the treatment of

orthomyxoviridae infections, such as influenza infections. In an embodiment, the inhibitor is a compound defined above. In other embodiments, the inhibitor is an agent for RNA interference, an antibody or a small molecule inhibitor.

Agents for RNA interference (siRNAs, shRNA, miRNA)

[00187] The present invention further concerns the use of RNA interference (RNAi) to decrease matriptase expression/activity in target cells (*e.g.*, respiratory epithelial cells). "RNA interference" refers to the process of sequence specific suppression of gene expression mediated by RNA molecules such as small interfering RNA (siRNA), microRNA (miRNA) or small/short hairpin RNA (shRNA) without generalized suppression of protein synthesis. While the invention is not limited to a particular mode of action, RNAi may involve degradation of messenger RNA (*e.g.*, matriptase mRNA, such as an mRNA comprising a sequence corresponding to the sequence of SEQ ID NO:1) by an RNA induced silencing complex (RISC), preventing translation of the transcribed targeted mRNA. Alternatively, it may involve methylation of genomic DNA, which shuts down transcription of a targeted gene. The suppression of gene expression caused by RNAi may be transient or it may be more stable, even permanent.

[00188] RNA interference is triggered by the presence of short interfering RNAs of about 20-25 nucleotides in length which comprise about 19 base pair duplexes. These siRNAs can be of synthetic origin or they can be derived from a ribonuclease III activity (*e.g.*, dicer ribonuclease) found in cells. The RNAi response also features an endonuclease complex containing siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates the cleavage of single stranded RNA having a sequence complementary to the antisense region of the siRNA duplex. Cleavage of the target RNA (*e.g.*, matriptase mRNA) takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir et al., 2001, Genes Dev., 15:188).

[00189] "Small interfering RNA" of the present invention refers to any nucleic acid molecule capable of mediating RNA interference "RNAi" or gene silencing (see for example, Bass, 2001, Nature, 411:428-429; Elbashir *et al.*, 2001, Nature, 411:494-498; Kreutzer *et al.*, International PCT publication No. WO 00/44895; Zernicka-Goetz *et al.*, International PCT publication No. WO 01/36646; Fire, International PCT publication No. WO99/32619; Mello and Fire, International PCT publication No. WO01/29058; Deschamps-Depaillette, International PCT publication No. WO99/07409; Han et al., International PCT publication No. WO 2004/011647; Tuschl *et al.*, International PCT publication No. WO 02/44321; and Li *et al.*, International PCT publication No. WO 00/44914). Specific embodiments of siRNA of the present invention are double stranded RNA molecules from about ten to about 30 nucleotides long that are named for their ability to specifically interfere with protein expression. In one embodiment, siRNA of the present invention are 12-28 nucleotides long, more preferably 15-25 nucleotides long, even more preferably 19-23 nucleotides long and most preferably 21-23 nucleotides long. Therefore preferred siRNA of the present invention are about 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 or 28 nucleotides in length. As used herein, siRNA molecules need not to be limited to those molecules containing only RNA, but further encompass chemically modified nucleotides and non-nucleotides. siRNA used in examples herein include s13520 (Ambion) Silencer®

Select siRNAs gene symbol ST14, and S2: EHU012511 (Sigma-Aldrich) MISSION® esiRNA targeting human ST14 (esiRNA1).

[00190] The length of one strand designates the length of an siRNA molecule. For example, a siRNA that is described as a 23 ribonucleotides long (a 23 mer) could comprise two opposite strands of RNA that anneal together for 21 contiguous base pairing. The two remaining ribonucleotides on each strand would form what is called an "overhang". In a particular embodiment, the siRNA of the present invention contains two strands of different lengths. In this case, the longer strand designates the length of the siRNA. For example, a dsRNA containing one strand that is 20 nucleotides long and a second strand that is 19 nucleotides long is considered a 20 mer.

[00191] siRNAs that comprise an overhang are desirable. The overhang may be at the 3' or 5' end. Preferably, the overhangs are at the 3' end of an RNA strand. The length of an overhang may vary but preferably is about 1 to 5 nucleotides long. Generally, 21 nucleotides siRNA with two nucleotides 3'-overhang are the most active siRNAs.

[00192] siRNA of the present invention are designed to decrease matriptase expression in a target cell by RNA interference. siRNA of the present invention comprise a sense region and an antisense region wherein the antisense region comprises a sequence complementary to a matriptase mRNA sequence and the sense region comprises a sequence complementary to the antisense sequence of matriptase mRNA. A siRNA molecule can be assembled from two nucleic acid fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of siRNA molecule. The sense region and antisense region can also be covalently connected via a linker molecule. The linker molecule can be a polynucleotide linker or a non polynucleotide linker.

Antibodies

[00193] The present invention further concerns the use of antibodies that inhibit matriptase activity in target cells (*e.g.*, respiratory epithelial cells), *e.g.*, that inhibit the cleavage of Influenza HA by matriptase. In an embodiment, the antibodies specifically recognize/bind to a matriptase polypeptide (*e.g.*, the matriptase polypeptide of SEQ ID NO:2). In an embodiment, the antibodies specifically recognize/bind to an epitope located in a region of matriptase that is involved in the cleavage of Influenza HA (the catalytic domain), for example an epitope located in the region spanning about residues 615 to 855, for example about residues 700 to 840 of matriptase. In an embodiment, the antibodies specifically recognizes/binds to an epitope located in a region spanning about residues 708 to 829 of matriptase. In embodiments, the antibodies specifically recognizes/binds to an epitope comprising one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or all) of the following residues of matriptase: Phe^{708}, Gln^{783}, Asp^{799}, Ser^{800}, Gln^{802}, Gly^{803}, Ser^{805}, Gly^{827}, Asp^{828} and/or Gly^{829}.

[00194] The term "antibody" is used in the broadest sense, and covers monoclonal antibodies (including full-length monoclonal antibodies), polyclonal antibodies, multispecific antibodies, chimeric antibodies and humanized antibodies, so long as they exhibit the desired biological activity (*e.g.*, inhibiting the binding of matriptase to influenza HA, and/or the matriptase-mediated cleavage of influenza HA). Antibody fragments comprise a portion of a full

length antibody, generally an antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments, diabodies, linear antibodies, single-chain antibody molecules, single domain antibodies (*e.g.*, from camelids), shark NAR single domain antibodies, and multispecific antibodies formed from antibody fragments. Antibody fragments can also refer to binding moieties comprising CDRs or antigen binding domains including, but not limited to, V_H regions (V_H, V_H-V_H), anticalins, PepBodies, antibody-T-cell epitope fusions (Troybodies) or Peptibodies.

[00195] In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody A Laboratory Manual, CSH Laboratories).

[00196] Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (s.c.), intravenous (i.v.) or intraperitoneal (i.p.) injections of the relevant antigen (*e.g.*, a peptide or polypeptide comprising a sequence of matriptase) with or without an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R_1N=C=NR$, where R and R_1 are different alkyl groups.

[00197] Animals may be immunized against the antigen, immunogenic conjugates, or derivatives by combining the antigen or conjugate (e.g., 100 µg for rabbits or 5 µg for mice) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with the antigen or conjugate (*e.g.*, with 1/5 to 1/10 of the original amount used to immunize) in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, for conjugate immunizations, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

[00198] Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256: 495 (1975), or may be made by recombinant DNA methods (*e.g.*, U.S. Patent No. 6,204,023). Monoclonal antibodies may also be made using the techniques described in U.S. Patent Nos. 6,025,155 and 6,077,677 as well as U.S. Patent Application Publication Nos. 2002/0160970 and 2003/0083293.

[00199] In the hybridoma method, a mouse or other appropriate host animal, such as a rat, hamster or monkey, is immunized (*e.g.*, as hereinabove described) to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the antigen used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as

polyethylene glycol, to form a hybridoma cell.

[00200] The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

[00201] Antibodies specific for the catalytic domain (residues 615 to 855) of human matriptase and exhibiting inhibitory activity against matriptase are described in U.S. Patent No. 7,572,444. Examples of such antibodies include those having the sequences depicted in Table 1 below.

Table 1: Sequences of representative anti-matriptase antibodies disclosed in U.S. Patent No. 7,572,444

Heavy chain

FR1	CDR1	FR2
EVQLLESGGGLVQPGGSLRLSCAAS	GFTFSSYAMS	WVRQAPGKGLEWVS
EVQLLESGGGLVQPGGSLRLSCAAS	GFTFSSYAMS	WVRQAPGKGLEWVS
EVQLLESGGGLVQPGGSLRLSCAAS	GVTFSSYAMS	WVRQAPGKGLEWVS

CDR2	FR3	CDR3	FR4
AISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQ MNSLRAEDTAVYYCAK		WGQGTLVTVSS
AISSSGVNTHYADSVKG	RFTISRDNSKNTLYLQ MNSLRAEDTAVYYCAT	IASIALRGYYFDY	WGQGTLVTVSS
AISSSGGNTHYADSVKG	RFTISRDNSKNTLYLQ MNSLRAEDTAVYYCAT	IASIATRGYFFNY	WGQGTLVTVSS

Light chain

FR1	CDR1	FR2
EIVLTQSPGTLSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY
EIVLTQSPGTLSLSPGERATLSC	RASQTFGSSYLA	WYQQKPGQAPRLLIY
DIVLTQSPGTLSLSPGERATLSC	RASQIFSSNSLA	WYQQKPGQAPSLLIY

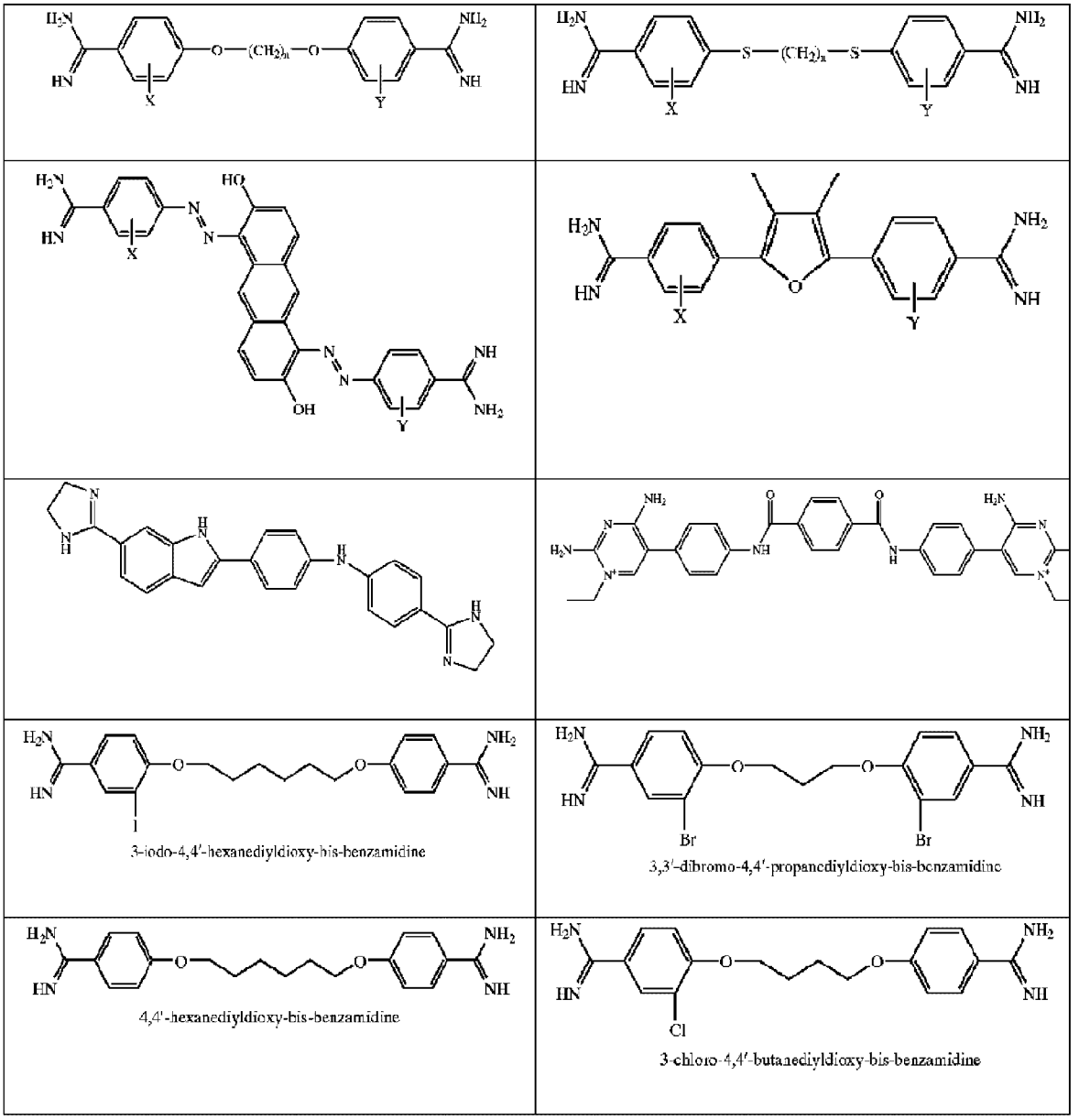
CDR2	FR3	CDR3	FR4
GASSRAT	GIPDRFSGSGSGTDFT LTISRLEPEDFAVYYC		FGQGTKVEIK
GASSRAT	VIPDRFSGSGSGTDFT LTISRLEPEDFAVYYC	QQYGSSPWT	FGQGTKVEIK
GASSRAT	GIPDRFSGSGSGTDFT LTISRLEPEDFAVYYC	QQYGSSPWT	FGQGTKVEIK

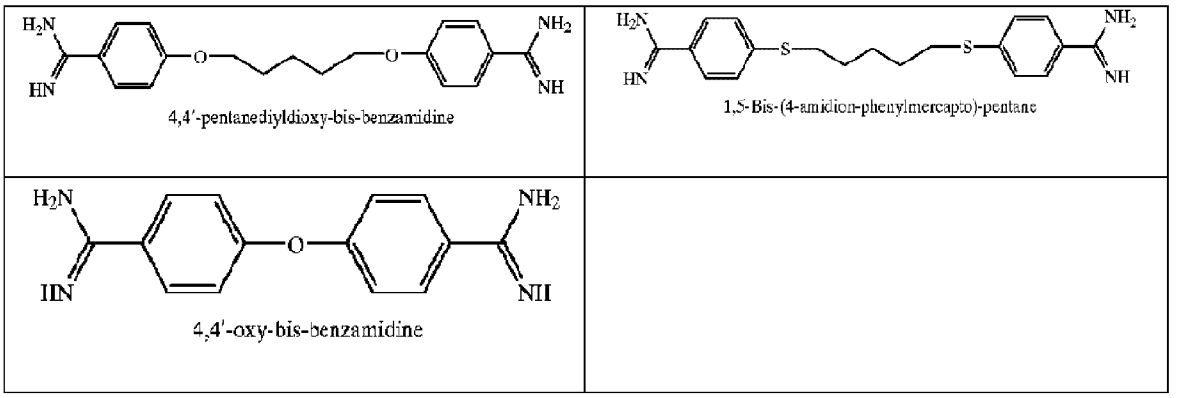
[00202] Other matriptase antibodies are disclosed in Sun *et al.*, 2003, *Biochemistry* **42**: 892-900 and Lin *et al.*, 1997, *Journal of Biological Chemistry*, **272**: 9147-9152.

Small molecules and other matriptase inhibitors

[00203] Inhibitors of matriptase activity that may be used in the methods of the present invention include those described in U.S. Patents Nos. 6,677,377 and 7,019,019. The structures of representative examples of such inhibitors are presented in Tables 2 and 3 below:

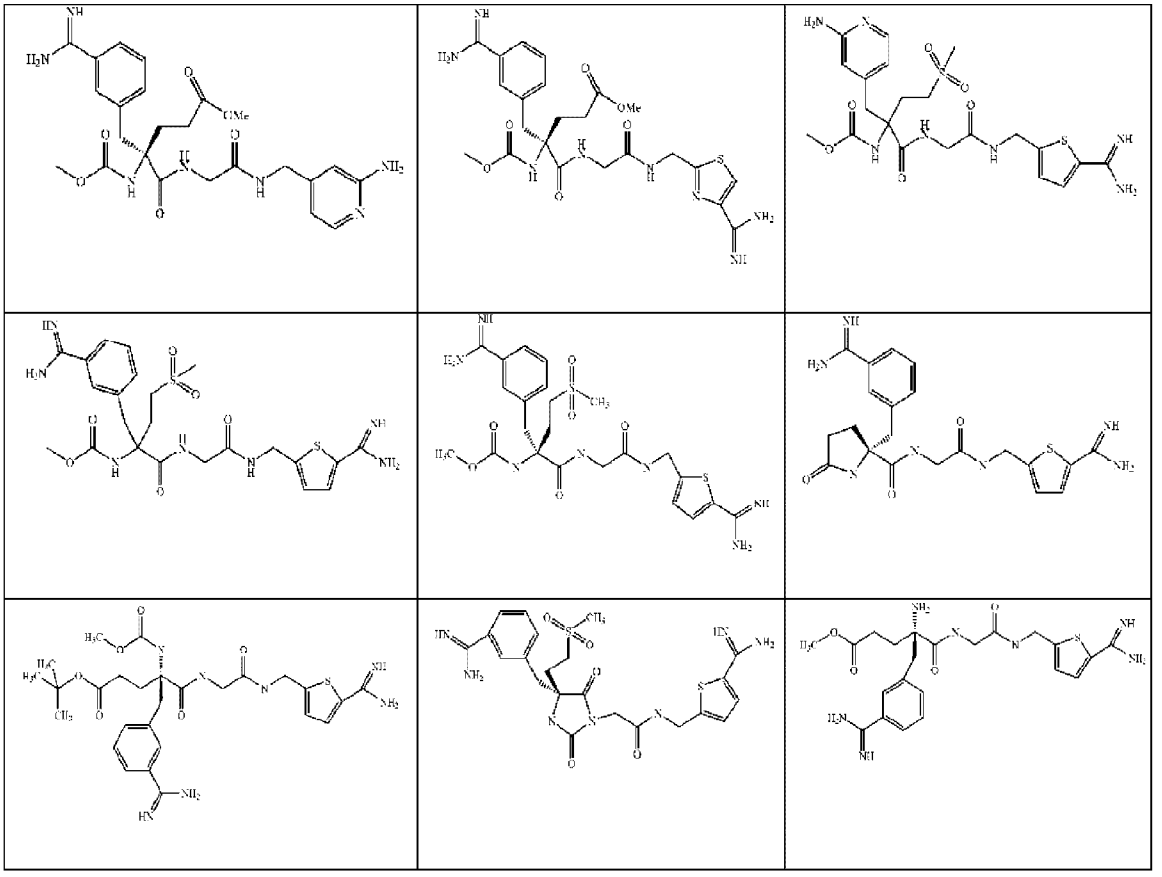
Table 2: Structures of representative matriptase inhibitors disclosed in U.S. Patent No 6,677,377

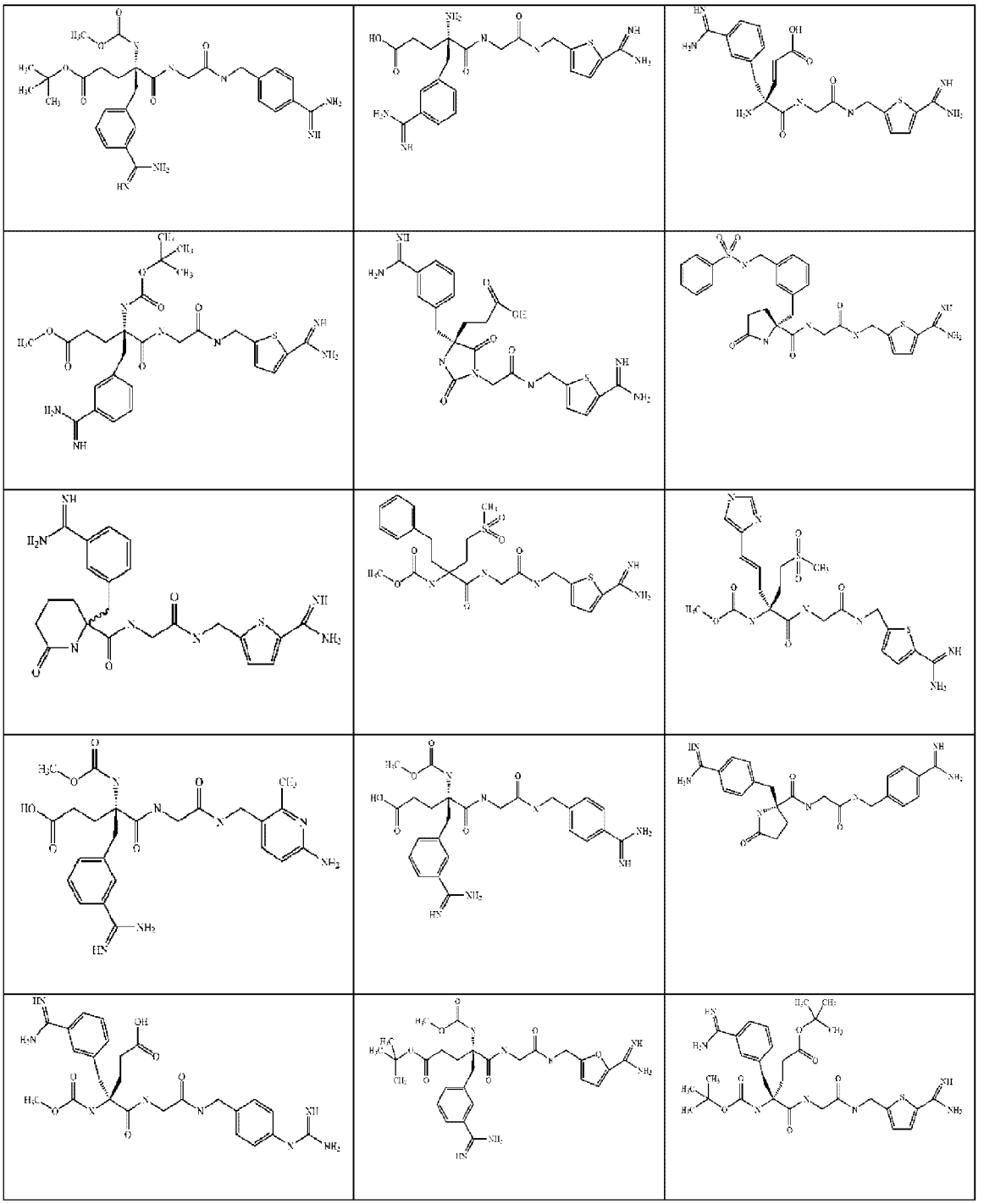


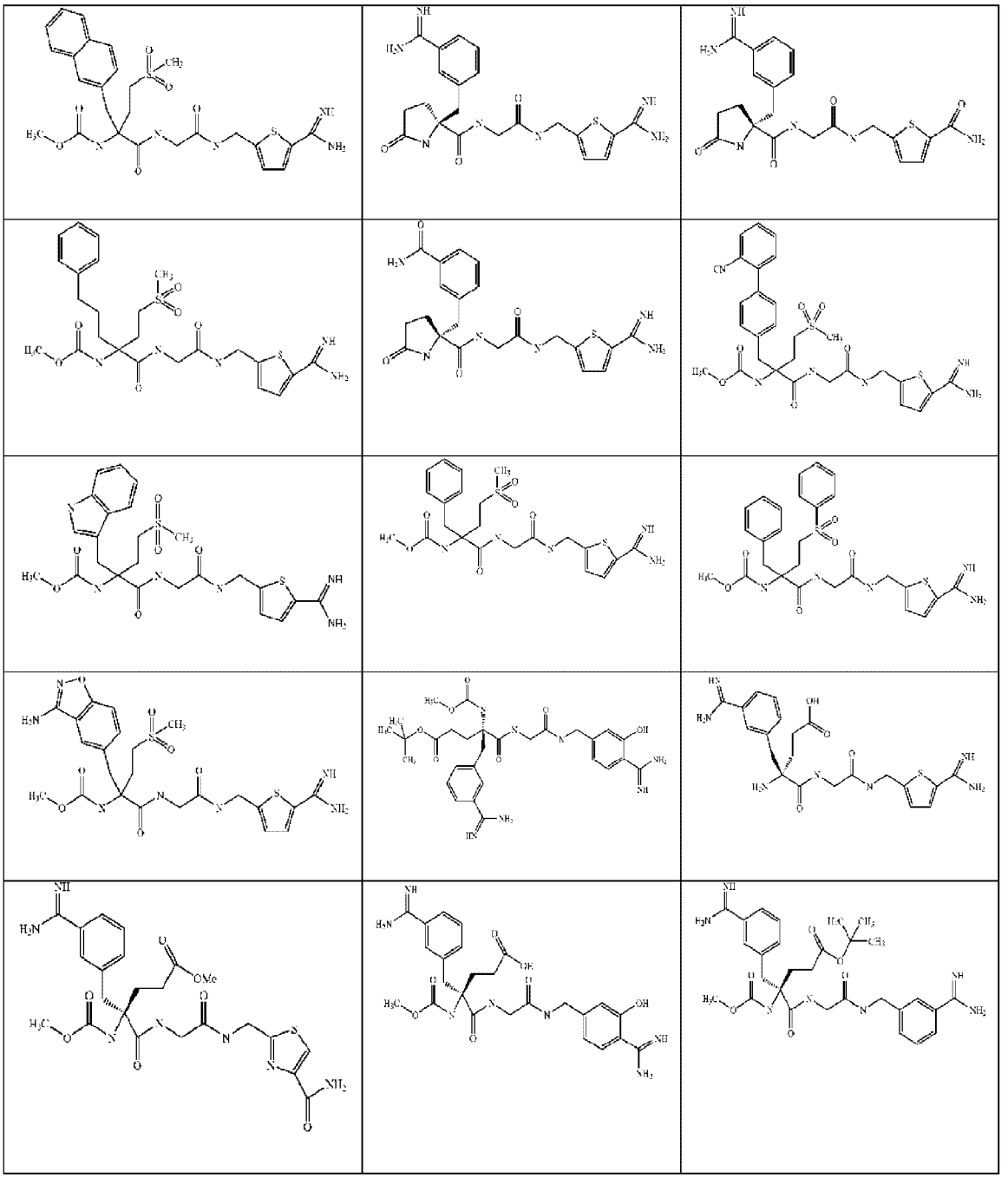


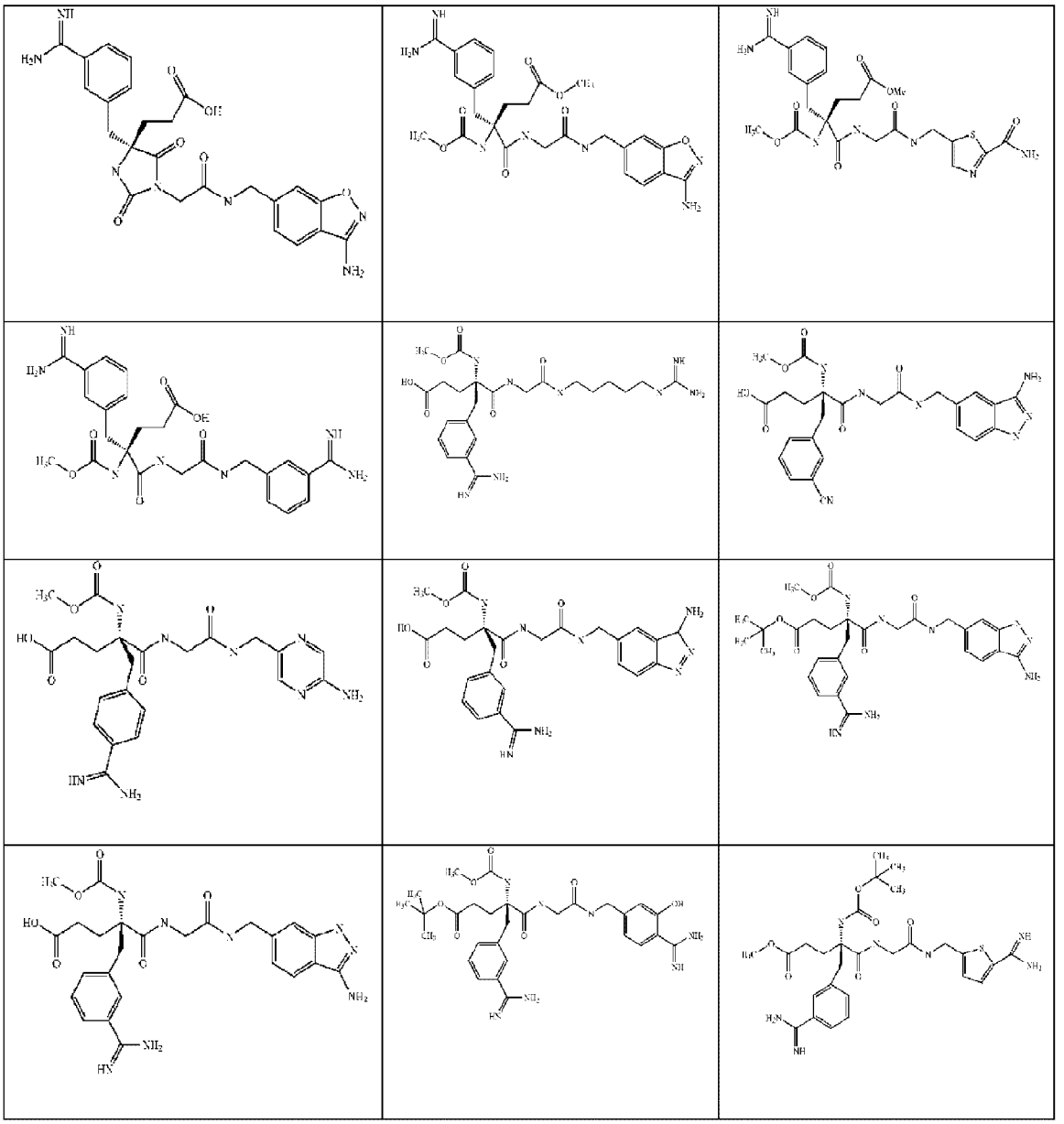
X and Y can be any substituent

Table 3: Structures of representative matriptase inhibitors disclosed in U.S. Patent No. 7,019,019



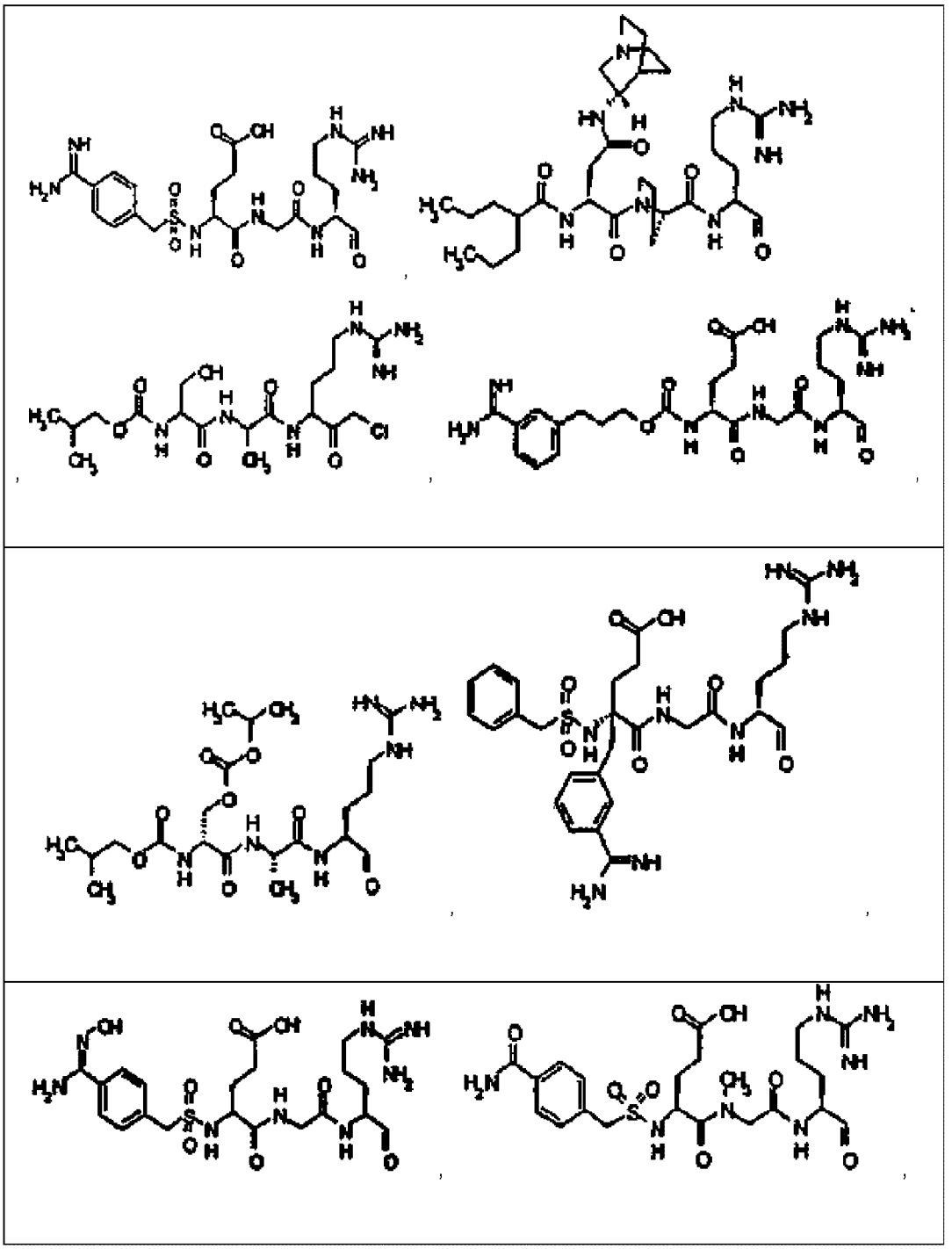


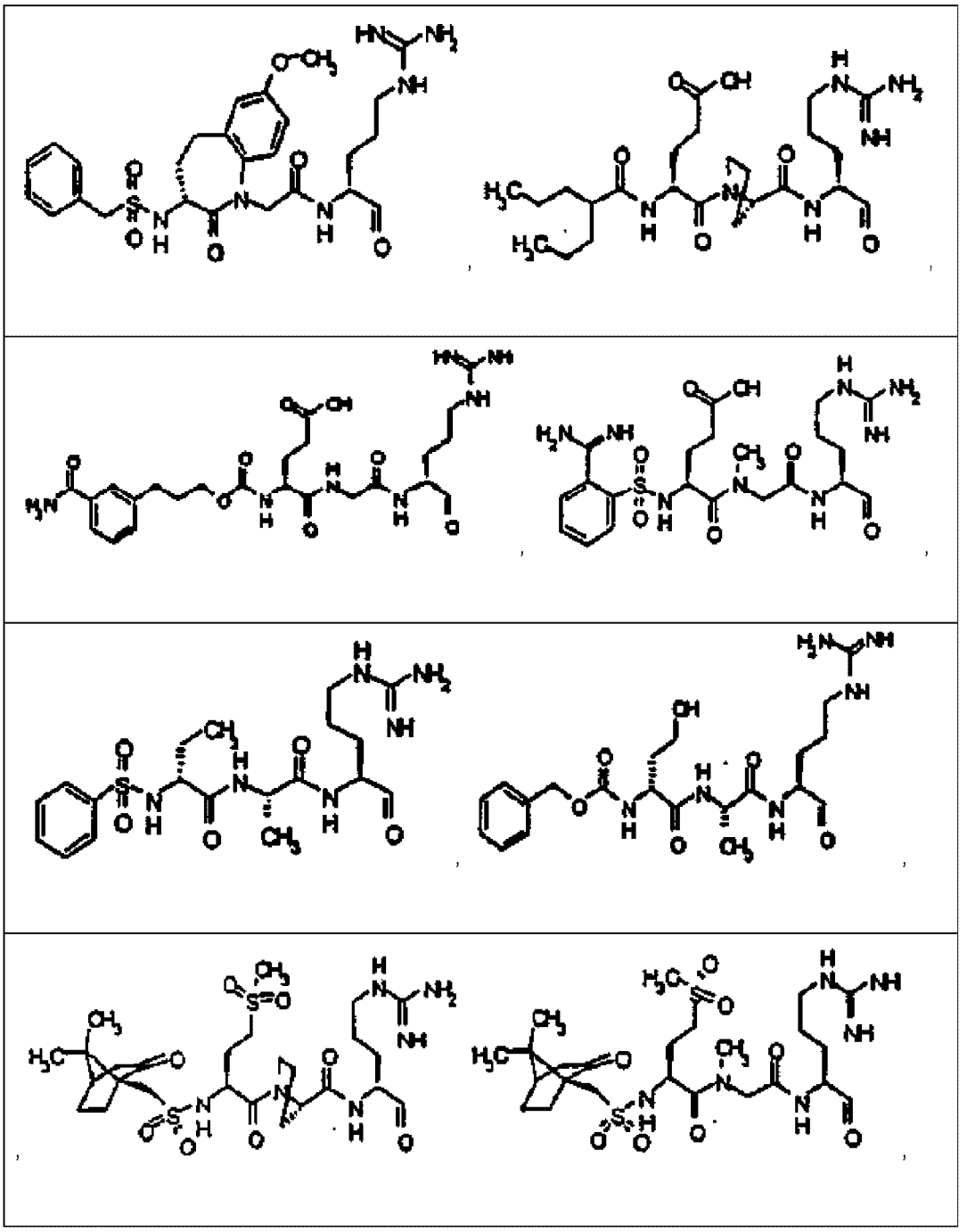


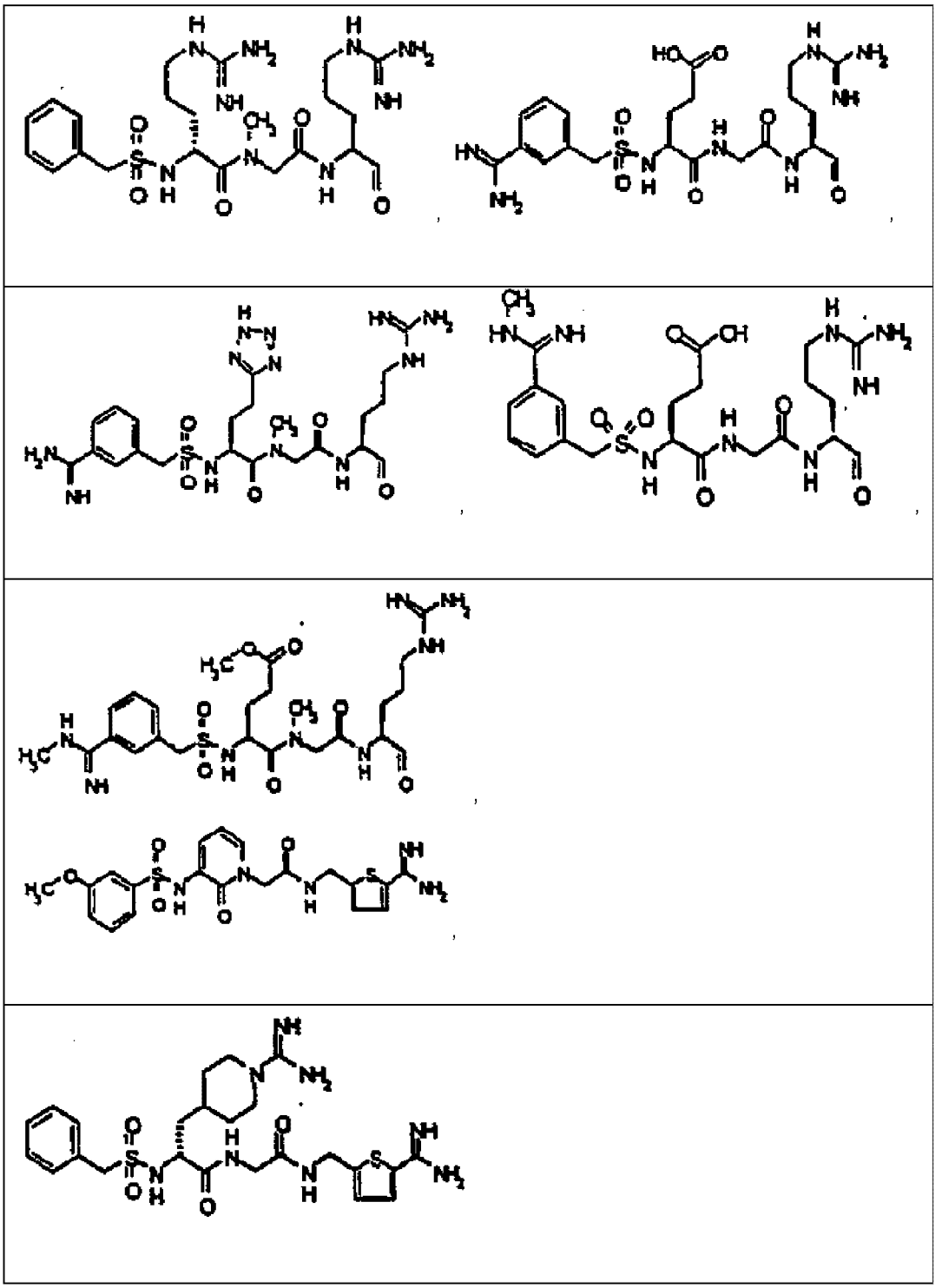


[00204] Matriptase inhibitors are also disclosed in PCT publication No. WO 2002/020475. The structures of representative examples of inhibitors disclosed therein are depicted in Table 4 below.

Table 4: Structures of representative matriptase inhibitors disclosed in WO 2002/020475

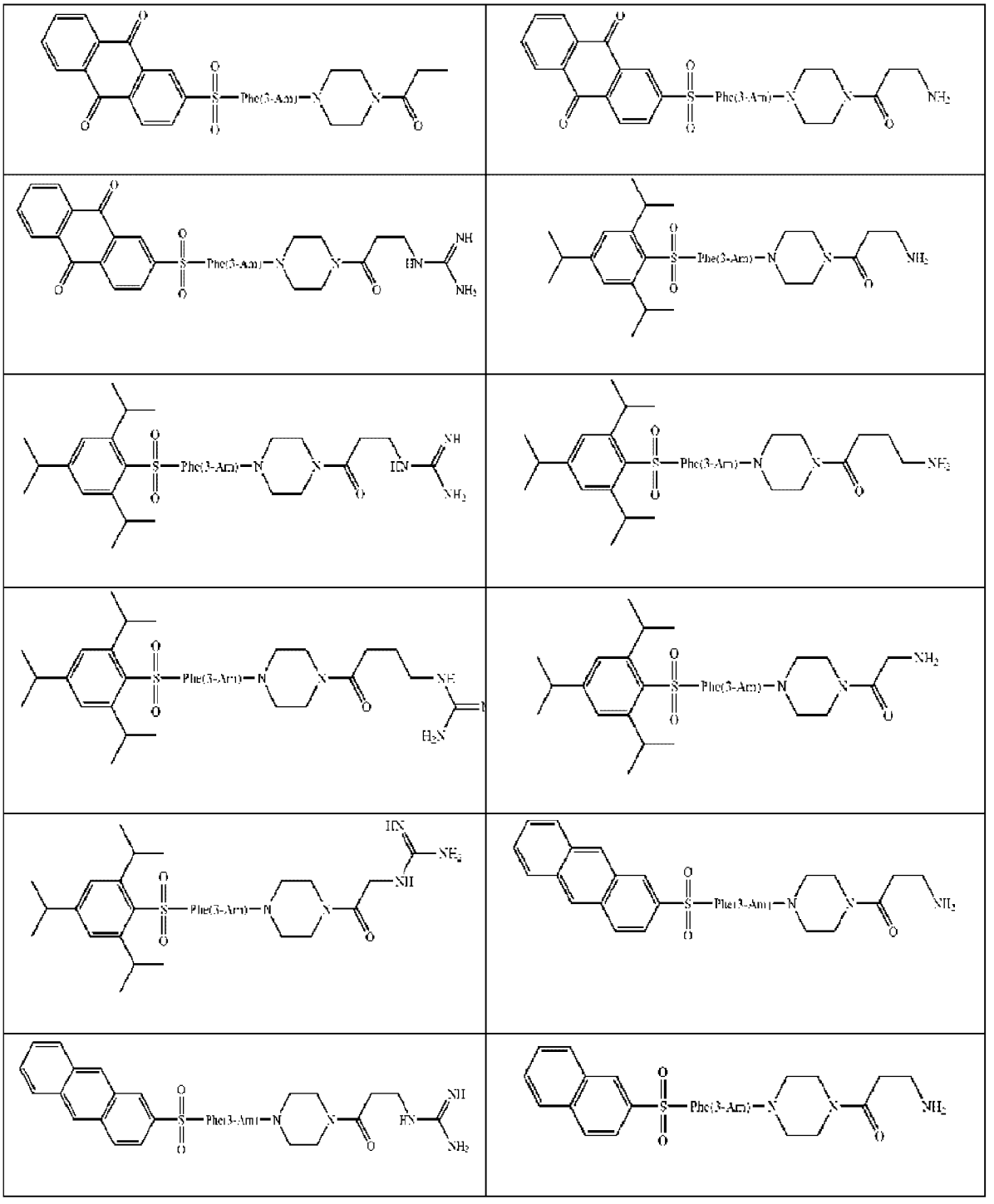


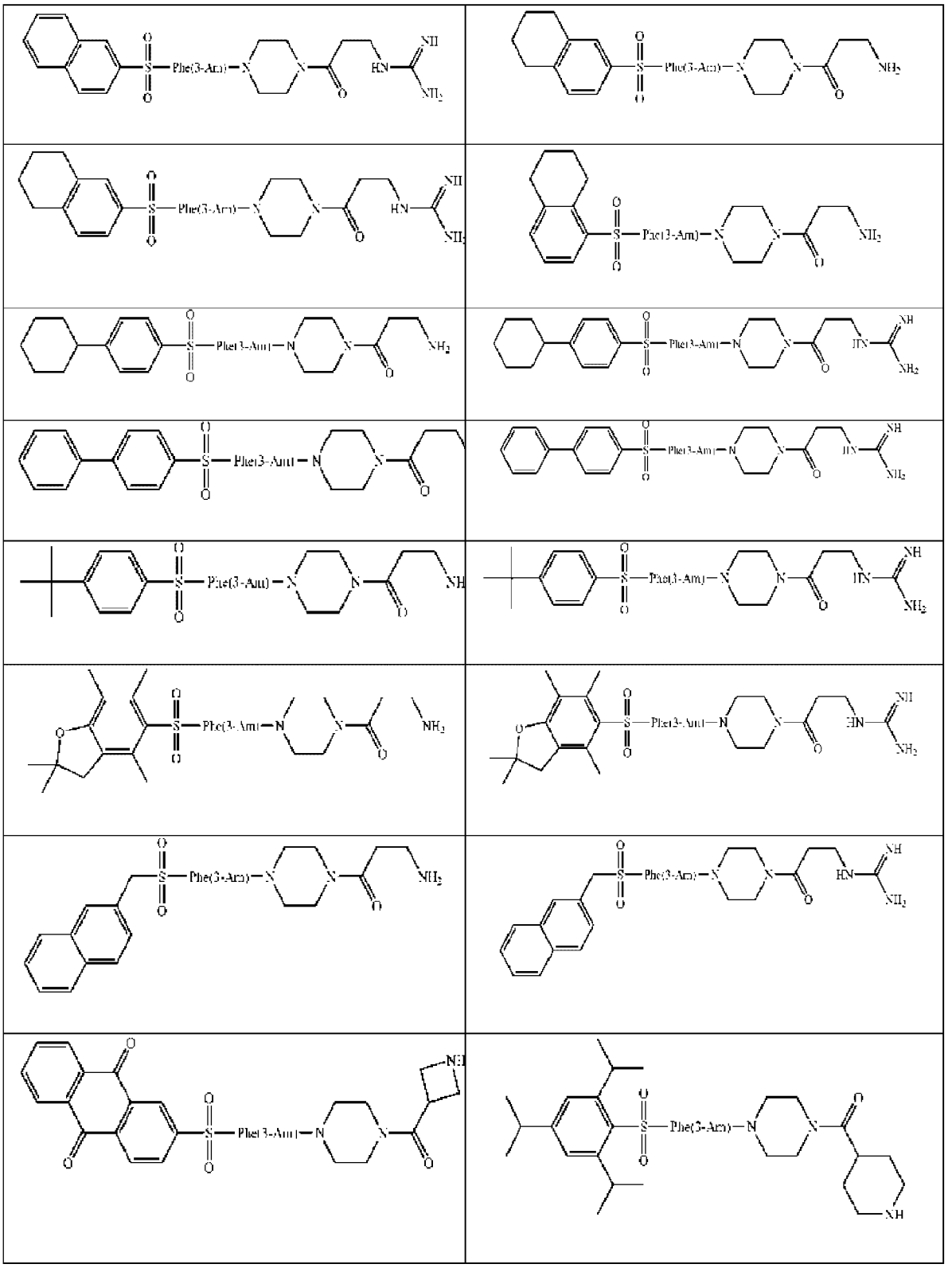


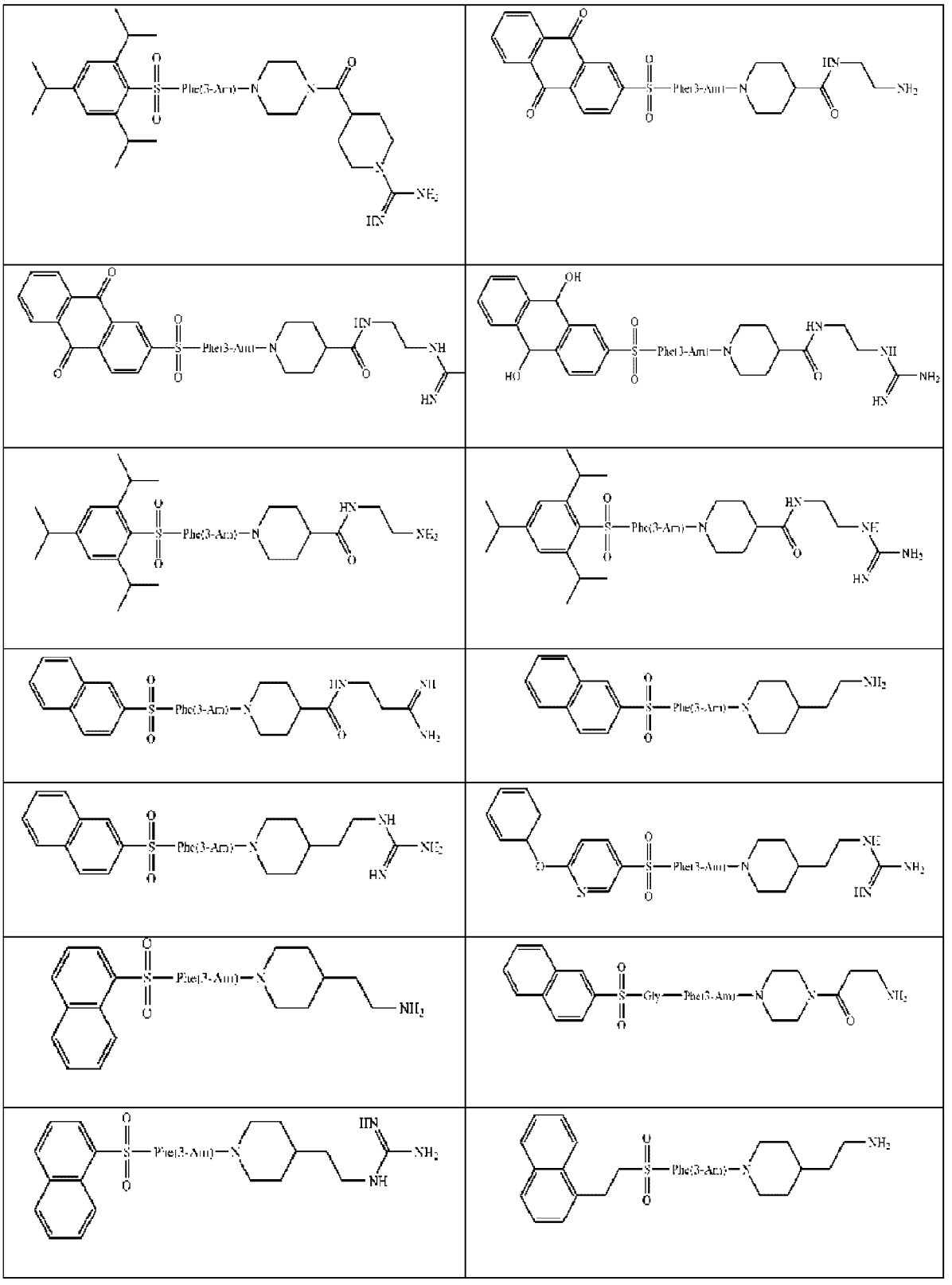


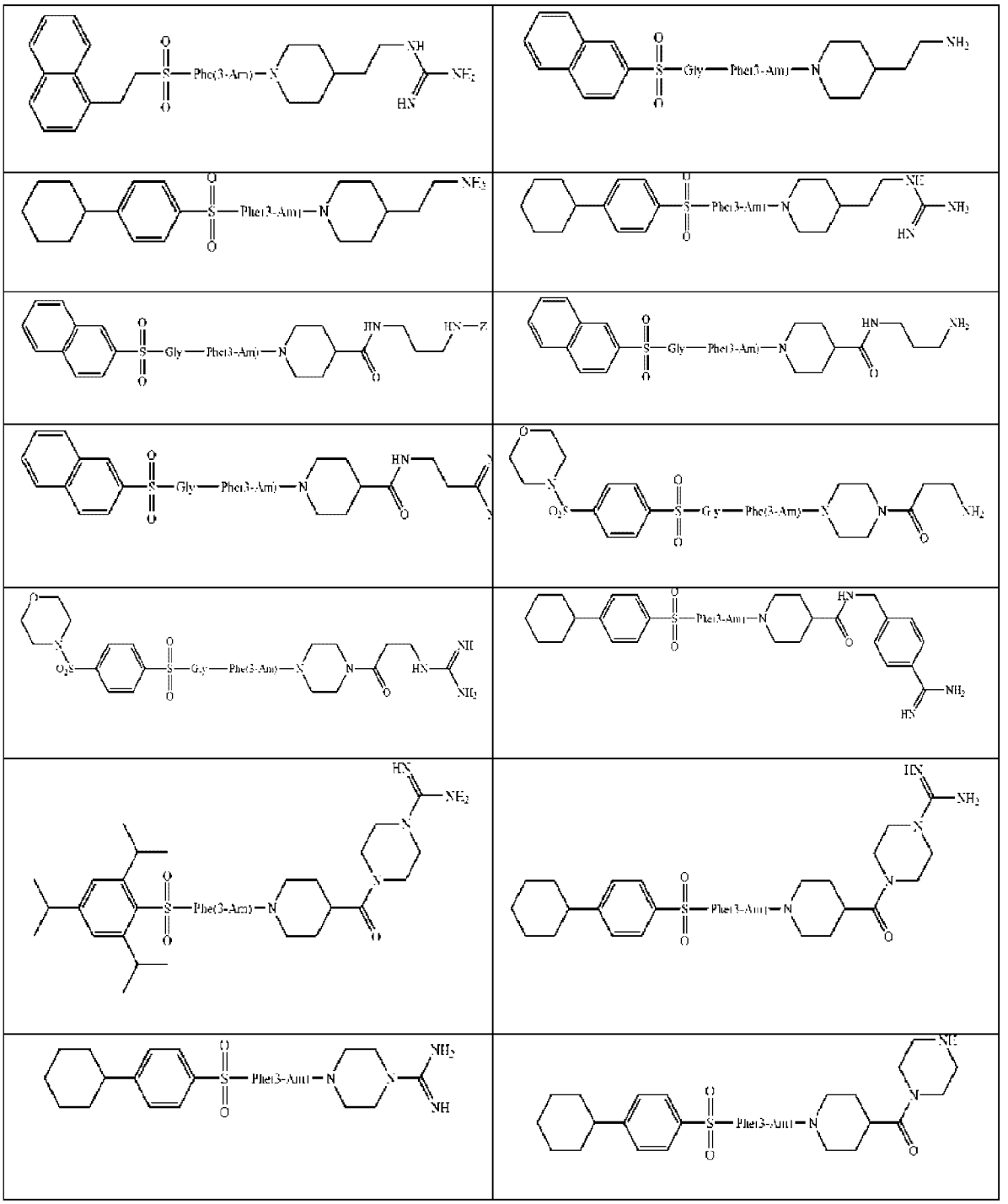
[00205] Matriptase inhibitors are also disclosed in US Patent No. 7,772,251. The structures of representative examples of inhibitors disclosed therein are depicted in Table 5 below.

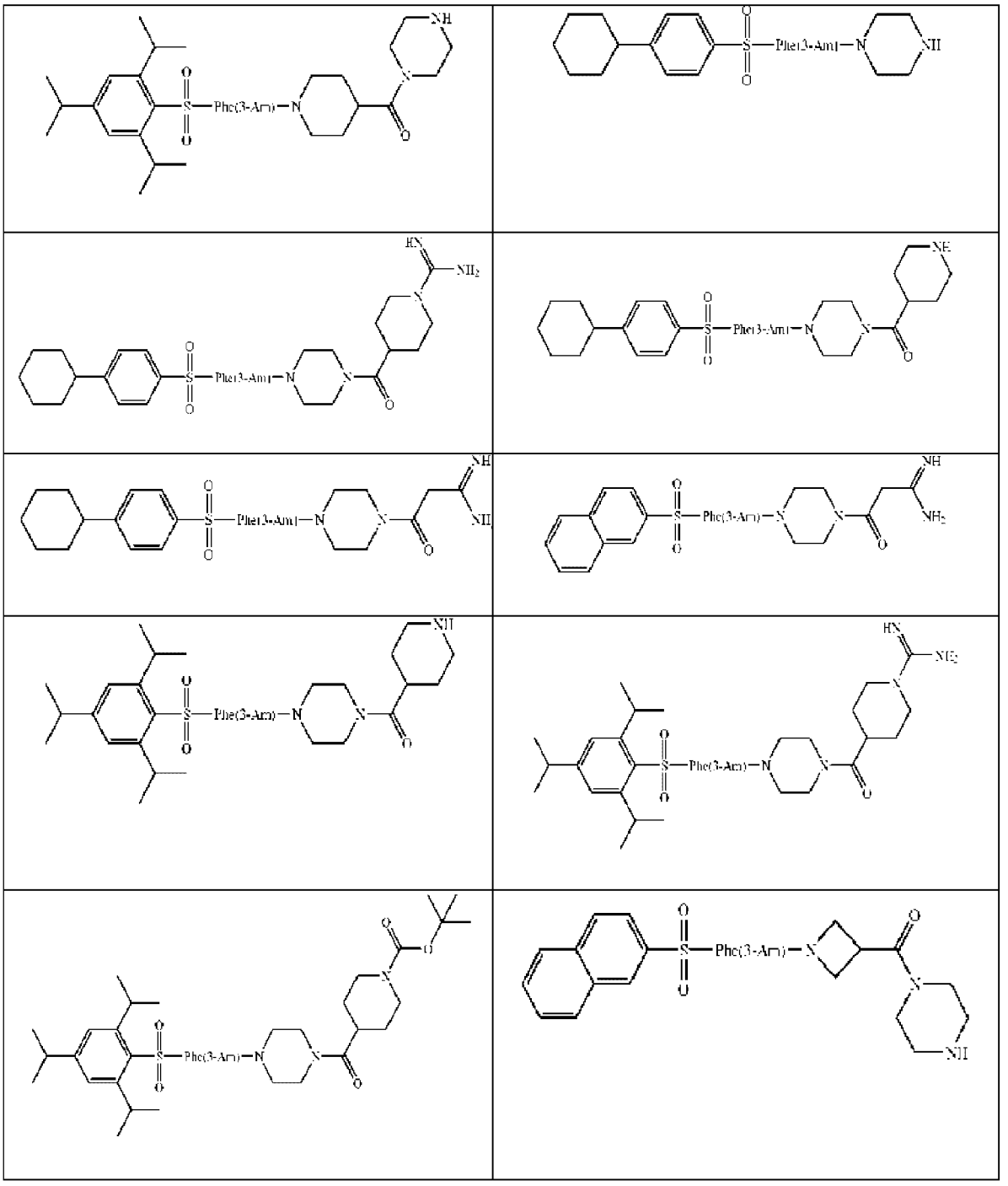
Table 5: Structures of representative matriptase inhibitors disclosed in US Patent No. 7,772,251

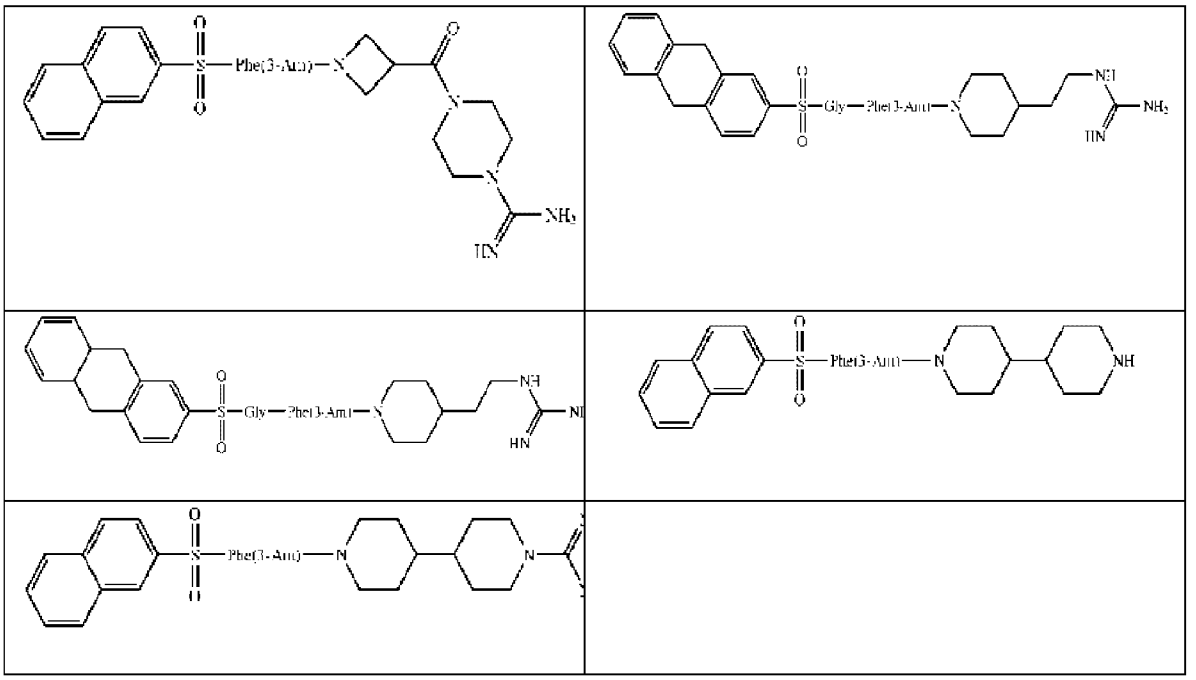






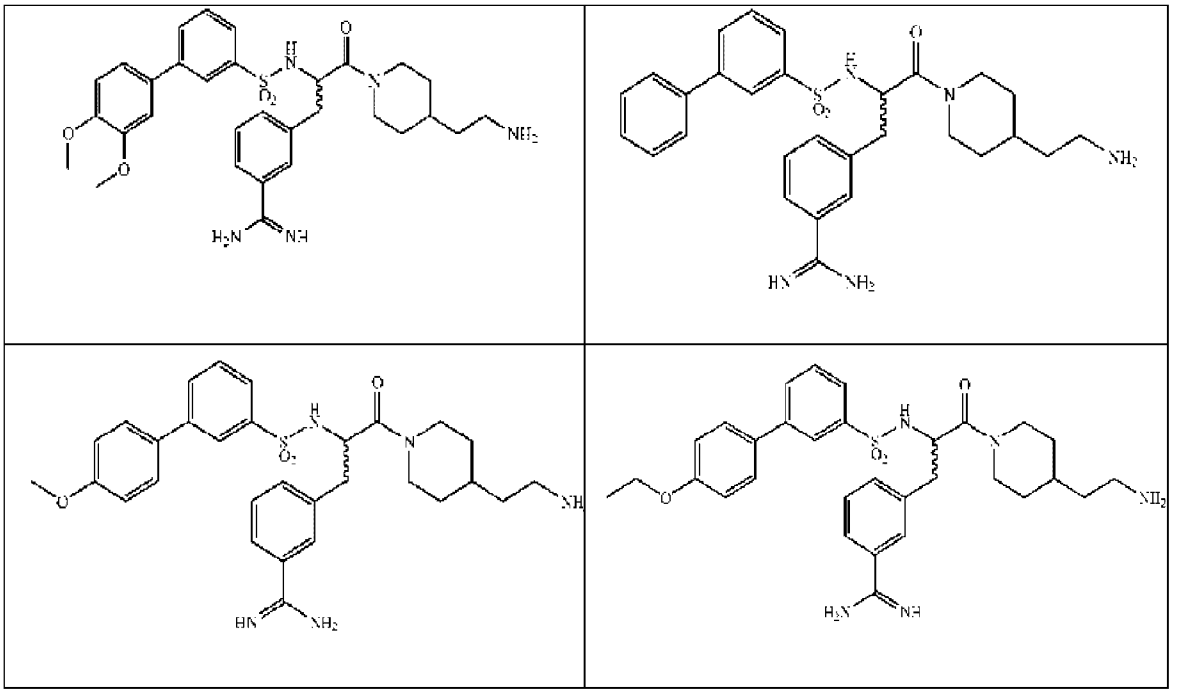


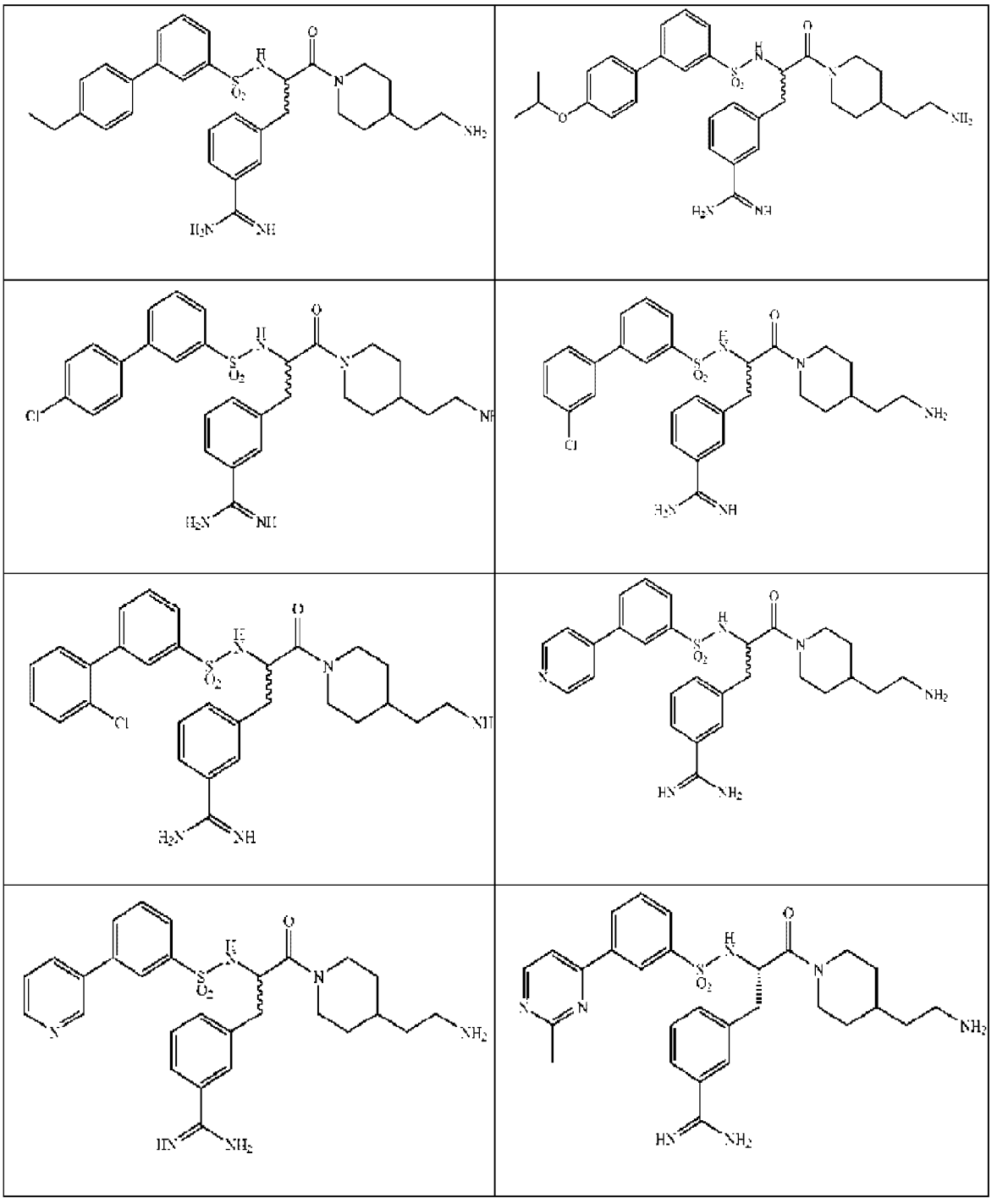


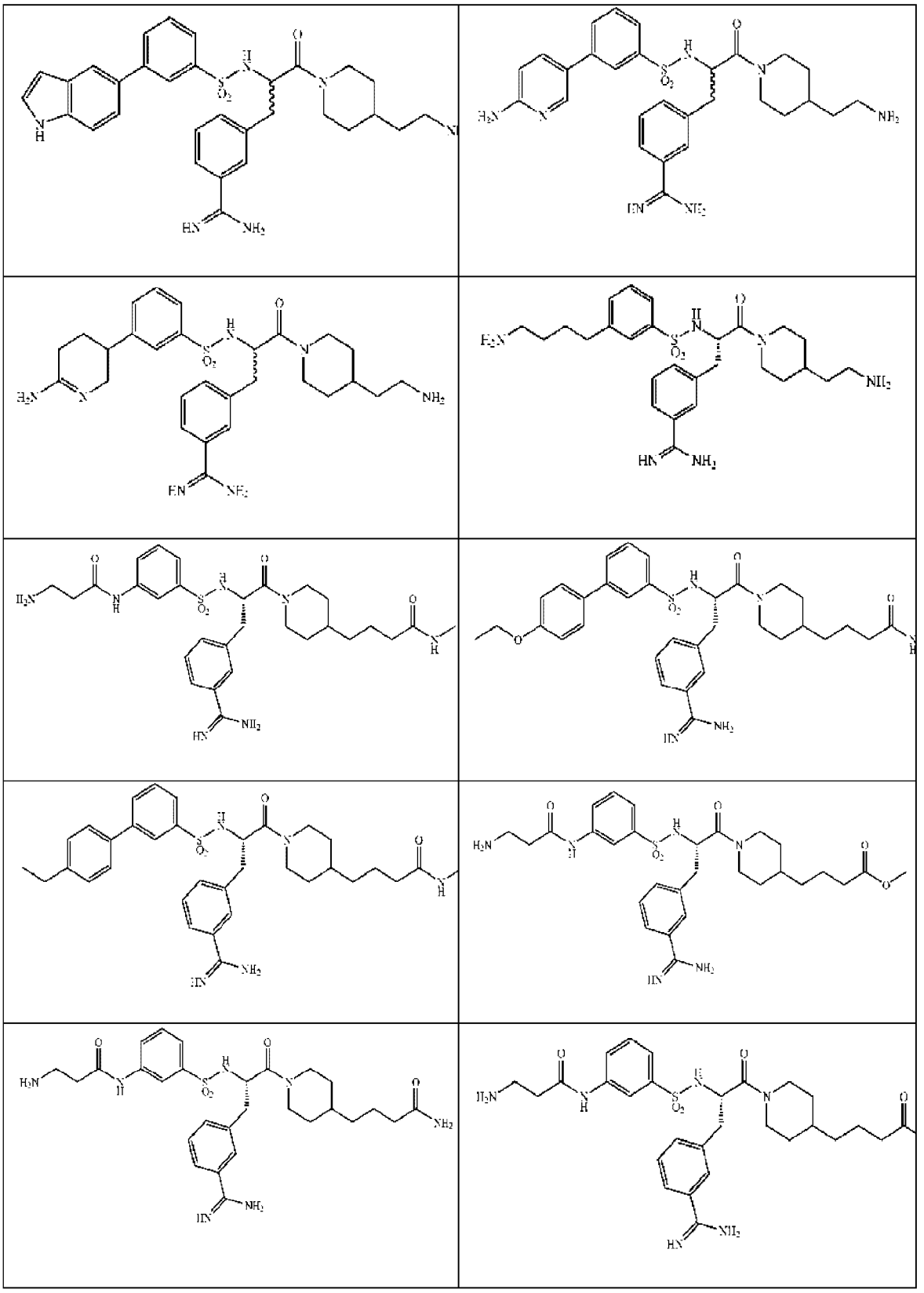


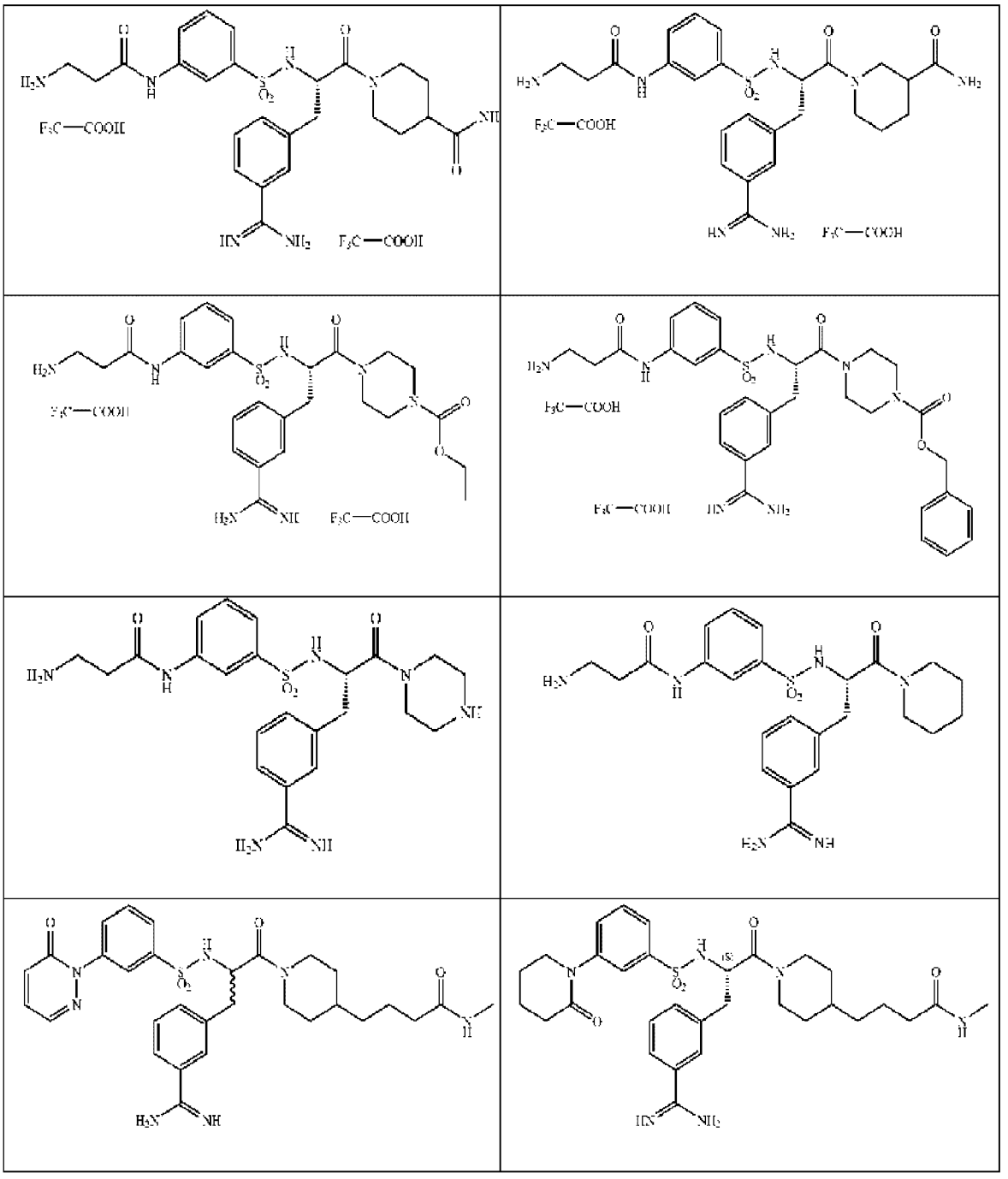
[00206] Matriptase inhibitors are also disclosed in US Patent Publication No. 2010/0305090. The structures of representative examples of inhibitors disclosed therein are depicted in Table 6 below.

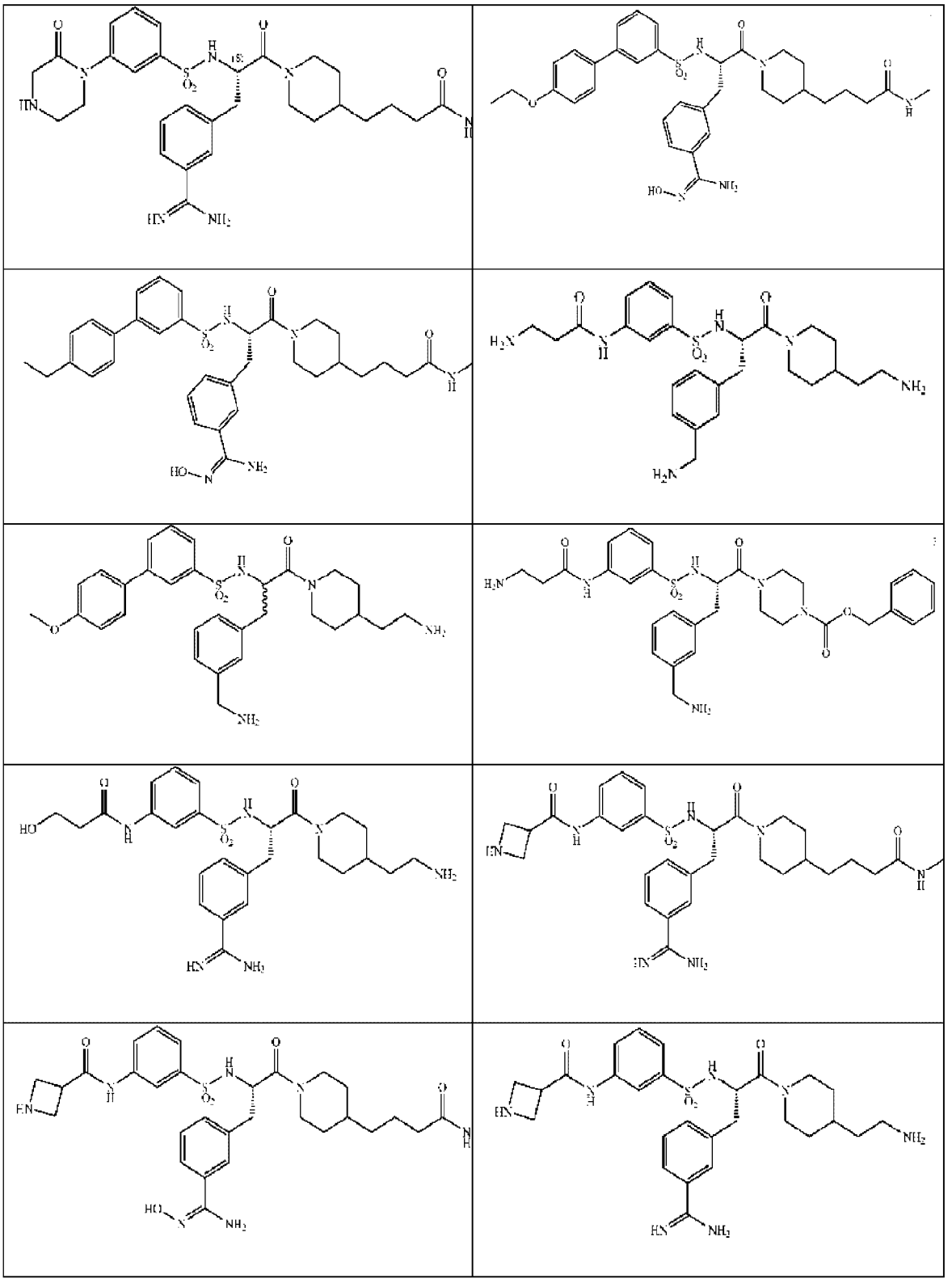
Table 6: Structures of representative matriptase inhibitors disclosed in US Patent Publication No. 2010/0305090

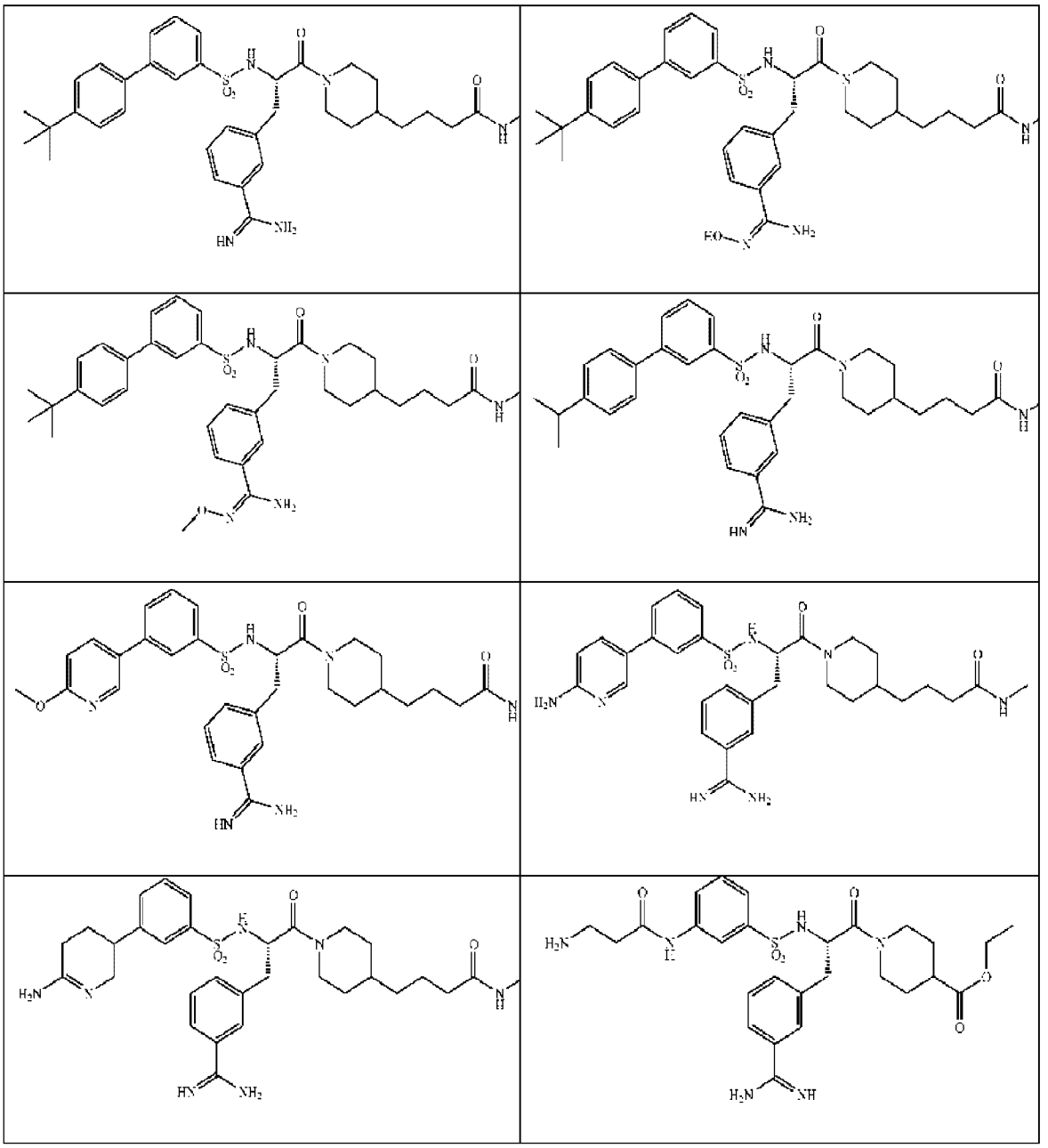


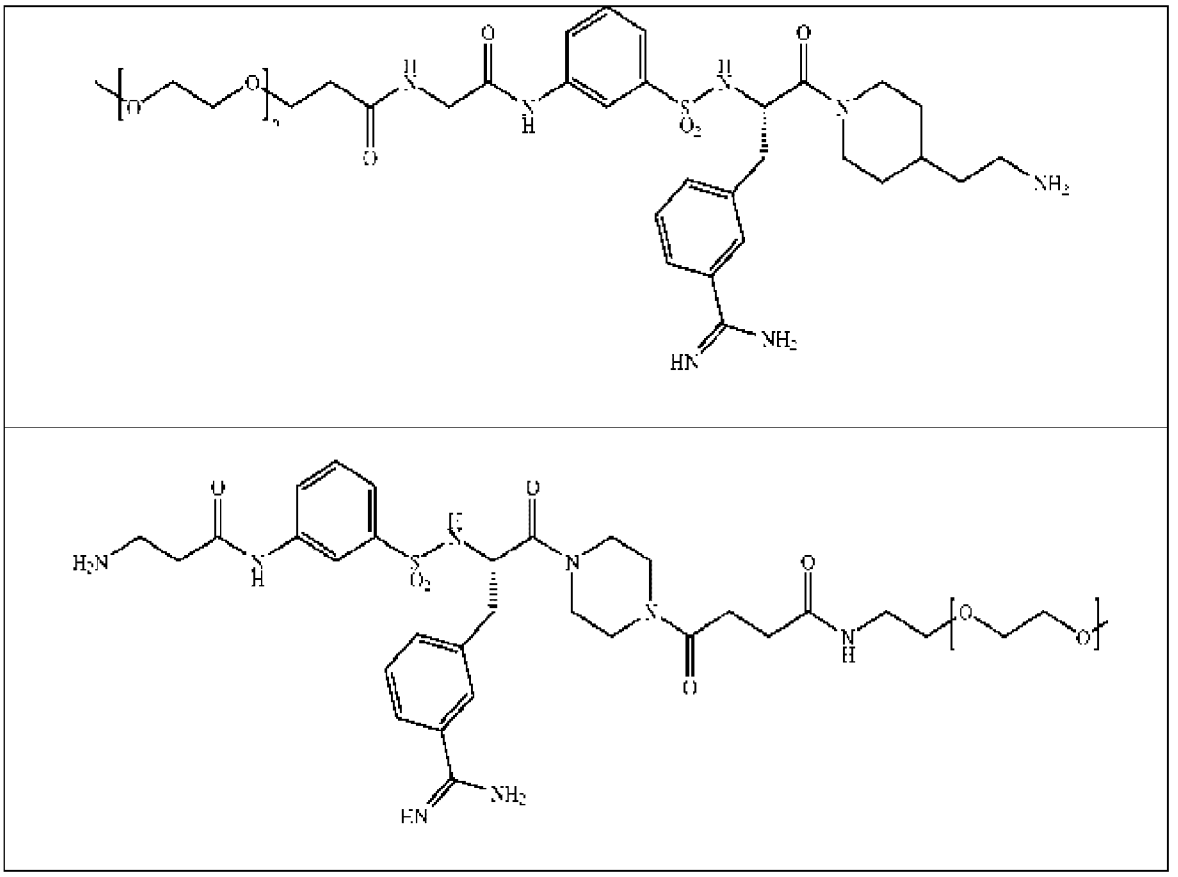












Combinations

[00207] As used herein the term "combination" when used in reference to the use of a matriptase inhibitor in combination with another therapeutic/active agent (*e.g.*, anti-flu) means i) simultaneously (*e.g.*, in separate compositions or a single composition); ii) simultaneously as a single dual action compound (*e.g.*, a conjugate of the two, the matriptase inhibitor chemically linked with another antiviral) in a single composition; or iii) subsequently (*e.g.*, in separate compositions wherein the matriptase inhibitor is administered before or after the other antiviral). The matriptase inhibitor and the therapeutic agent can act additively or, more preferably, synergistically. Other therapeutic agents which may be used with matriptase inhibitors include but are not limited to a viral M2 ion channel inhibitor or a neuraminidase inhibitor. More specifically, such agents may be a siRNA, Tamiflu™ (oseltamivir), Relenza™ (zanamivir), laninamivir, peramivir, amantadine, rimantadine, ribavirin vitamin C, Cold Fx™, Echinacea and ginseng.

[00208] The present invention encompasses therefore the use of a combination of two, three or more active ingredients including a matriptase inhibitor. A combination of three compounds in accordance of the present invention can include a matriptase inhibitor (*e.g.*, a compound as defined above), another anti-influenza agent and a

decongestant.

Routes of administration

[00209] Matriptase inhibitors may be administered in a pharmaceutical composition. Pharmaceutical compositions may be administered in unit dosage form. The route of administration can depend on a variety of factors, such as the environment and therapeutic goals, and particulars about the subject. Any appropriate route of administration may be employed, for example, nasally, transdermal (topical), parenteral, subcutaneous, intramuscular, intramammary, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraarticular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Examples of specific routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, intramammary; oral (*e.g.*, inhalation, by nebulizer); transdermal (topical); transmucosal, and rectal administration. A preferred route of administration is with a nebulizer or inhaler.

[00210] Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer such compositions to patients. Methods well known in the art for making pharmaceutical compositions and formulations are found in, for example, Remington: The Science and Practice of Pharmacy, (20th ed.) ed. A. R. Gennaro A R., 2000, Lippincott: Philadelphia.

Excipients/carriers

[00211] As indicated above, the composition may further comprise a pharmaceutically acceptable carrier or excipient. As used herein, the terms "pharmaceutically acceptable" refer to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to animals (*e.g.*, cows, humans). Preferably, as used herein, the term "pharmaceutically acceptable" means approved by regulatory agency of the federal or state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans.

[00212] The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compounds of the present invention may be administered. Sterile water or aqueous saline solutions and aqueous dextrose and glycerol solutions may be employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[00213] The pharmaceutical compositions of the present invention may therefore contain non-limiting pharmaceutically acceptable excipients/carriers such as solubilizing/diluting agents, antioxidants, enteric coatings, absorption enhancers, pH adjusting agents and buffers, dispersing agents, coatings, antibacterial, antifungal agents, absorption delaying agents (controlled time-release), osmolarity adjusters, isotonic agents, preservative agents, stabilizers, surfactants, emulsifiers, sweeteners, thickening agents, solvents, emollients, coloring agents, wetting

agents, as well as colors and flavors and salts for the variation of osmotic pressure. As mentioned earlier, they may also contain other therapeutically valuable agents. Methods for preparing appropriate formulations are well known in the art (see *e.g.*, Hendrickson, 2005),

[00214] The carrier/excipient is selected for administration by the selected route of administration. The use of such media and agents for pharmaceutically active substances is well known in the art (Rowe et al., Handbook of pharmaceutical excipients, 2003, 4th edition, Pharmaceutical Press, London UK). Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated.

[00215] Therapeutic formulations for oral administration, may be in the form of tablets or capsules; for transmucosal (*e.g.*, rectal, intranasal) or transdermal/percutaneous administration may be in the form of ointments, powders, nasal drops, sprays/aerosols or suppositories; for topical administration, may be in the form of ointments, creams, gels or solutions; for parenteral administration (*e.g.*, intravenously, intramuscularly, intradermal, intramammary, subcutaneously, intrathecally or transdermally), using for example injectable solutions. Furthermore, administration can be carried out sublingually or as ophthalmological preparations or as an aerosol, for example in the form of a spray. Intravenous, intramuscular or oral administration is a preferred form of use.

Oral

[00216] For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used for example in the form of tablets, troches, dragees, hard or soft gelatin capsules, solutions (*e.g.*, syrups), aerosols, emulsions or suspensions, or capsules. For the preparation of formulations for oral administration, the compounds of the present invention may be admixed with pharmaceutically inert, inorganic or organic excipients (*e.g.*, pharmaceutically compatible binding agents, and/or adjuvant). The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring. Examples of suitable excipients for tablets, dragees or hard gelatin capsules for example include lactose, maize starch or derivatives thereof, talc or stearic acid or salts thereof. Suitable excipients for use with soft gelatin capsules include for example vegetable oils, waxes, fats, semi-solid or liquid polyols etc.; according to the nature of the active ingredients it may however be the case that no excipient is needed at all for soft gelatin capsules.

[00217] For the preparation of solutions and syrups, excipients which may be used include for example water, polyols, saccharose, invert sugar and glucose.

Nasal/Inhalation

[00218] A inhibitory compound and/or composition of the invention may also be administered directly to the lung by inhalation. For administration by inhalation, a compound and/or composition of the invention may be conveniently delivered to the lung by a number of different devices. For example, a Metered Dose Inhaler ("MDI"), which utilizes canisters that contain a suitable low boiling propellant, (*e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or any other suitable gas) may be used to deliver compounds of the invention directly to the lung. An aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

[00219] Alternatively, a Dry Powder Inhaler ("DPI") device may be used to administer a compound and/or composition of the invention to the lung. DPI devices typically use a mechanism such as a burst of gas to create a cloud of dry powder inside a container, which may then be inhaled by the patient. DPI devices are also well known in the art. A popular variation is the multiple dose DPI ("MDDPI") system, which allows for the delivery of more than one therapeutic dose. For example, capsules and cartridges of gelatin for use in an inhaler or insulator may be formulated containing a powder mix of a compound of the invention and a suitable powder base such as lactose or starch for these systems.

[00220] Another type of device that may be used to deliver a compound and/or composition of the invention to the lung is a liquid spray device. Liquid spray systems use extremely small nozzle holes to aerosolize liquid drug formulations that may then be directly inhaled into the lung.

[00221] In one embodiment, a nebulizer is used to deliver a compound and/or composition of the invention to the lung. Nebulizers create aerosols from liquid drug formulations by using, for example, ultrasonic energy to form fine particles that may be readily inhaled. Examples of nebulizers include devices supplied by Sheffield/Systemic Pulmonary Delivery Ltd. (see, Armer et al., U.S. Pat. No. 5,954,047; van der Linden er al., U.S. Pat. No. 5,950,619; van der Linden et al., U.S. Pat. No. 5,970,974), Aventis, and Batelle Pulmonary Therapeutics.

[00222] In another embodiment, an electrohydrodynamic ("EHD") aerosol device is used to deliver a compound and/or composition of the invention to the lung. EHD aerosol devices use electrical energy to aerosolize liquid drug solutions or suspensions (see *e.g.*, Noakes et al., U.S. Pat. No. 4,765,539). Elm aerosol devices may deliver drugs to the lung more efficiently than other pulmonary delivery technologies.

[00223] Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Transmucosal or transdermal (topical)

[00224] For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal

administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art. For suppositories, and local or percutaneous application, excipients which may be used include for example natural or hardened oils, waxes, fats and semi-solid or liquid polyols.

Parenteral

[00225] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Solutions or suspensions used for parenteral application can include the following components: a sterile diluent such as water for injection (where water soluble), saline solution, fixed oils (*e.g.*, paraffin oil), polyalkylene glycols such as polyethylene glycols, glycerine, propylene glycol or other synthetic solvents, oils of vegetable origin, or hydrogenated napthalenes; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; reducing agents such as dithiothreitol, buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for compounds of the invention include ethylenevinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. The parenteral preparation can also be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00226] For intravenous or intramammary administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS).

[00227] Liposomal suspensions (including liposomes targeted to specific cell types) can also be used as pharmaceutically acceptable carriers. A variety of liposomal formulations suitable for delivering a compound to an animal have been described and demonstrated to be effective in delivering a variety of compound, including, *e.g.*, small molecules, nucleic acids, and polypeptides.

[00228] As mentioned earlier, medicaments containing the compounds of the present invention are also an object of the present invention, as is a process for the manufacture of such medicaments, which process comprises bringing one or more of the compounds of the present invention to, if desired, one or more other therapeutically valuable substances into a galenical administration form.

Kits

[00229] The present invention also encompasses kits comprising the compounds of the present invention. For

example, the kit can comprise one or more compounds inhibiting influenza virus. The kit may optionally include one or more control samples and a device (*e.g.*, inhaler, nebulizer, etc.). The compounds or agents can be packaged in a suitable container. The kit can further comprise instructions for using the kit.

Applications of the compounds and compositions

[00230] As used herein, the terms "treat/treating/treatment" and "prevent/preventing/prevention", refer to eliciting the desired biological response, i.e. a therapeutic and prophylactic effect, respectively. In accordance with the subject invention, the therapeutic effect comprises a decrease/reduction in the progress of the infection or in the severity of associated symptoms or a complete cure of the infection and/or associated symptoms. In accordance with the invention, a prophylactic effect may comprise a delay or decrease in the onset of, progression of, or the severity of flu infection and associated symptoms (*e.g.*, viral titer in the subject's blood, cells or nasopharingeal swabs, runny nose, plugged nose, sneezing, sore throat, scratchy throat, cough, hoarseness, head congestion, chest congestion, feeling tired, fever, muscle pain, loss of appetite, headache and chills), following administration of a inhibitor of the present invention. In an embodiment, the composition of the present invention, comprising a inhibitor of formula (1), (1.1), (1.2), (2), (2.1) or (3), prevents the subject from contracting an orthomyxovirus infection. The methods, compositions formulations and uses described herein are suitable for both humans and animals (including birds), preferably mammals.

[00231] As used herein, the term "flu" and "flu infection" refers to an infectious disease caused by certain RNA viruses from the orthomyxoviridae (*e.g.*, influenza virus) family. It includes infections by types A, B and C influenza viruses. It affects birds and mammals. The most common symptoms of the disease are chills, fever, sore throat, scratchy throat, muscle pains, headache, chest congestion, head congestion, coughing, weakness, exhaustion, loss of appetite and general discomfort.

[00232] Matriptase is known to be expressed in a variety of cancers, notably epithelia-derived human tumors such as prostate, breast, colon, stomach, ovarian, renal and cervical carcinomas (reviewed in Lee, *J. Cancer Mol.* **2(5)**: 183-190, 2006). Accordingly, in another aspect, the present invention provides a method for inhibiting tumor growth, progression and/or metastasis in a subject in need thereof, the method comprising administering to the subject an effective amount of the inhibitor of formula (1), (1.1), (1.2), (2), (2.1) or (3) defined above. In another aspect, the present invention provides the use of the inhibitor of formula (1), (1.1), (1.2) (2), (2.1) or (3) defined above for inhibiting tumor growth, progression and/or metastasis in a subject. In another aspect, the present invention provides the use of the inhibitor of formula (1), (1.1), (1.2), (2), (2.1) or (3) defined above for the preparation of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.

Dosages

[00233] Compositions and formulations of the present invention are administered in amounts and at a frequency sufficient to prevent or treat flu infections and/or ameliorate any symptom associated with flu. The skilled artisan will

appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health, weight and/or age of the subject, the requirements of the patient and the mode of application, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a compound of the present invention can include a series of treatments.

[00234] Any amount of a composition of the present invention can be administered to a subject, provided it is not associated with important adverse effects and it is in a non-toxic dose. Typically, the amount of inhibitor contained within a single dose will be an amount that effectively prevents or treats an orthomyxovirus infection (*e.g.*, flu) and one or more associated symptoms, without inducing significant toxicity i.e., the composition improves or reduces one or more of : viral titer in the subject's blood or cells, runny nose, plugged nose, sneezing, sore throat, scratchy throat, cough, hoarseness, head congestion, chest congestion, general weakness, fever, muscle pain, loss of appetite, headache and chills. Generally, the effective dose will not exceed CA, US and EP pharmacopeia for each product individually.

[00235] Thus, in one aspect of the present invention, the composition comprising the inhibitors of the present invention, and/or one or more other matriptase inhibitors, is administered prior to the onset of flu symptoms as a preventive measure. In another aspect of the present invention, the pharmaceutical composition of the present invention is administered in combination with a drug or drugs used to treat flu symptoms. In a further aspect, the composition of the present invention is administered once the subject has been diagnosed with flu infection or after the onset of flu symptoms. In another embodiment, the composition of the present invention is administered in combination with one or more other drugs used for the prevention and/or treatment flu infections such as Tamiflu™ (oseltamivir), Relenza™ (zanamivir), laninamivir, peramivir, amantadine, rimantadine, ribavirin, vitamin C, vitamin C, Cold Fx™, echinacea, ginseng, etc.

[00236] As used herein the expression "effective amount" or "therapeutically effective amount" is meant to refer to an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects, such as an improvement of the condition of the patient. An effective amount can be administered in one or more doses. For the purposes of this invention, an effective amount on the composition of the present invention is an amount that induces a therapeutic or prophylactic response against flu infections. Such amount may vary according to the nature of the infection (specific type of viral infection), the severity of the infection, the mode of administration, the age, weight and sex of the affected subject, etc. One skilled in the art can easily and without difficulty determine what will be such effective amount. Generally, the effective amount will be between about 1mg and about 5000mg, preferably between 5 to 500mg of inhibitor of the present invention. In an embodiment, the effective amount is between about 5 mg and about 400 mg of inhibitor. In another embodiment, the above-mentioned composition comprises between about 5 mg and about 300 mg of inhibitor.

[00237] The effective amount may be given daily in a single or several doses (*e.g.*, twice daily, three times per

day or 4 times per day). It may also be given every 2 days, every 3 days or once a week, as prescribed. Preferably, the effective amount is given once daily.

[00238] The effective amount to be administered to a human subject may be calculated from studies in animals. The dose may be scaled up to a human equivalent dose (HED) for starting clinical trials using published conversion tables which provide a conversion factor from mice to human of 12.3.

[00239] These are simply guidelines since the actual dose must be carefully selected and titrated by the attending physician based upon clinical factors unique to each patient. The optimal daily dose will be determined by methods known in the art and will be influenced by factors such as the age of the patient as indicated above and other clinically relevant factors.

Toxicity and therapeutic efficacy

[00240] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00241] Data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography in tandem with mass spectrometry.

Viral Targets

[00242] Compounds of the present invention may be used as anti-viral agents. In this respect, the compounds of the present invention are used against viruses that require proteolytic cleavage of HA for their activation. In a specific embodiment, viral targets of the present invention are viruses of the orthomyxoviridae family such as influenza (*e.g.*, type A, B or C).

Subjects

[00243] The term "subject" in the context of the present invention relates to any mammal including a mouse, rat, cat, dog, cow, pig, monkey, a bird and a horse. In a specific embodiment, it refers to a human. A "subject in need thereof " or a "patient" in the context of the present invention is intended to include any subject that will benefit or that is likely to benefit from the inhibitor of the present invention. In an embodiment, a subject in need thereof is a subject diagnosed with an orthomyxovirus infection (*e.g.*, flu). In another embodiment, a subject in need thereof is a subject that is likely to catch an orthomyxovirus infection (*e.g.*, flu) or in which an orthomyxovirus infection (*e.g.*, flu) infection is likely to have important health consequences (*e.g.*, young children, elderly or immune deficient subjects). The likelihood of contracting an orthomyxovirus infection (*e.g.*, flu) can be determined for instance with the prevalence of the disease in the subject's environment including close members of the family (sisters, brothers, parents, grandparents, uncles and aunts, spouse, colleagues, friends, etc.). In an embodiment, a subject in need thereof is a subject suffering from the orthomyxovirus infection (*e.g.*, flu) or any associated symptoms.

[00244] The present invention is illustrated in further details by the following non-limiting examples.

EXAMPLE 1

MATRIPTASE EXPRESSION IN HUMAN AIRWAY EPITHELIAL CELLS

[00245] RNA extracts from various human respiratory system cell lines including Calu-3, H292, A549 and Beas-2B, from normal human lungs (Ambion) were obtained using the Trizol™ extraction method to probe for matriptase mRNA expression. cDNA was obtained after reverse transcription of RNA using random primers. Next, specific primers for matriptase (FW-ctaggatgagcagctgtgga (SEQ ID NO:3); RV-aagaatttgaagcgcacctt (SEQ ID NO:4)) were used to amplify by PCR any RNA message for the protease. Water instead of RNA was used as a negative control (-) and a plasmid containing the matriptase sequence (coding for residues 1-855 of SEQ ID NO:2) was used as a positive control (+). The results showed that mRNA for matriptase was present in Calu-3, H292 and Beas-2B cells as well as in normal human lung cells (Figure 2). These results showed that human respiratory system cells can produce the messenger RNA for the matriptase serine-protease, thus suggesting that these cells can express the enzyme in protein form.

[00246] It was next determined by Western blot whether the matriptase protein was expressed by human respiratory cells. Protein extracts were obtained from each cell line (Calu-3, H292, A549, Beas-2B, NHBE and 16-HBE) and subjected to electrophoresis in a polyacrylamide gel (SDS-PAGE). Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane. Membranes were blocked using non-fat dry milk in Tris-Buffered Saline (TBS) with Tween™ 20. Membranes were then labeled with a rabbit anti-human matriptase/ST14-specific antibody (Bethyl laboratories, Inc.). Labeled proteins were then revealed using a donkey anti-rabbit secondary antibody coupled to horseradish peroxidase (GE Healthcare UK Ltd.) and enhanced chemiluminescence (ECL). Results showed that matriptase protein is expressed in human respiratory epithelial cell lines Calu-3 and H292, and to a lesser extent in NHBE and 16-HBE cells (Figure 3). Predicted molecular weight of matriptase is about 75 kDa.

[00247] The cellular localization of matriptase was also analyzed by immunofluorescence. A monolayer of Calu-3 cells was grown on cover slips and stained with Hoechst (staining of the nucleus; Invitrogen Corp.), DiI (staining of membranes; Invitrogen Corp.) and rabbit anti-human matriptase/ST14 antibody (Bethyl Laboratories Inc.) and goat anti-rabbit Alexa Fluor™-488 antibody (Invitrogen Corp.). Cover slips were analyzed using a Nikon™ TE2000-Ufluorescence microscope and the appropriate filters (Figure 4, upper panel). Also, Calu-3 cells were grown in transwell chambers to confluence (tight junctions; elevated electrical resistance). Transwell membranes were removed and placed in paraffin blocks. Five-micron sections were cut and stained with DAPI (staining of the nucleus) and rabbit anti-human matriptase antibody and anti-rabbit Alexa-647. Fluorescence was detected using confocal microscopy (Figure 4, lower panel). Results confirmed that the matriptase protein is indeed expressed by airway epithelial cells and that it is localized at least in the plasma membrane of these cells (Figure 4). These results are in accordance with matriptase being a type II transmembrane serine protease.

EXAMPLE 2

MATRIPTASE PROMOTES INFLUENZA HEMAGGLUTININ CLEAVAGE/ACTIVATION AND MULTICYCLE VIRAL REPLICATION

[00248] Experiments were performed to determine whether matriptase has the potential to cleave influenza HA sequences. Internally quenched ortho-Aminobenzoic acid (Abz) fluorescent peptide substrates (IQFPs) were designed based on consensus sequences of influenza HAs most relevant to humans (H1 and H3) with the general structure Abz-XXXR↓GLFG-Tyr(3-NO_2) (SEQ ID NO: 6). H1 sequence was Abz-IQSR↓GLFG-Tyr(3-NO_2) (SEQ ID NO: 7) and H3 sequence was Abz-KQTR↓GLFG-Tyr(3-NO_2) (SEQ ID NO: 8). Downward pointing arrow represents cleavage site between enzyme recognition site (P4-P1) and first four amino acids of the influenza fusion peptide (P1'-P4'; conserved among all influenza strains). IQFPs containing HA sequences of H1 or H3 at fixed concentration were incubated with recombinant matriptase as previously described (Beliveau, F., A. Desilets, and R. Leduc. 2009. *Febs J* **276**:2213-2226). The data presented in Figure 5 indicate that matriptase has the potential to cleave influenza H1 and H3 sequences.

[00249] Next it was also tested whether the recombinant form of matriptase containing residues 596-855 of SEQ ID NO:2 produced in the inventors' laboratories (16), could cleave the HA of influenza A/Puerto Rico/8/34 (PR8) virus (for this assay a virus with non-cleaved HA was generated). HA0 viruses were generated as described in Klenk *et al.* 1984. HA0 viruses were incubated with matriptase 500nM (or trypsin 200nM as positive control) for 2h at 37°C and viral particles were disrupted using Triton X-100™ at a final concentration of 1%. Viral proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Using a H1 (H1N1/Pan)-specific rabbit anti-viral protein antibody and anti-rabbit-HRP secondary antibody, bands were revealed using ECL reagent. HA0 indicates uncleaved HA and HA1 represents the HA1 fragment of cleaved HA. Results are presented in Figure 6 and show that matriptase was able to cleave viral HA.

[00250] It was then tested whether the above-cited recombinant form of matriptase, could substitute for trypsin

in the MDCK cell model of influenza replication using the PR8 virus. This cell model necessitates an exogenous protease to activate the influenza HA and to support multicycle replication.

[00251] MDCK cells were cultured to confluence and infected with 25 PFU of the PR8 virus for 1h (to allow viral attachment) in incomplete medium containing BSA. Cells were then washed and a 1.8% Avicel microcrystalline cellulose solution in MEM containing 43nM of matriptase (or 43nM trypsin; positive control) was added to allow viral plaque formation. 48h later, cells were washed and cells/viral plaques were fixed with Carnoy fixative and revealed with a solution of crystal violet. Viral plaques were counted and results were compared. Results presented in Figure 7 demonstrate that substituting trypsin for matriptase (residues 596-855 which includes the catalytic domain) as the exogenous protease in the assay resulted in influenza HA activation and multicycle replication of the virus. The efficiency of matriptase compared to trypsin was approximately 80%. The lower left panel shows a compilation of results and efficacy of IN-1 at inhibiting matriptase in this assay (no effect on trypsin indicating selectivity). Panel C shows a concentration-response curve of inhibition of PR8 plaque formation in the presence of IN-1 in MDCK cells supplemented with Matriptase (43nM). Thus, these results show that matriptase is able to support multicycle replication of influenza virus with high efficiency as compared to trypsin.

[00252] Since the above assay involved the addition of exogenous enzymes, it was sought to evaluate whether matriptase is involved in supporting multicycle replication of influenza virus in a system that does not require exogenous proteases. The Calu-3 airway epithelial cell line was used to test this hypothesis.

[00253] Using RNA interference technology, siRNAs specific to human matriptase were targeted in these cells. Calu-3 cells were transfected with two different siRNAs (50nmol, S1: s13520 (Ambion) Silencer® Select siRNAs gene symbol ST14; S2: EHU012511 (Sigma-Aldrich) MISSION® esiRNA targeting human ST14 (esiRNA1)) targeted toward matriptase mRNA (target sequence of EHU012511: ACGTCCTGCTCATCACACTGATAACCAACACTGAGCGGCGGCATCCCGGCTTTGAGGCCACCTTCTTCCAGCT GCCTAGGATGAGCAGCTGTGGAGGCCGCTTACGTAAAGCCCAGGGGACATTCAACAGCCCCTACTACCCAGG CCACTACCCACCCAACATTGACTGCACATGGAACATTGAGGTGCCCAACAACCAGCATGTGAAGGTGCGCTTC AAATTCTTCTACCTGCTGGAGCCCGGCGTGCCTGCGG (SEQ ID NO:5)) using various concentrations of Lipofectamine RNAiMAX™ (0.5 to 1.5µl). Scrambled siRNAs were used as controls. 48h after transfection, cellular protein extracts were prepared. Extracts were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were probed for matriptase protein expression using a rabbit anti-human matriptase-specific antibody. Membranes were blocked using non-fat dry milk in Tris-Buffered Saline (TBS) with Tween™ 20. Membranes were then labeled with a rabbit anti-human matriptase-specific antibody (Bethyl Laboratories Inc.). Labeled proteins were revealed using a donkey anti-rabbit secondary antibody coupled to horseradish peroxidase (GE Healthcare UK Ltd.) and enhanced chemiluminescence (ECL).

[00254] Results presented in Figure 8 demonstrate that the two independent matriptase-targeted siRNAs significantly block matriptase protein expression. Using 1.5µl of Lipofectamine RNAiMAX™ for transfection,

scrambled siRNA controls (AllStars™ negative controls, Cat # 1027280; Qiagen) had little or no effect on matriptase protein expression, whereas both siRNAs 1 and 2 significantly blocked matriptase protein expression in Calu-3 cells. Densitometric analysis of blots presented in Figure 8A revealed that matriptase-targeted siRNAs significantly inhibited matriptase protein expression in Calu-3 cells 48h after transfection. Matriptase protein knock-downs ranged between 80-90% using these siRNAs (Figure 8B).

[00255] Further experiments were conducted to test whether this knockdown of matriptase would inhibit influenza replication. Calu-3 cells were transfected (Lipofectamine RNAiMAX™ 1.5µl) with siRNAs S1 and S2 described above targeted to matriptase 48h before infection (scrambled siRNAs were used as controls). Following transfection, cells were washed and infected with influenza A/Puerto Rico/8/34 (1000 PFU) for 1h to allow viral attachment. Cells were then washed and incubated for 48h. Supernatants were collected and assayed for infectious viral particles using the MDCK/trypsin system. Influenza virus replicated to high titers in untransfected and in scrambled siRNA-transfected cells, but replication was significantly blocked in matriptase siRNA-transfected cells (Figure 9). Taken together these results show that matriptase, a type II transmembrane serine protease, located in the plasma membrane of airway epithelial cells (Figure 4) is capable of promoting multicycle influenza replication (Figures 6, 7, and 9) and is responsible for influenza hemagglutinin activation in airway epithelial cells, which are the target of influenza viruses.

EXAMPLE 3

SYNTHESIS OF MATRIPTASE INHIBITORS

[00256] Specific embodiments of the inhibitors of the present invention have a peptide backbone and include a ketobenzothiazole warhead moiety. The ketobenzothiazole moiety is involved the inhibitory action of these compounds while the peptide backbone play a role in the selectivity for matriptase over other enzymes. Synthesis of ketobenzothiazole inhibitors is exemplified below by IN-1 (compound **1**), a potent inhibitor of matriptase identified by the present inventors. Of course the synthesis described below can be adapted to produce variants of peptide-ketobenzothiazole or peptide-ketoheterocyclic inhibitors or other peptide derivatives, which are within the scope of the present invention.

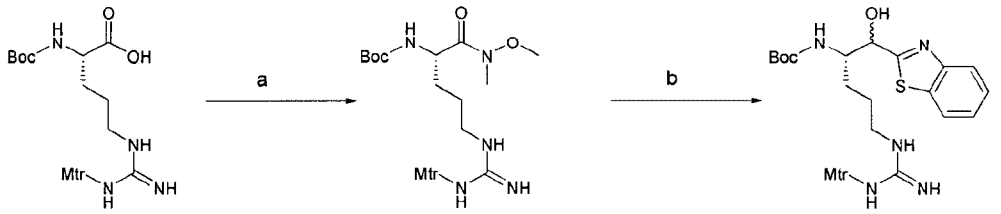
[00257] Materials & Methods (chemistry). Amino acids and coupling reagents were obtained from ChemImpex International (USA) and used as received. Reagents and solvents were purchased from commercial vendors and used as received unless otherwise specified. Solvents were dried as follows: THF from sodium benzophenone, DCM from phosphorus pentoxide, and MeOH from magnesium. Flash chromatography was performed on 60F254™ silica from Silicycle, thin layer chromatography from glass CCM plates from Silicycle. Analytical HPLC experiments were performed on a Agilent™ 1100 series instrument equipped with UV detector set at 223 nm and an Agilent™ Eclipse Plus C18 column (3.0 x 50 mm, 1.8 mm spherical particle size column) with a linear gradient of 2-50% CH_3CN and H_2O containing 0.1% TFA (10 min), 50-100% (4 min), 100% (4 min), 100-2% (1 min) and 2% (3 min). Final products

were purified to > 95% purity (HPLC-UV) by preparative HPLC (Beckman 126™ instrument) using a Vydac™ C18, 250 x 22 mm ID, 5 µm particle size column and a linear gradient of acetonitrile containing 0.1% TFA at a flow rate of 7 mL/min. All inhibitors were obtained as trifluoroacetic acid (TFA) salts after lyophilisation. Molecular weights of compounds were confirmed by mass spectrometry (Electrospray micromass ZQ-2000™ from Waters). ^{1}H and ^{13}C NMR spectra were recorded on an ECX-300™ (Bruker Inc., USA) at 300 MHz, and are referenced to internal solvent signals.

[00258] Synthetically, compound **1** (IN-1, RQAR-ketobenzothiazole) and its analogues were assembled similarly to the method reported by Costanzo *et al.* (*J. Med. Chem.* **2005**, *48*, 1984-2008) by peptide coupling of warhead-functionalized P1 fragment with protected P4-P2 fragment (Schemes 1 to 4). The synthesis of IN-1 is shown as an example. Firstly, fragment **1-C** carrying the serine trap was prepared from the corresponding Weinreb amide **1-B**, by addition of *in situ* generated 2-lithio-benzothiazole. The resulting ketobenzothiazole was reduced in the same operation with $NaBH_4$ as a means of protecting the electrophilic keto group, then the Boc group was deprotected by acidolysis. In parallel, L-Ala benzyl ester tosylate was coupled with Fmoc-Gln-OH using HATU to afford dipeptide **1-f**. After Fmoc removal, the crude dipeptide was coupled with Boc-Arg(Mtr)-OH in the presence of EDC and HOBt to give the corresponding fully protected tripeptide. Benzyl ester hydrogenolysis then generated the desired fragment **1-H**. Subsequent coupling of tripeptide **1-H** with warhead **1-C** provided intermediate **1-K** (Scheme 3). The tetrapeptide scaffold **1-K** was then oxidized with IBX (Frigerio, *et al.*, *J. Org. Chem.* **1995**, *60*, 7272-7276) followed by final acidolysis of protective groups with HF. Compounds were generally obtained as a 8:2 mixtures of epimers (Costanzo *et al.*, *supra*), which were separated by reverse-phase preparative HPLC. Structural analogs **2-7** (Table 7, below) were obtained according to the same synthetic method.

[00259] **SYNTHESIS OF IN-1 (RQAR-ketobenzothiazole, compound 1 in Table 7 below)**

Scheme 1



1-A 1-B 1-C

[00260] **Step a:**

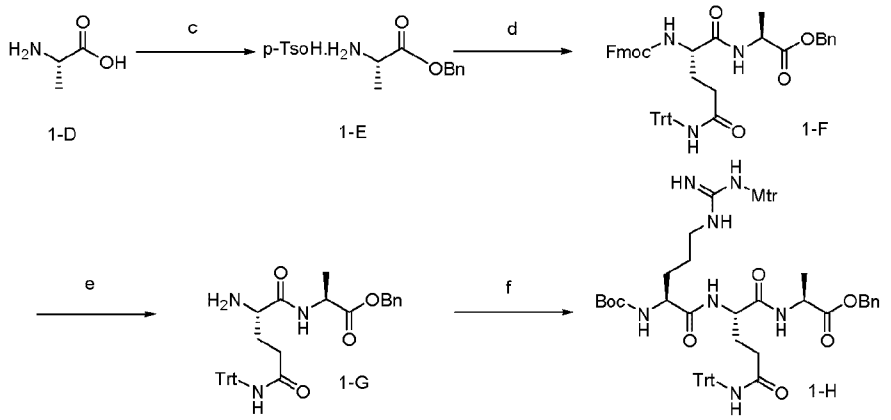
[00261] To a solution of Boc-Arg(Mtr)-OH (Scheme 1, compound 1-A, 1357.4mg, 2.79 mmol, 1eq) in anhydrous THF (93 mL), were added under nitrogen (i) 1273.3 mg of HATU (3.35 mmol, 1.2 eq); (ii) 326.8 mg of HN(Me)OMe•HCl (3.35 mmol, 12 eq) and (iii) 2.4 mL of DIPEA (14 mmol, 5 eq). The reaction mixture was stirred over 3h at ambient temperature. After completion of the reaction, the mixture was concentrated *in vacuo* and directly

purified by flash chromatography, eluting with EtOAc/hexane (80:20 to 100:0) to give the Weinreb amide (Compound **1-B**) as a white solid (1.4 g, 93 %). HPLC: 10.98 min, 90%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (s, 9H), 1.55-1.71 (m, 4H), 2.59 (s, 3H), 2.67 (s. 3H), 2.80 (s,3H), 3.22 (s, 3H), 3.29-3.33 (m, 1H), 3.72 (s, 3H), 3.82 (s, 3H), 4.63 (t, 1H), 5.47 (d, 2H), 6.20-6.31 (m,2H) 6.53 (s, 3H).^{13}C NMR (75.4 MHz, $CDCl_3$) δ 11.94,18.20, 24.12,24.59, 28.34, 32.13, 41.00, 49.31, 55.46, 61.65, 80.38, 111.77, 120.20, 124.91, 136.25, 138.77, 155.74, 156.62, 158.54. MS (ES) m/z 530.2 $(M+H)^+$.

[00262] **Step b:**

[00263] A solution of benzothiazole (1.7 mL, 15.5 mmol, 18 eq) in 52 mL of anhydrous THF was cooled to -75 °C with stirring under nitrogen. Butyllithium (1.02 M in hexane, 14.8 mL, 15.1 mmol, 17.50 eq) was added dropwise over 30 min at -75 to -70 °C and the mixture was stirred for an additional 30 min -75 to -70 °C. To this solution was added, dropwise over 50 min, a solution of the Weinreb amide **1-B** (Scheme 1; 456 mg, 0.86 mmol, 1 eq) in 27 mL of THF dried at -75 to -70 °C. The reaction mixture was stirred at -75 °C for 2 h. The reaction was quenched with 20 mL of saturated aqueous NH_4Cl, and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed successively with water (3 x 50 mL) and brine (2 x 50 mL, dried ($MgSO_4$)), filtered through fritted glass, and concentrated *in vacuo*. The residue was dissolved in dried MeOH (17.2 mL), cooled to -20 °C while stirring and treated with $NaBH_4$ (198 mg, 5.2 mmol, 6 eq). After 1 h, acetone (20 mL) was added and the reaction mixture was warmed to room temperature over 30 min. The reaction mixture was concentrated *in vacuo* and partitioned between water and EtOAc. The aqueous layer was extracted with EtOAc (2 x 20 mL), and the combined organic extracts were washed with brine (2 x 20 mL), dried ($MgSO_4$), filtered through fritted glass and concentrated *in vacuo*. The residue was purified by flash chromatography, eluting with EtOAc/hexane (80:20 to 100:0) to give hydroxybenzothiazole (Scheme 1, intermediate **1-C**) as a yellow solid (322 g, 63 %). HPLC: 12.09 min, 100% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (s, 4H), 1.37 (s, 5H), 1.40-1.72 (m, 4H), 2.58 (s, 3H), 2.64 (s,3H), 2.66 (s,3H), 3.08-3.35 (m, 2H), 3.80 (s, 3H), 4.13 (t, 1H), 5.16 (s, 1H), 5.52 (d, 2H), 6.21-6.38 (m, 3H), 6.50 (s, 3H), 7.90 (d, 1H), 7.95 (d, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 11.9, 19.1, 23.2,24.1, 26.9, 28.8, 40.9, 55.5, 60.4, 80.2, 112.1, 121.6, 122.5, 124.8, 124.9, 125.0, 126.9, 134.7, 136.7, 138.6, 152.7, 156.6, 157.1, 158.5, 173.0. MS (ES) m/z 606.1$(MH)^+$.

Scheme 2



[00264] **Step c:**

[00265] To a solution of L-alanine (Scheme 2, compound **1-D**) (1151.2 mg, 12.9 mmol, 1.00 eq) in 51 mL of toluene, were added (i) 23 mL of BnOH; and (ii) 2457.6 g of APTS.H_2O (14.2 mmol, 1.10 eq). Overcoming a Dean-Stark assembly and a condenser, the mixture was stirred at reflux overnight. After completion of the reaction, the mixture was concentrated in vacuo, dissolved in EtOAc (20 mL) and extracted with 1M HCl (3 x 20 mL). The combined extracts were washed with ether (4 x 20 mL), adjusted to pH 8-9 with $NaHCO_3$ and extracted with EtOAc (4 x 20 mL). The combined EtOAc extracts were dried ($MgSO_4$), filtered through fritted glass and concentrated in vacuo. The residue was crystallized in a solution of APTS.H_2O (2457.6 mg, mmol, 1 eq) in 20 mL of diethyl ether to give intermediate compound **1-E** (Scheme 2) as a white solid (3260.0 mg, (72 %; MS (ES) m/z 180.1 (MH) +).

[00266] **Step d**:

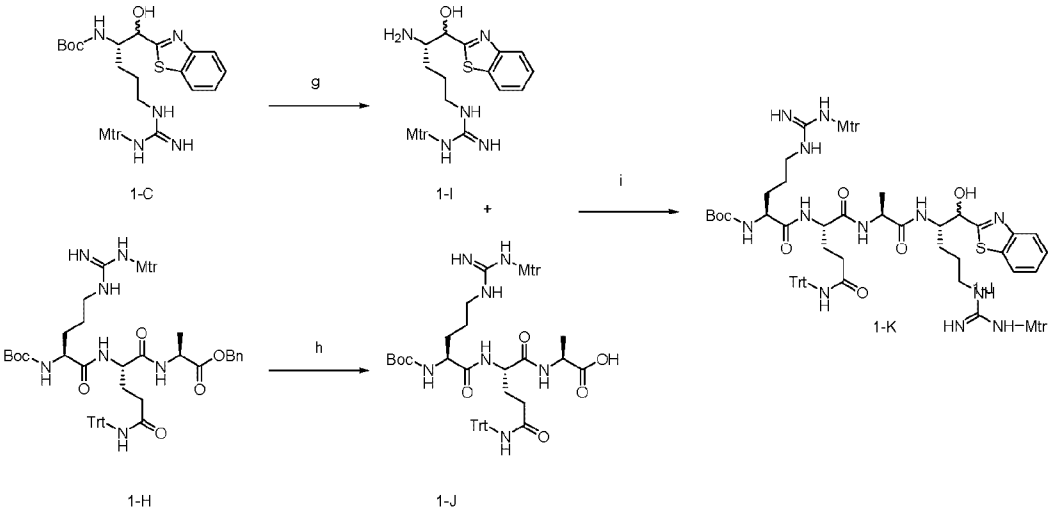
[00267] To a solution of Fmoc-Gln(Trt)-OH (1.83 g, 3.0 mmol, 1 eq) in 30 mL anhydrous THF were added 1.37 g HATU (3.6 mmol, 1.2 eq), 1.05 g H_2N-Ala-OBn (1.42 mmol, 1.0 eq), and 2.6 mL DIPEA (15.0 mmol, 5.0 eq) under nitrogen. The reaction mixture was stirred over 3h at room temperature. After completion of the reaction, the mixture was concentrated *in vacuo* and purified by flash chromatography, (EtOAc/Hexane 50:50 to 70:30) to give compound **1-F** as a white solid (2.07 g, 90%). HPLC: 15.19 min, 100% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (d, 1H), 1.95-2.00 (m, 4H), 4.10-4.20 (m, 2H), 4.22-4.37 (m, 1H), 4.47-4.52 (m, 1H), 5.05-5.17 (m, 2H), 7.10-7.41 (m, 26H), 7.76 (d, 2H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 17.56, 30.00, 33.29, 47.16, 48.28, 53.35, 66.99, 77.49, 119.97, 125.19, 127.08, 128.22, 128.68, 141.30, 144.47, 144.52, 170.97. MS (ES) m/z 794 $(M+Na)^+$.

[00268] **Step e-f:**

[00269] A solution of Fmoc-Gln(Trt)-Ala-OBn (1.06 mg, 1.37 mmol, 1 eq) in 13.2 ml of CH_2Cl_2/Et_2NH (90:10) was stirred for 3 h at room temperature, then concentrated *in vacuo*. The residue was diluted with CH_2Cl_2 (3 x 20 mL) and concentrated under *in vacuo* to give the intermediate free amine as a colorless oil. To a solution of 798 mg Boc-Arg(Mtr)-OH (1.64 mmol, 1.2 eq) in 14 mL of CH_2Cl_2 was added 314 mg EDC (1.64 mmol, 1.2 eq), 1.37 mmol

H_2N-Gln(Trt)-Ala-OBn (compound **1-G**, 1.00 eq) and 222 mg HOBt (1.64 mmol, 1.2 eq). The mixture was stirred at room temperature overnigth. After completion, the reaction mixture was concentrated *in vacuo* and purified by flash chromatography (EtOAc/Hexane 80:20 to 100:0) to yield intermediate **1-H** as a white solid (813 mg, 77%). HPLC-UV: 14.48 min, 80% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28 (d, 2H), 1.36 (s, 10H), 1.58-1.66 (m, 1H), 2.42 (s, 3H), 2.59 (s,3H), 2.63 (s,3H), 3.02-3.18 (m, 1H), 3.77 (s, 3H), 4.08-4.15 (m, 1H), 4.34-4.41 (m, 2H), 4.95 (dd, 2H), 5.35 (d, 2H),6.08- 6.30 (s, 3H), 6.47 (s, 3H), 7.14-7.36 (m, 20H). ^{13}CNMR (75.4 MHz, $CDCl_3$) δ 11.0, 17.1, 18.4, 24.2, 25.2, 28.3, 29.8, 48.5, 52.7, 53.6, 55.4, 66.9, 77.5, 79.9, 111.7, 121.1, 126.9, 127.9, 128.9, 131.3, 135.3, 138.7, 144.3, 155.0, 155.6, 158.2, 172.5. MS (ES) m/z 1018.0 $(M+H)^+$.

Scheme 3:



[00270] **Step g:**

[00271] A solution of Boc-Arg(Mtr)-benzothiazole, compound **1-C** (Scheme 3; 188.8 mg, 0.31 mmol, 1 eq) in 3.1 mL CH_2Cl_2/TFA (8:2), was stirred at room temperature for 1h and concentrated *in vacuo*. The residue was concentrated many times with CH_2Cl_2 (3 x 20 mL) to eliminate TFA and to give intermediate **1-I** (Scheme 3) as yellow oil.

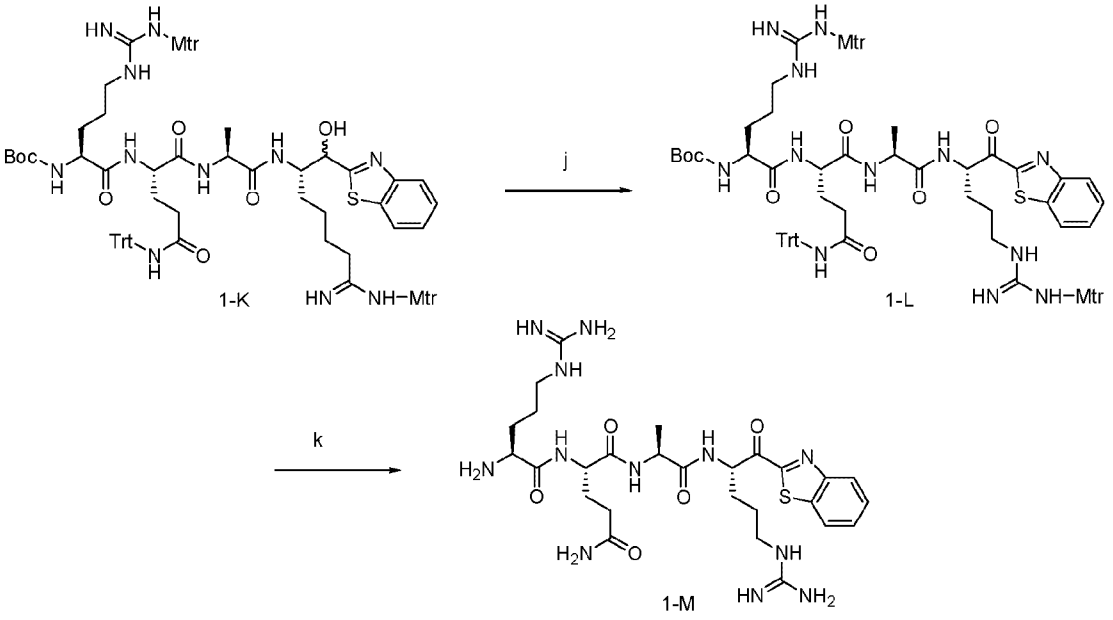
[00272] **Step h:**

[00273] The intermediate **1-H** (320.7 mg, 0.31 mmol, 1 eq), 10% Pd on activated carbon (140 mg), and absolute EtOH (3.1 mL) were stirred under an atmospheric pressure of hydrogen for 1 h at ambient temperature. The resulting mixture was filtered on diatomaceous earth through fritted glass and concentrated *in vacuo* to give compound crude compound **1-J**, which was used as such in the following step.

[00274] **Step i:**

[00275] To a solution of Boc-Arg(Mtr)-Gln(Trt)-Ala-OH, compound **1-J** (Scheme 3; 0.31 mmol, 1 eq in anhydrous DMF, were added: (i) 353.6 mg of HATU (0.93 mmol, 3 eq); (ii) 0.31 mmol of hydroxybenzothiazole, compound **1-I** (Scheme 3, 1.00 eq); and (iii) 0.3 mL of DIPEA (1.55 mmol, 5 eq). The mixture was stirred overnight, concentrated *in vacuo* and purified by flash chromatography, eluting with MeOH/EtOAc (95:5) to give compound **1-K** (Scheme 3; 366.2 g, 84 %) HPLC: 14.59 min, 83% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ (s, 9H), 1.39 (d, 3H), 1.47-1.60 (m, 6H), 2.04-2.15 (m, 2H), 2.33-2.58 (m, 10H), 2.84 (s, 6H), 2.97(s, 6H), 3.03-3.11 (m,2H), 3.82 (s, 9H), 4.04-4.10 (m, 1H), 4.18-4.28 (m, 2H), 4.30-4.49 (m,1H), 5.15 (d, 1H), 6.49 (s, 1H), 7.07-7.53 (m, 15H), 7.79-7.92 (m, 2H), 8.32 (d, 1H), 8.65 (d, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 11.97, 17.23, 18.59, 24.17, 28.06, 43.24, 55.51, 76.59, 77.43, 127.02, 127.94, 128.94, 128.60, 128.73, 144.10. MS (ES) m/z 1415.0 $(M+H)^+$.

Scheme 4



[00276] **Step j:**

[00277] The reactant IBX (84 mg, 0.3 mmol, 1.2 eq) was added to a solution of compound **1-K** (355 mg, 0.25 mmol, 1 eq) in 2.5 mL DMSO. The resulting mixture was stirred overnight at ambient temperature. The reaction mixture was washed with water (2 x 10 mL) and brine (2 x 10 mL), dried ($MgSO_4$), filtered through fritted glass, and concentrated *in vacuo*. The residue was purified by flash chromatography (EtOAc/Hexane 80:20 to 100:0) to give compound **1-L** as a yellow solid (170 mg, 48%). HPLC: 14.94 min, 95% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (s, 9H), 1.41 (d, 3H), 1.44-1.87 (m, 4H), 2.00-2.15 (m, 4H), 2.30-2.61 (m, 10H), 2.87 (s, 6H), 2.95 (s, 6H), 3.03-3.36 (m, 2H), 3.79 (s, 9H), 4.03-4.15 (m, 2H), 4.30-4.99 (m, 2H), 6.46 (s, 1H), 7.03-7.20 (m, 15H), 7.41-7.52 (m, 2H), 7.86 (d,

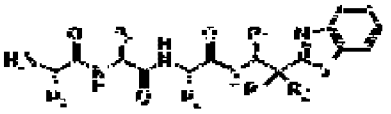
1H), 8.16 (d, 1H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 11.97, 17.23, 18.59, 24.17, 28.06, 43.24, 55.51, 76.59, 77.43, 127.02, 127.94, 128.60, 128.73, 144.10. MS (ES) m/z 1414.0 $(M+H)^+$.

[00278] **Step K:**

[00279] A mixture of Boc-Arg(Mtr)-Gln(Trt)-Ala-Arg(Mtr)benzothiazole (compound 1-L, 206 mg, 0.15 mmol, 1 eq) and anhydrous anisole (200 µL) was placed in a teflon reaction tube of an HF apparatus and cooled to -78 °C. Anhydrous HF (15-20 mL) was condensed into the tube, and the temperature was increased to 0 °C. The reaction mixture was stirred at 0 °C for 3 h, concentrated *in vacuo*, and triturated with Et_2O (3 x 20 mL) to give a yellow solid. This solid was purified by reverse-phase HPLC, (water/MeCN/TFA 70:30:0.1), which allowed the separation of two diastereoisomers (88:12). The major diastereomer **1-M (IN-1,** Compound **1**) was obtained as a white solid after lyophilisation (56 mg, 35%). HPLC: 7.31 min, 94% purity. The second diastereomer was also isolated as a pure product (8 mg, 6.87 min, 95% purity). ^1H NMR (300 MHz, D_2O) δ 1.38 (d, 3H) 1.49-1.58 (m, 2H), 1.61-1.69 (m, 2H), 1.73-1.92 (m, 2H), 2.19-2.25 (m, 4H), 3.09-3.23 (m, 1H), 3.98 (t, 1H), 4.23-4.32 (m, 2H),7.56-7.68 (m, 2H), 8.11 (d, 1H), 8.19 (d, 1H). ^{13}C NMR (75.4 MHz, D_2O) δ 17.44, 24.12, 24.73, 25.02, 27.94, 33.22. 39.74, 54.66, 55.49, 74.38, 76.60, 121.76, 124.80, 125.94, 144.09, 173.49, 177.48, 177.96. HRMS calculated for $C_{27}H_{42}N_{12}O_5S$: 647.3200; found: 647.3228 $(M+H)^+$.

[00280] **SYNTHESIS OF STRUCTURAL ANALOGS OF IN-1 (compounds 2 to 7 in Table 7 below)**

Table 7: Structure of structural analogs of IN-1



Compound #	P_4-P_3-P_2-P_1	R_1, R_2
1	R-Q-A-R	= 0
2	R-Q-A-R	H, OH*
3	R-Q-A-K	= 0
4	Q-A-R	= 0
5	A-R	= 0
6	R	= 0
7	R-Q-A-(D)R	= 0

* 3:2 mixture of diastereoisomers, absolute stereochemistry undetermined.

[00281] **2-(2-amino-5-carbamimidamidopentanamido)-N-(1-{[1-(1,3-benzothiazol-2-yl)-5-carbamimidamido-1-hydroxypentan-2-yl]carbamoyl}ethyl)pentanediamide (compound 2)**

[00282] The same method used for compound **1** (IN-1) described above was used to synthesize compound **2**,

which was obtained as a white solid (7.2 mg, 30%). HPLC: 6.08 min, 94%. ^{1}H NMR (300 MHz, D_2O). δ 1.07 (d, 3H), 1.43-1.61 (m, 4H), 1.70-1.99 (m, 4H), 2.15-2.29 (m,4H), 3.00-3.18 (m,1H), 3.93 (t, 1H), 4.04-4.13 (m, 1H), 4.14-4.24 (m, 1H), 5.02 (d, 1H), 7.39-7.55 (m, 2H), 7.89 (d, 1H), 7.97-8.00 (m,1H).^{13}C NMR (75.4 MHz, D_2O) δ 17.62, 23.37, 24.37, 26.86, 27.94, 30.82, 40.33, 52.50, 53.07, 73.44, 172.36. HRMS calculated for $C_{27}H_{44}N_{12}O_5S$: 649.3351; found: 649.3366 $(M+H)^+$.

[00283] N-(1-{[6-amino-1-(1,3-benzothiazol-2-yl)-1-oxohexan-2-yl]carbamoyl}ethyl)-2-(2-amino-5-carbamimidamidopentanamido)pentanediamide (RQAK-ketobenzothiazole, IN-2, compound 3 in Table 7)

[00284] The same method as used for compound **1** was also used to synthesize compound **3** with appropriate adaptations (Lysine instead of Arginine), which was obtained as a white solid (6 mg, 42%). HPLC: 5.11 min, 91%. ^{1}H NMR (300 MHz, D_2O) δ 1.36 (d, 6H), 1.42-1.54 (m,4H), 1.61-1.80 (m, 4H), 1.98-2.12 (m, 4H), 2.21-2.31 (m, 4H), 2.94 (t, 1H), 3.84-3.94 (m, 1H), 4.23-4.32 (m, 1H), 4.34-45 (m, 1H), 7.59-7.70 (m, 2H), 8.11 (m, 1H), 8.19 (d, 1H).^{13}CNMR (75.4 MHz, D_2O) δ 17.64, 22.42, 26.31, 26.80, 30.35, 30.94, 39.06, 40.20, 52.44, 54.98, 122.96, 124.89. HRMS calculated for $C_{27}H_{42}N_{10}O_5S$: 310.1603; found: 310.1603 ($M2H^+/2$).

[00285] 2-amino-N-(1-{[1-(1,3-benzothiazol-2-yl)-5-carbamimidamido-1-oxopentan-2-yl]carbamoyl}ethyl)pentanediamide (compound 4):

[00286] The same method as used for compound **1** was used to synthesize compound **4** (with appropriate adaptations), which was obtained as a white solid (10.0 mg, 30%). The second diastereomer was also isolated as a pure product (2.1 mg, 6.38 min, 93% purity). HPLC: 7.31 min, 94% purity. ^{1}H NMR (300 MHz, D_2O) δ 1.36 (d, 3H) 1.58-1.62 (m, 2H), 1.91-1.96 (m, 2H), 2.11 (d, 2H), 2.20- 2.25 (m, 2H), 3.17 (d, 2H), 3.97(d, 1H), 4.14-4.25 (m, 1H), 4.35 (q, 1H), 7.43-7.57 (m, 2H), 8.09 (d, 1H), 8.14 (d,1H). ^{13}C NMR (75.4 MHz, D_2O) δ 16.47, 24.62, 26.44, 29.89, 30.22, 40.61, 52.13, 122.52, 122.89, 124.85, 127.81, 136.71, 152.61, 156.66, 163.98, 174.58, 176.80, 193.38. HRMS calculated for $C_{27}H_{42}N_{10}O_5S$: 491.2189; found: 491.2161$(M+H)^+$.

[00287] N-[1-(1,3-benzothiazol-2-yl)-5-carbamimidamido-1-oxopentan-2-yl]-2-acetamidopropanamide (compound 5)

[00288] The same method as used for compound **1** was used to synthesize compound **5** (with appropriate adaptations), which was obtained as a white solid (10.8 mg, 30%). The second diastereomer was also isolated as a pure product (5.1 mg, 8.04 min, 99% purity). HPLC: 8.29 min, 97% purity. ^{1}H NMR (300 MHz, D_2O) δ 1.29 (d, 3H), 1.52-1.63 (m, 2H), 1.77-1.82 (m, 2H), 1.86 (s, 3H), 3.15 (t, 2H), 4.07 (d, 1H), 4.21 (q, 1H), 7.43-7.59 (m, 2H), 8.06 (d, 1H), 8.12 (d,1H).^{13}C NMR (75.4 MHz, D_2O) δ 16.51, 21.49, 24.34, 27.56, 40.36, 49.93, 55.22, 122.85, 124.77, 125.12, 136.66, 152.53, 156.66, 163.92, 173.99, 175.41, 193.26. HRMS calculated for $C_{18}H_{24}N_6O_3S$: 405.1709; found: 405.1683 $(M+H)^+$.

[00289] N-[1-(1,3-benzothiazol-2-yl)-5-carbamimidamido-1-oxopentan-2-yl]acetamide (compound 6)

[00290] The same method as used for compound **1** was used to isolate compound **6** (with appropriate adaptations), which was obtained as a white solid (6.2 mg, 48%). HPLC: 8.22 min, 99% purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.52-1.68 (m, 2H), 1.97-2.08 (m, 2H), 2.11(s, 3H), 3.16 (t, 2H), 5-35-5.50 (m, 1H), 7.43-7.59 (m, 2H), 8.08 (d, 1H), 8.17 (d, 1H).^{13}C NMR (75.4 MHz, $CDCl_3$) δ 21.47, 24.26, 27.76, 40.33, 55.36, 122.89, 124.84, 127.83, 136.78, 148.14, 152.66, 163.80, 174.27, 193.91). HRMS calculated for $C_{15}H_{19}N_5O_2S$: 334.1332; found: 334.1340 $(M+H)^+$.

EXAMPLE 4

KETOBENZOTHIAZOLE TETRAPEPTIDES INHIBIT MATRIPTASE ENZYMATIC ACTIVITY

[00291] Experimental procedures

[00292] Materials

[00293] Purified recombinant human matriptase, matriptase-2, hepsin and furin were prepared as described (1,2). Bovine trypsin and thrombin are from Sigma-Aldrich.

[00294] General Kinetic Methods

[00295] Enzymatic assays were performed in the following reaction buffer: 50mM HEPES, pH 7.4 containing 500 µg/ml bovine serum albumin. Enzyme activities were monitored by measuring the release of fluorescence of an amido methyl coumarin (AMC) moiety (excitation, 360 nm; emission, 441 nm) from Boc-Gln-Ala-Arg-AMC (50 µm) (Bachem Biosciences, King of Prussia, PA) for 20 min at 37 °C in a FLX-800 TBE™ microplate reader (Bio-Tek Instruments, Winooski, VT).

[00296] Matriptase inhibition by ketobenzothiazole tetrapeptides

[00297] Recombinant purified human matriptase (amino acids 596-855) (1nM) was incubated with vehicle (1% DMSO) or 1µM of inhibitors IN-1 (RQAR-ketobenzothiazole, Compound **1**) and IN-2 (RQAK-ketobenzothiazole, compound **3**) for 10 minutes at room temperature. Enzyme activity was measured for 20 minutes at 37°C using 50µM of Boc-Gln-Ala-Arg-AMC as a substrate. Reaction was performed in a 50mM HEPES buffer (pH 7.4) containing 5µg/ml BSA.

[00298] For IC_{50} determination, residual enzymatic activity (V_i/V_o) was plotted as a function of the log transformation of inhibitor concentration (log I). Data were fitted by non-linear regression analysis using GraphPad Prism 5™ software. IC_{50} corresponds to the concentration of inhibitor needed to reduce the maximal enzymatic activity by 50%.

[00299] Results show that matriptase is effectively inhibited by IN-1 and to a lesser extent by IN-2 (Figure 10). IN-1 inhibited almost completely matriptase activity compared to the control, while IN-2 inhibited about 25% of matriptase activity at 1µM.

[00300] To further evaluate the inhibitor profile, the dissociation of the enzyme: inhibitor complex (EI) was investigated using dilution experiments (Bieth, J. G. *Methods. Enzymol.* **1995**, *248*, 59-84). Dissociation of the enzyme-inhibitor (EI) complex was investigated using dilution recovery experiments. High concentration of matriptase (0,5 µM) with variable concentration of A) IN-1 (Compound **1**) or B) EGR-CMK was incubated for 20 minutes at room temperature in reaction buffer to allow for the formation of the EI complex. Complexes were rapidly (1:2000) diluted in a reaction buffer containing 400µM of Boc-Gln-Ala-Arg-AMC (~7 times Km). Activity was continuously recorded for 75 minutes and fluorescence plotted as a function of time.

[00301] Figures 11A and 11B reports the comparison of the dissociation curves for IN-1 (compound **1**) (Fig. 11A) and irreversible inhibitor Glu-Gly-Arg chloromethyl ketone (EGR-CMK) (Fig. 11B). The dissociation curve of EGR-CMK displays a linear product versus time relationship, indicative of irreversible inhibition. Conversely, the dissociation curve of IN-1 (compound **1**) shows an exponential shape, suggesting dissociation of the enzyme: inhibitor complex. Together, these data confirm the formation of a slow, tight-binding and reversible complex between inhibitor and enzyme. This provides the advantage of a better control on inhibition.

[00302] To further characterize matriptase inhibition by IN-1 (compound **1**), the inhibition constant (K_i) was determined using the Morrison equation for reversible tight-binding inhibition (Bieth, J. G. *Methods. Enzymol.* **1995**, *248*, 59-84). To evaluate inhibition potency of the ketobenzothiazoles compounds towards proteases, 1 nM of enzymes was added to the reaction buffer containing 0 nM, 10 nM or 10µM of the different ketobenzothiazoles (0.4 µM, 4 µM and 400 µM for the reduced form of RQAR-ketobenzothiazole (Compound **2**)) and 100µM of Boc-Arg-Arg-Val-Arg-AMC for furin and 100µM of Boc-Gln-Ala-Arg-AMC for the other proteases. If substantial inhibition occurred using a ratio of I/E < 10, compounds were treated as tight-binding inhibitors (Bieth, J.G., *Methods. Enzymol.* **1995**, 248, 59-84). For Ki determination of tight-binding inhibitors, enzymes diluted to concentration ranging from 0, 25 to 1 nM were pre-equilibrated with appropriate dilutions of the compounds for 15 minutes at room temperature. Residual enzyme activity was then measured by following the hydrolysis of the fluorogenic substrate Boc-Gln-Ala-Arg-AMC for 30 minutes at room temperature. Data from at least three independent experiments were averaged and residual velocities were plotted as a function of inhibitor concentration. Data were fitted by non-linear regression analysis to the Morrison Ki equation (1):

$$vi/vo = vo*(1-((((Eo + Io + Kiapp) - (((Eo + Io + Kiapp)2) -4*Eo*Io)½)) / (2*Eo))), \quad (1)$$

[00303] where vo and vi are the steady-state rates of substrate hydrolysis in the absence and presence of inhibitor, respectively, Eo, the initial concentration of enzyme, Io, the initial concentration of inhibitor and Kiapp the substrate-dependent equilibrium dissociation constant. The substrate-independent constant Ki was calculated using the equation (2):

$$Kiapp = (Ki*(1+(S/Km))), \quad (2)$$

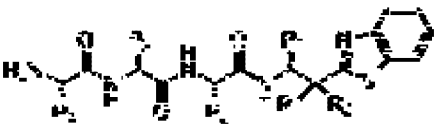
[00304] where S is the initial concentration of substrate and Km the Michaelis–Menten constant for the

enzyme–substrate interaction.

[00305] If substantial inhibition occurred only when using a ratio of I/E > 10, compounds were treated as classical reversible inhibitors. In this case, inhibition assays used for Ki determination were initiated by the addition of 1 nM of protease to a reaction mixture containing the inhibitor and the fluorogenic substrate. Data generated from plots of enzyme velocity versus substrate concentration at several fixed inhibitor concentration were fitted by non-linear regression analysis to equations describing competitive, uncompetitive, noncompetitive and mixed model inhibition (Copeland, R. A Practical Introduction to Structure, Mechanism and Data Analysis. Wiley-VCH, New-York, 2000, chapter 10, pp 1-397.). For inhibition of matriptase by compound **6** (R-Benzo) and thrombin by compound **1** (RQAR-Benzo), the mixed model inhibition was the preferred model as determined by the goodness of fit (R2) and the Akaike's information criteria (AICc) and therefore used for Ki determination. All non-linear regression and statistical analysis were performed using GraphPad™ Prisms version 5.04 for Windows (GraphPad Software, San Diego, CA, USA).

[00306] In these conditions, the RQAR-ketobenzothiazole compound **1** showed high potency for matriptase, with a K_i of 0.011 nM (Table 8). A preliminary analysis of Structure-Activity Relationships (SAR) was subsequently performed by exploring the P1 position. In order to confirm the role of the keto group for matriptase inhibition, the inhibitory activity of a reduced form of the RQAR-ketobenzothiazole toward matriptase was measured (compound **2**, Table 8). Reduced compound **2** (3:2 mixture of diastereoisomers at the alcohol position, undetermined absolute stereochemistry) displayed weak inhibition. Indeed, a stoichiometric ratio of I/E > 1000 was needed in order to observe substantial inhibition, as testified by a K_i of 6.1 µM, which contrasts with the much more potent oxidized form of compound **1** and is consistent with a functional serine trap mechanism.

Table 8: Structure-activity relationships of matriptase inhibitors



Compound #	P_4-P_3-P_2-P_1	R_1, R_2	K_i (nM)
1	R-Q-A-R	= 0	0.011^{tb} ± 0.0004
2	R-Q-A-R	H, OH*	6124^{mm} ± 2702
3	R-Q-A-K	= 0	9.5^{tb} ± 1.3
4	Q-A-R	= 0	0.088^{tb} ± 0.010
5	A-R	= 0	1.4^{tb} ± 0.3
6	R	= 0	457^{mm} ± 132
7	R-Q-A-(D)R	= 0	4.6^{tb} ± 0.8

K_i values were determined as described above (tb: tight-binding, mm: mixed model). Measurements of enzymatic activity were performed in triplicate and represent the means ± standard deviation of at least three independent

experiments. * 3:2 mixture of diastereoisomers, absolute stereochemistry undetermined.

[00307] Table 8 reports the influence of structural variations of compound **1** on matriptase inhibition. In order to ascertain the importance of stereochemistry at the P1 position, analogue R-Q-A-(D)R (compound **7**) was tested and was still active in the nanomolar range, but displayed a 400-fold lower inhibition relative to compound **1**. An Arg to Lys substitution at position P1 resulted in a decrease of the Ki of about 3 orders of magnitude (compare compounds **1** and **3**). Furthermore, in order to better ascertain the respective contribution of the P4, P3 and P2 residues on inhibitory profile, the peptidic portion was truncated by one, two and three residues starting from the N-terminal extremity (compounds **4**-**6**). Deletion of the P4 residue gave a compound that conserves the profile of a tight binding inhibitor, yet with an 8-fold decreased potency compared to compound **1** (compound **4**, K_i = 0.088 nM *vs.* 0.011 nM for compound **1**). Compound **5**, in which the P4 and P3 moieties were simultaneously deleted, remains a tight binding inhibitor with 127-fold reduced potency compared to compound **1** (K_i = 1.4 nM vs 0.011 nM). Finally, compound **6**, in which the P4-P2 tripeptide portion is removed, is significantly less potent, with a K_i of 457 nM, over 30,000-fold less potent than compound **1**. Additionally, compound **6** no longer behaves as a tight-binding inhibitor but as an inhibitor possessing a mixed mode of inhibition as determined by global fitting analysis for different modes of inhibition.

[00308] Matriptase inhibition by ketobenzothiazole tetrapeptides *in cellulo*

[00309] Next, the ability of IN-1 (compound **1**) to inhibit matriptase activity in human cells was tested. Human embryonic kidney cells (HEK293) were transfected with 6 µg of pcDNA3.1™ (Mock) or pcDNA3.1™-matriptase (Matriptase) using Lipofectamine 2000™ reagent (Invitrogen) in 10-cm plates. After a 24-h transfection, the medium was replaced by serum-free media (293 SFM II™ media (Invitrogen)) in the presence or absence (vehicle) of the IN-1 (compound **1**). After 24h incubation, media was incubated with Boc-Gln-Ala-Arg-AMC substrate (50µM) and fluorescence measured for 20 minutes at 37°C. Enzyme activity was determined in terms of released fluorescence units per minutes per µl of media for each condition.

[00310] Results show that IN-1 (compound **1**) efficiently inhibits matriptase activity in cultured cells at 1 µM (about 50% inhibition) and 10 µM (almost 100% inhibition; matriptase activity is returned to background level) (Figure 12).

EXAMPLE 5

SELECTIVITY OF MATRIPTASE INHIBITORS

[00311] The selectivity/specificity of ketobenzothiazole peptides of the present invention for matriptase activity was tested. Various proteases (matriptase; matriptase-2; hepsin; thrombin; trypsin and furin at concentration of 1nM), were incubated with vehicle (1% DMSO) or 1µM of IN-1 compound for 10 minutes at room temperature. Proteolytic activity was monitored by addition of the Boc-Gln-Ala-Arg-AMC peptide substrate (50µM) for 20 minutes at 37°C. Data are presented as relative activity compared to the control reaction (vehicle = 1) (Figure 13A).

[00312] The IC_{50} of IN-1 for matriptase and hepsin were also determined (Figures 13B and C). For the IC_{50} determination of IN-1 for matriptase (Figure 13B) and hepsin (Figure 13C), different concentrations of inhibitors were incubated with 1nM of enzyme. Relative activities (V_i/V_o) were plotted as a function of the log transformation of inhibitor concentration (log I). Data were fitted by non-linear regression analysis using GraphPad Prism 5™ software. IC_{50} corresponds to the concentration of inhibitor that results in a response half way between the maximal response and the maximally inhibited response. The IC_{50} value of the inhibitor was about 29 nM for matriptase while that for hepsin was about 540 nM (i.e. more than 18 times that for matriptase).

[00313] The selectivity profile of compound **1** for matriptase *vs.* other serine proteases, including type II transmembrane serine proteases (TTSPs), was also determined by measuring the experimental K_i, using the method described above. Results are presented in Table 9 below. Selectivity was expressed as the ratio of K_is. Compound **1** was found to be selective for matriptase *vs.* other enzymes: trypsin (88-fold-protease #5 in Table 9), hepsin (100-fold-protease #3 in Table 9), matriptase-2 (300-fold-protease #2 in Table 9), TMPRSS11D (764-fold-protease #4 in Table 9), thrombin (>30000-fold -protease #6 in Table 9) and furin (no inhibition (NI)- protease #7 in Table 9). These results demonstrate that IN-1 has a good selectivity for matriptase and inhibit other related proteases with significantly less efficacy.

Table 9: Selectivity profile

Proteases	K_i (nM)	Selectivity K_i other/K_i matriptase
1	0.011^{tb} ± 0.0004	---
2	3.3^{tb} ± 1.0	300
3	1.1^{tb} ± 0.3	100
4	8.4^{tb} ± 2.6	764
5	0.97^{tb} ± 0.17	88
6	637^{mm} ± 131	> 300000
7	NI (10 µM)	---

K_i values were determined as described above (tb: tight-binding, mm: mixed model). Measurements of enzymatic activity were performed in triplicate and represent the means ± standard deviation of at least three independent experiments.

[00314] In order to understand the preferred mode of docking of compound **1** in the active site of matriptase and rationalize Structure-Activity Relationships, a molecular model of compound **1** docked in the published X-ray structure of matriptase was built (Figure 18). According to this docking model, the side chain of residue Arg in P1 is highly stabilized in a network of hydrogen bonds, which includes a salt bridge with matriptase residue Asp^{799}, a hydrogen bond with Ser^{800} and hydrogen bonds with the backbone amide of Gly^{827} and Gly^{829}. This may account for the preference of Arg over Lys in P1, particularly since the S1 pocket seems to be best suited to accommodate the side chain of Arg instead of Lys, which is longer and possesses reduced hydrogen bond capability compared to Arg.

The side chain of residue Gln in P3 of compound **1** bridges over the Arg residue in P1 to interact with Gln^{802} in the S3 pocket. Next, the side chain of the Arg residue in P4 of compound **1** interacts with the side chain of Asp^{826} of matriptase via a salt bridge. It also interacts via hydrogen bonding with Gln^{783}. Finally, the catalytic Ser^{805} residue is adequately positioned in the vicinity of the carbonyl moiety of the ketobenzothiazole group to form a covalent, reversible bond in the form of a hemiacetal. The oxygen atom of the carbonyl group is stabilized via hydrogen bonding with the backbone amides of residues Gly^{803} and Ser^{805}.

EXAMPLE 6

KETOBENZOTHIAZOLE TETRAPEPTIDES INHIBIT INFLUENZA VIRAL REPLICATION IN HUMAN AIRWAY EPITHELIAL CELLS

[00315] Next was tested whether IN-1 (compound **1**) was able to block influenza replication *in vitro*, using the MDCK/exogenous matriptase system (Figures 14 and 15) and the Calu-3 system that does not require exogenous proteases (Figure 16).

[00316] In the MDCK cell system, IN-1 (compound **1**) was added either 1h or 5h after viral attachment in an experiment identical to that presented in Figure 7 and described in Example 2 above. MDCK cells were cultured to confluence and infected with 25 PFU of influenza A/Puerto Rico/8/34 virus for 1h (to allow viral attachment) in incomplete medium containing BSA. Cells were then washed and a 1.8% Avicel microcrystalline cellulose solution in MEM containing 43 nM of matriptase (or 43 nM trypsin; positive control) was added to allow viral plaque formation. Matriptase inhibitor IN-1 was added to the assay at 1h post-viral attachment. 48h after infection, cells were washed and viral plaques were fixed with Carnoy fixative and revealed with a solution of crystal violet. Viral plaques were counted and results were compared. Similar results were obtained when IN-1 was added at 5h post-viral attachment.

[00317] The results showed that at concentration of 1μM and 10μM, IN-1 was able to completely abrogate influenza replication supported by matriptase (Figures 14 and 15).

[00318] In a separate experiment using Calu-3 cells, IN-1 blocked influenza replication by more than 50% at 72h post-infection (Figure 16, upper panel), and the inhibition is achieved in a dose-dependent manner (Figure 16, lower panel). Calu-3 cells were cultured to confluence, washed and infected with influenza A/Puerto Rico/8/34 (1000 PFU) for 1h to allow viral attachment. Cells were then washed and incubated for 72h in the presence or absence of 1μM IN-1 (or DMSO as the vehicle control). Supernatants were collected and assayed for infectious viral particles using the MDCK/trypsin viral plaque assay system. Influenza virus replicated to high titers in untreated or DMSO-treated cells, but replication was significantly blocked in IN-1 (1μM)-treated cells. Interestingly, IN-1 had no effect on influenza replication when trypsin was used as the activating protease indicating a selective inhibitory activity for IN-1 (Figure 15). Figure 17 shows inhibitory concentration-response curve of IN-1 in Calu-3 cells infected with influenza viruses A/Hong Kong/X-31 or a strain of the 2009 pandemic H1N1 virus isolated in Quebec during the pandemic

(A/California-like/04/09). These results show that inhibition of matriptase inhibits the replication of different influenza strains.

[00319] Although the present invention has been described hereinabove by way of specific embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

REFERENCES

1. Cox, N. J., and K. Subbarao. 2000. Global epidemiology of influenza: past and present. *Annu Rev Med* 51:407-421.

2. De Clercq, E. 2006. Antiviral agents active against influenza A viruses. *Nat Rev Drug Discov* 5:1015-1025.

3. Hayden, F. G., and A. J. Hay. 1992. Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine. *Curr Top Microbiol Immunol* 176:119-130.

4. Steinhauer, D. A., J. Martin, Y. P. Lin, S. A. Wharton, M. B. Oldstone, J. J. Skehel, and D. C. Wiley. 1996. Studies using double mutants of the conformational transitions in influenza hemagglutinin required for its membrane fusion activity. *Proc Natl Acad Sci U S A* 93:12873-12878.

5. Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature* 371:37-43.

6. Sollner, T. H. 2004. Intracellular and viral membrane fusion: a uniting mechanism. *Curr Opin Cell Biol* 16:429-435.

7. Ferreira, D. F., M. P. Santo, M. A. Rebello, and M. C. Rebello. 2000. Weak bases affect late stages of Mavaro virus replication cycle in vertebrate cells. *Journal of Medical Microbiology* 49:313-318.

8. Kido, H., T. Towatari, Y. Niwa, Y. Okumura, and Y. Beppu. 1996. Cellular proteases involved in the pathogenicity of human immunodeficiency and influenza viruses. *Adv Exp Med Biol* 389:233-240.

9. Garten, W., and H. D. Klenk. 2008. *Cleavage activation of the influenza virus hemagglutinin and its role in pathogenesis*. Karger, Basel, Switzerland.

10. Klenk, H. D., and W. Garten. 1994. Host cell proteases controlling virus pathogenicity. *Trends Microbiol.* 2:39-43.

11. Skehel, J. J., and D. C. Wiley. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* 69:531–569.

12. Steinhauer, D. A. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* 258:1-20.

13. Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H. D. Klenk, and W. Garten. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *Embo J* 11:2407-2414.

14. Zambon, M. C. 2001. The pathogenesis of influenza in humans. *Rev Med Virol* 11:227-241.

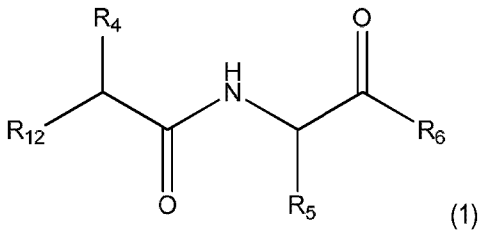
15. Bottcher, E., T. Matrosovich, M. Beyerle, H. D. Klenk, W. Garten, and M. Matrosovich. 2006. Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. *J Virol* 80:9896-9898.

16. Désilets, A., J. M. Longpré, M. E. Beaulieu, and R. Leduc. 2006. Inhibition of human matriptase by eglin c variants. *FEBS Letters* 580:2227-2232.

17. Klenk HD, Garten W, Rott R. Inhibition of proteolytic cleavage of the hemagglutinin of influenza virus by the calcium-specific ionophore A23187. EMBO J. 1984 Dec 1;3(12):2911–2915.

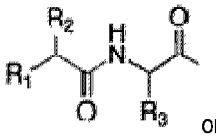
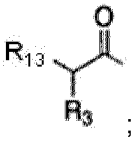
CLAIMS

1. A compound of formula (1):



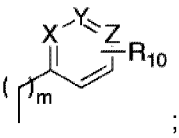
(1)

or a pharmaceutically acceptable salt, hydrate, solvate, or stereoisomer thereof, wherein

R_{12} is NHR_7; wherein R_7 is  or  ;

R_{13} is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl or one or more amino acids residues;

R_1 is H, NH_2 or NHR_7; wherein R_7 is an alkyl, aryl, (C=O)-alkyl, (C=O)-aryl, SO_2-alkyl, SO_2-aryl, C(=NH)-NH_2, C(=NH)-NHalkyl, C(=NH)-NHaryl or one or more amino acids residues;

R_2 and R_5 are independently -CH_2-R_8 or  ;

wherein R_8 is (CH_2)n-NH(C=NH)NH- R_{14} or (CH_2)n-NH-R_{14}, wherein,

n is 1 to 4;

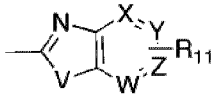
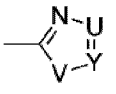
m is 0 to 3;

R_{10} is -C(=NH)-NH_2, NH_2 or NH alkyl; and

R_{14} is H, alkyl, NH_2, NO_2 or COalkyl;

R_3 is a linear alkyl; an alkyl substituted with $(CO)NH_2$, (CO)OH, NH_2, NHCO-alkyl, NHCO-aryl, $NHSO_2$-alkyl, $NHSO_2$-aryl or heteroaryl; an aryl, a substituted aryl, an heteroaryl or a substituted heteroaryl;

R_4 is an alkyl, an aryl, a substituted alkyl, a substituted aryl, an heteroaryl, a substituted heteroaryl, a side chain of an amino acid or a substituted side chain of an amino acid;

R_6 is  ,  , CF_3, CO_2H, $CONH_2$, CONH-alkyl, CONH-aryl or CO-(amino acid residue)$_p$, wherein p is 1 to 3;

wherein W, X, Y and Z are independently N or CH;

V and U are independently O, NH, NCH_3 or S; and

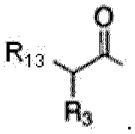
R_{11} is H, CO_2H, CONH-alkyl, CONH-aryl, aryl, heteroaryl, halide or CO-(amino acid residue)$_q$, wherein q is 1 or 2;

wherein unless indicated otherwise:

the substituted aryl, heteroaryl or alkyl is an aryl, heteroaryl or alkyl substituted with alkyl; alkenyl; alkynyl; hydroxy; alkoxy; amide; halide; heterocyclic; aryl; aryl substituted on any or all possible aryl positions by hydroxyl, amide, halides, nitrile, nitro, alkyl, amino, amides or sulfonamides; guanidine; amidine; heteroaryl; aryloxy; halogen; haloalkyl; cyano; nitro; amino ($-NH_2$); lower alkylamino; lower dialkylamino; NHCO-alkyl; NHCO-aryl; $NHSO_2$-alkyl; $NHSO_2$-aryl; amido (-(CO)NR_a); azido; acyl (—(CO)R_a); alkoxymethyl; mercapto (—S—R_a); sulfoxy (—SO—R_a); sulfonyl —$(SO)_2$—R_a); sulfonamide (—$S(O)_2N(R_a)_2$); carbonate (—O(CO)—O—R_a); oxyacyl (—O(CO)—R_a); carboxyl (—CO_2H); ester (—(CO)OR_6); carbamate (—O(CO)—$N(R_a)_2$); wherein R_a is H or lower alkyl, lower alkenyl, lower alkynyl, aryl, heteroaryl or heterocycle; a side chain of a natural amino acid or a substituted side chain of a natural amino acid; and

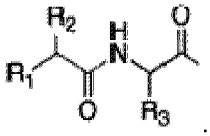
the substituted side chain of an amino acid is a side chain of an amino acid substituted with alkoxy, hydroxyl, halide, aminoalkyl, amide, sulfonamide, aryl, alkyl, heteroaryl, amidine.

2. The compound of claim 1, wherein

R_7 is 

.

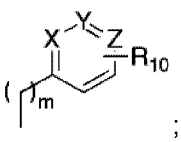
3. The compound of claim 1, wherein

R_7 is 

.

4. The compound of any one of claims 1 to 3, wherein

(i) R_1 is -H or $-NH_2$;

(ii) R_2 is 

;

(iii) m is 1;

(iv) W is CH;

(v) X is CH;

(vi) Y is CH;

(vii) R_{10} is -C(=NH)NH_2; or

(viii) any combination of (i) to (vii).

5. The compound of any one of claims 1 to 3, wherein

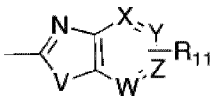
(i) R_1 is -NH_2;

(ii) R_2 is -CH_2-R_8;

(iii) R_3 is a alkyl substituted with alkyl; alkenyl; alkynyl; hydroxy; alkoxy; amide; halide; heterocyclic; aryl; aryl substituted on any or all possible aryl positions by hydroxyl, amide, halides, nitrile, nitro, alkyl, amino, amides or sulfonamides; guanidine; amidine; heteroaryl; aryloxy; halogen; haloalkyl; cyano; nitro; amino (-NH_2); lower alkylamino; lower dialkylamino; NHCO-alkyl; NHCO-aryl; $NHSO_2$-alkyl; $NHSO_2$-aryl; amido (-(CO)NR_a); azido; acyl (—(CO)R_a); alkoxymethyl; mercapto (—S—R_a); sulfoxy (—SO—R_a); sulfonyl —$(SO)_2$—R_a); sulfonamide (—$S(O)_2N(R_a)_2$); carbonate (—O(CO)—O—R_a); oxyacyl (—O(CO)—R_a); carboxyl (—CO_2H); ester (—(CO)OR_6); carbamate (—O(CO)—$N(R_a)_2$); wherein R_a is H or lower alkyl, lower alkenyl, lower alkynyl, aryl, heteroaryl or heterocycle; a side chain of a natural amino acid or a side chain of a natural amino acid substituted with alkoxy, hydroxyl, halide, aminoalkyl, amide, sulfonamide, aryl, alkyl, heteroaryl or amidine;

(iv) R_4 is an alkyl;

(v) R_5 is -CH_2-R_8;

(vi) R_6 is 

;

(vii) R_8 is -$(CH_2)_3NH(C=NH)NH_2$;

(viii) W is CH;

(ix) X is CH;

(x) Y is CH;

(xi) Z is CH;

(xii) V is S;

(xiii) R_{11} is H; or

(xiv) any combination of (i) to (xiii).

6. The compound of any one of claims 1 to 5, wherein R_3 is a substituted alkyl, which is substituted with an alkyl, aryl, $(CO)NH_2$, (CO)OH, NH_2, NHCO-alkyl, NHCO-aryl, $NHSO_2$-alkyl, $NHSO_2$-aryl or heteroaryl.

7. The compound of claim 6, wherein R_3 is a substituted C_1 to C_6 alkyl.

8. The compound of claim 7, wherein R_3 is a substituted C_1 to C_3 alkyl.

9. The compound of any one of claims 1 to 8, wherein R_3 is -$(CH_2)_2C(=O)NH_2$.

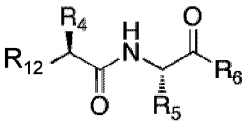
10. The compound of any one of claims 1 to 9, wherein R_4 is a C_1 to C_6 alkyl.

11. The compound of claim 10, wherein R_4 is a C_1 to C_3 alkyl.

12. The compound of claim 11, wherein R_4 is $-CH_3$.

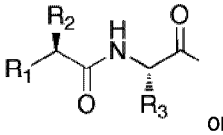
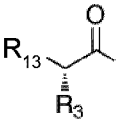
13. The compound of any one of claims 1 to 12, wherein the carbon to which R_2 and R_5 are bonded have the (S) configuration.

14. The compound of any one of claims 1 to 13, wherein the compound is of formula (1.1):



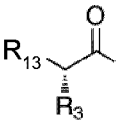
wherein

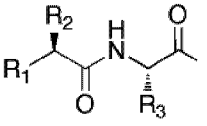
R_{12} is NHR_7, wherein R_7 is

 or ,

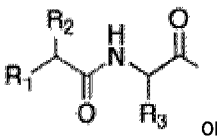
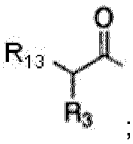
and

wherein R_1, R_2, R_3, R_4, R_5, R_6, R_8, R_{10}, R_{11} and R_{13} are as defined in any one of claims 1 to 13.

15. The compound of claim 14, wherein R_7 is .

16. The compound of claim 14, wherein R_7 is .

17. The compound of claim 1, wherein

R_7 is  or ;

R_{13} is NH_2;

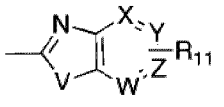
R_1 is NH_2;

R_2 is the side chain of arginine;

R_5 is the side chain of arginine or lysine;

R_3 is the side chain of glutamine;

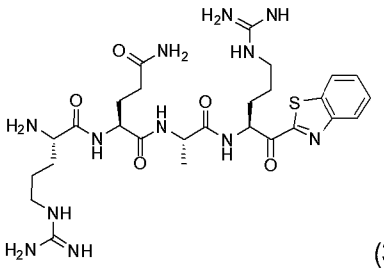
R_4 is the side chain of alanine;

R_6 is  ;

wherein W, X, Y and Z are CH; and V is S; and

R_{11} is H.

18. The compound of claim 1, wherein the compound is of formula (3):



(3).

19. A composition comprising the compound as defined in any one of claims 1 to 18, and

(a) a further therapeutic agent for treating or preventing an orthomyxovirus infection;

(b) a pharmaceutically acceptable excipient; or

(c) a combination of (a) and (b).

20. The composition of claim 19, wherein said orthomyxovirus infection is an influenza infection.

21. The composition of claim 19 or 20, wherein said further therapeutic agent is a viral M2 ion channel inhibitor or a neuraminidase inhibitor.

22. The composition of any one of claims 19 to 21, wherein said further therapeutic agent is Tamiflu™ (oseltamivir), Relenza™ (zanamivir), laninamivir, peramivir, amantadine, rimantadine, ribavirin, vitamin C, Cold Fx™, echinacea, ginseng or any combination thereof.

23. The composition of any one of claims 19 to 22, formulated for direct administration into lungs.

24. The composition of claim 23, formulated for administration by an inhaler or nebulizer.

25. Use of the compound according to any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for treating or preventing an influenza type A, B or C infection in a subject.

26. Use of the compound according to any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for the manufacture of a medicament for treating or preventing an influenza type A, B or C infection in a subject.

27. The use of claim 25 or 26, wherein the influenza infection is an influenza type A infection.

28. Use of (i) the compound as defined in any one of claims 1 to 18; or (ii) the composition as defined in any one of claims 19 to 24, for inhibiting matriptase activity in a cell.

29. Use of (i) the compound as defined in any one of claims 1 to 18; or (ii) the composition as defined in any one of claims 19 to 24, for the manufacture of a medicament for inhibiting matriptase activity in a cell.

30. The use of claim 28 or 29, wherein said cell is an epithelial cell of the respiratory system.

31. The compound as defined in any one of claims 1 to 18, or the composition as defined in any one of claims 19 to 24, for treating or preventing an influenza type A, B or C infection in a cell.

32. The compound as defined in any one of claims 1 to 18, or the composition as defined in any one of claims 19 to 24, for the manufacture of a medicament for treating or preventing an influenza type A, B or C infection in a cell.

33. The compound or composition of claim 31 or 32, wherein the infection is an influenza type A infection.

34. The compound of any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for inhibiting matriptase activity in a cell.

35. The compound of any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for the manufacture of a medicament for inhibiting matriptase activity in a cell.

36. The compound or composition of claim 34 or 35, wherein said cell is an epithelial cell of the respiratory system.

37. A kit comprising a compound as defined in any one of claims 1 to 18, or a composition as defined in any one of claims 19 to 24, and instructions to use same for treating or preventing an influenza type A, B or C infection in a subject.

Date Reçue/Date Received 2020-08-06

38. The kit of claim 37, wherein the infection is an influenza type A infection.

39. Use of the compound of any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for inhibiting tumor growth, progression and/or metastasis in a subject.

40. Use of the compound of any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.

41. The compound of any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for inhibiting tumor growth, progression and/or metastasis in a subject.

42. The compound of any one of claims 1 to 18, or the composition of any one of claims 19 to 24, for the manufacture of a medicament for inhibiting tumor growth, progression and/or metastasis in a subject.

Date Reçue/Date Received 2020-08-06

A

(start)

atggggagcgatcgggcccgcaagggcggaggggggcccgaaggacttcggcgcgggactcaagtacaactcccggcacgagaaagtgaatg
gcttggaggaaggcgtggagttcctgccagtcaacaacgtcaagaaggtggaaaagcatggcccggggcgctgggtggtgctggcagccgtgct
gatcggcctcctcttggtcttgctggggatcggcttcctggtgtggcatttgcagtaccgggacgtgcgtgtccagaaggtcttcaatggctacatgag
gatcacaaatgagaattttgtggatgcctacgagaactccaactccactgagtttgtaagcctggccagcaaggtgaaggacgcgctgaagctgctgt
acagcggagtcccattcctgggcccctaccacaaggagtcggctgtgacggccttcagcgagggcagcgtcatcgcctactactggtctgagttcag
catcccgcagcacctggtggaggaggccgagcgcgtcatggccgaggagcgcgtagtcatgctgccccgcgggcgcgctccttgaagtcctttg
tggtcacctcagtggtggctttccccacggactccaaaacagtacagaggacccaggacaacagctgcagctttggcctgcacgcccgcggtgtgg
agctgatgcgcttcaccacgcccggcttccctgacagcccctaccccgctcatgcccgctgccagtgggccctgcgggggacgccgactcagtg
ctgagcctcaccttccgcagctttgaccttgcgtcctgcgacgagcgcggcagcgacctggtgacggtgtacaacaccctgagccccatggagccc
cacgccctggtgcagttgtgtggcacctaccctccctcctacaacctgaccttccactcctcccagaacgtcctgctcatcacactgataaccaacact
gagcggcggcatcccggctttgaggccaccttcttccagctgcctaggatgagcagctgtggaggccgcttacgtaaagcccaggggacattcaac
agcccctactacccaggccactacccacccaacattgactgcacatggaacattgaggtgcccaacaaccagcatgtgaaggtgcgcttcaaattctt
ctacctgctggagcccggcgtgcctgcgggcacctgccccaaggactacgtggagatcaatggggagaaatactgcggagagaggtcccagttcg
tcgtcaccagcaacagcaacaagatcacagttcgcttccactcagatcagtcctacaccgacaccggcttcttagctgaatacctctcctacgactcca
gtgacccatgcccggggcagttcacgtgccgcacggggcggtgtatccggaaggagctgcgctgtgatggctgggccgactgcaccgaccacag
cgatgagctcaactgcagttgcgacgccggccaccagttcacgtgcaagaacaagttctgcaagcccctcttctgggtctgcgacagtgtgaacgac
tgcggagacaacagcgacgagcaggggtgcagttgtccggcccagaccttcaggtgttccaatgggaagtgcctctcgaaaagccagcagtgcaa
tgggaaggacgactgtggggacgggtccgacgaggcctcctgccccaaggtgaacgtcgtcacttgtaccaaacacacctaccgctgcctcaatg
ggctctgcttgagcaagggcaaccctgagtgtgacgggaaggaggactgtagcgacggctcagatgagaaggactgcgactgtgggctgcggtc
attcacgagacaggctcgtgttgttgggggcacggatgcggatgagggcgagtggccctggcaggtaagcctgcatgctctgggccagggccaca
tctgcggtgcttccctcatctctcccaactggctggtctctgccgcacactgctacatcgatgacagaggattcaggtactcagaccccacgcagtgga
cggccttcctgggcttgcacgaccagagccagcgcagcgcccctggggtgcaggagcgcaggctcaagcgcatcatctcccacccctttcttcaatg
acttcaccttcgactatgacatcgcgctgctggagctggagaaaccggcagagtacagctccatggtgcggcccatctgcctgccggacgcctccca
tgtcttccctgccggcaaggccatctgggtcacgggctggggacacacccagtatggaggcactggcgcgctgatcctgcaaaagggtgagatcc
gcgtcatcaaccagaccacctgcgagaacctcctgccgcagcagatcacgccgcgcatgatgtgcgtgggcttcctcagcggcggcgtggactcct
gccagggtgattccgggggaccccctgtccagcgtggaggcggatgggcggatcttccaggccggtgtggtgagctggggagacggctgcgctca
gaggaacaagccaggcgtgtacacaaggctccctctgtttcgggactggatcaaagagaacactggggta**tag (stop)**

Figure 1A

B

```
        10         20         30         40         50         60
MGSDRARKGG GGPKDFGAGL KYNSRHEKVN GLEEGVEFLP VNNVKKVEKH GPGRWVVLAA

        70         80         90        100        110        120
VLIGLLLVLL GIGFLVWHLQ YRDVRVQKVF NGYMRITNEN FVDAYENSNS TEFVSLASKV

       130        140        150        160        170        180
KDALKLLYSG VPFLGPYHKE SAVTAFSEGS VIAYYWSEFS IPQHLVEEAE RVMAEERVVM

       190        200        210        220        230        240
LPPRARSLKS FVVTSVVAFP TDSKTVQRTQ DNSCSFGLHA RGVELMRFTT PGFPDSPYPA

       250        260        270        280        290        300
HARCQWALRG DADSVLSLTF RSFDLASCDE RGSDLVTVYN TLSPMEPHAL VQLCGTYPPS

       310        320        330        340        350        360
YNLTFHSSQN VLLITLITNT ERRHPGFEAT FFQLPRMSSC GGRLRKAQGT FNSPYYPGHY

       370        380        390        400        410        420
PPNIDCTWNI EVPNNQHVKV RFKFFYLLEP GVPAGTCPKD YVEINGEKYC GERSQFVVTS

       430        440        450        460        470        480
NSNKITVRFH SDQSYTDTGF LAEYLSYDSS DPCPGQFTCR TGRCIRKELR CDGWADCTDH

       490        500        510        520        530        540
SDELNCSCDA GHQFTCKNKF CKPLFWVCDS VNDCGDNSDE QGCSCPAQTF RCSNGKCLSK

       550        560        570        580        590        600
SQQCNGKDDC GDGSDEASCP KVNVVTCTKH TYRCLNGLCL SKGNPECDGK EDCSDGSDEK

       610        620        630        640        650        660
DCDCGLRSFT RQARVVGGTD ADEGEWPWQV SLHALGQGHI CGASLISPNW LVSAAHCYID

       670        680        690        700        710        720
DRGFRYSDPT QWTAFLGLHD QSQRSAPGVQ ERRLKRIISH PFFNDFTFDY DIALLELEKP

       730        740        750        760        770        780
AEYSSMVRPI CLPDASHVFP AGKAIWVTGW GHTQYGGTGA LILQKGEIRV INQTTCENLL

       790        800        810        820        830        840
PQQITPRMMC VGFLSGGVDS CQGDSGGPLS SVEADGRIFQ AGVVSWGDGC AQRNKPGVYT

       850
RLPLFRDWIK ENTGV
```

Figure 1B

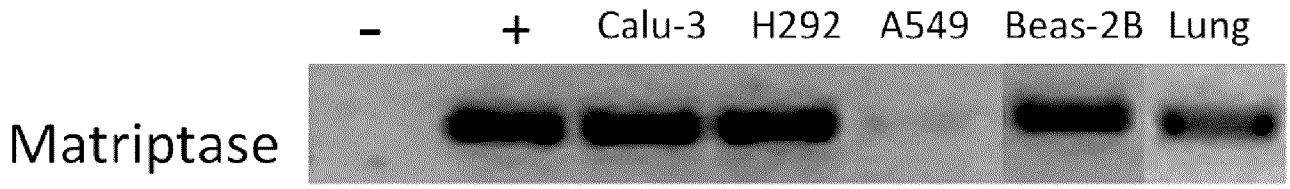


Figure 2

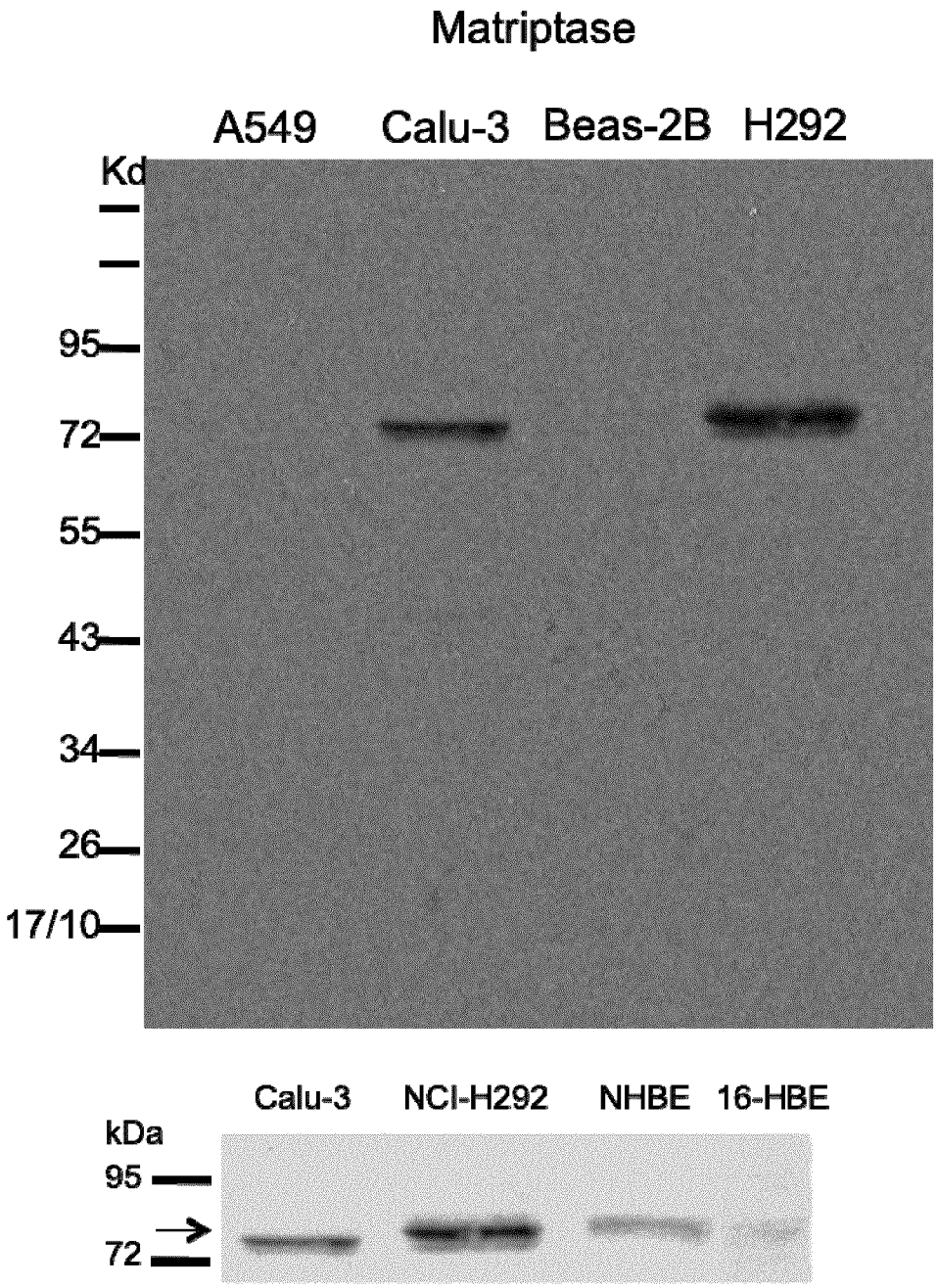

Matriptase
A549
Calu-3
Beas-2B
H292
Kd
95
72
55
43
34
26
17/10
Calu-3
NCI-H292
NHBE
16-HBE
kDa
95
72

Figure 3

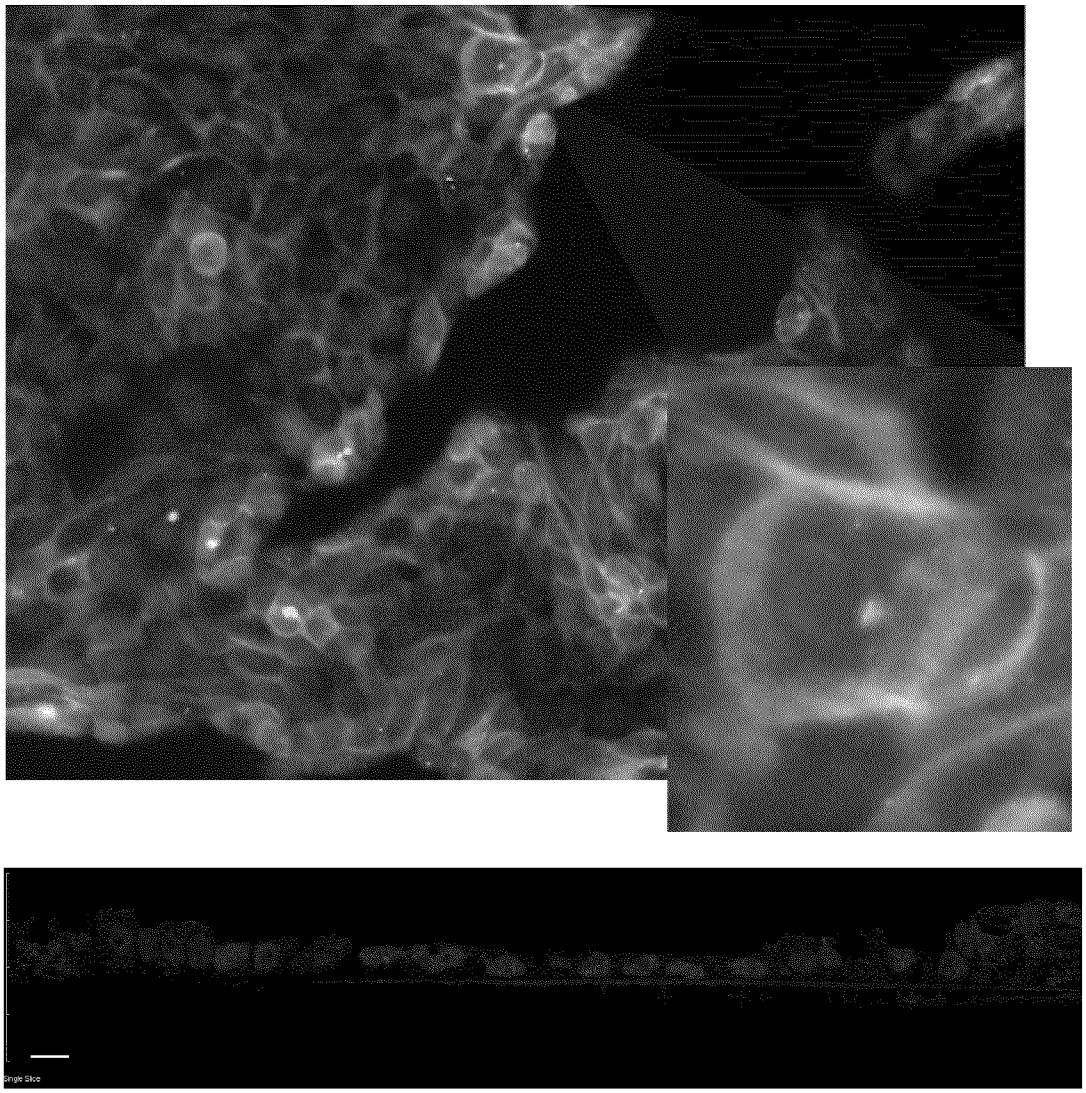


Figure 4

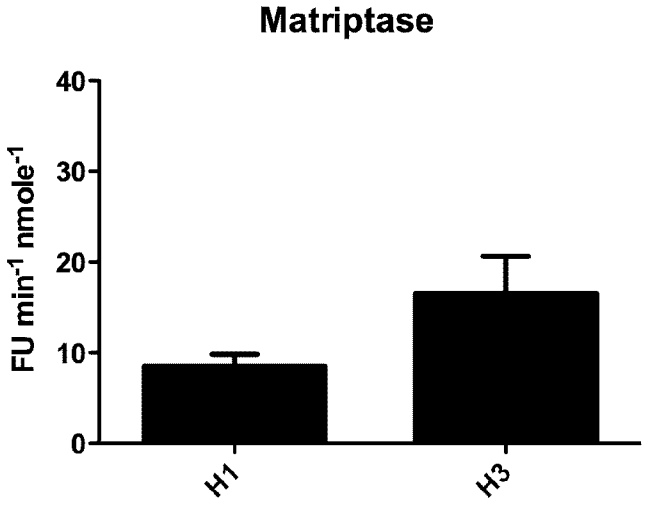


Figure 5

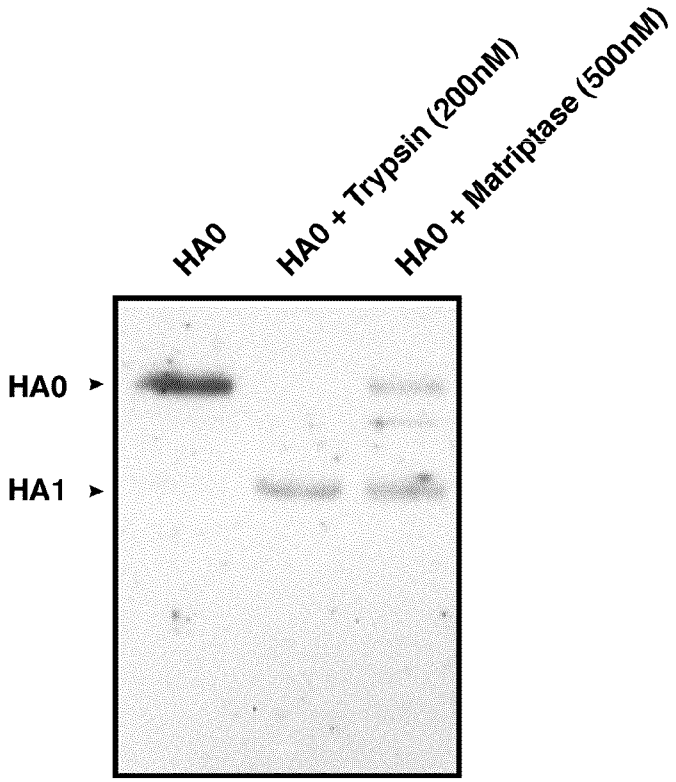


Figure 6

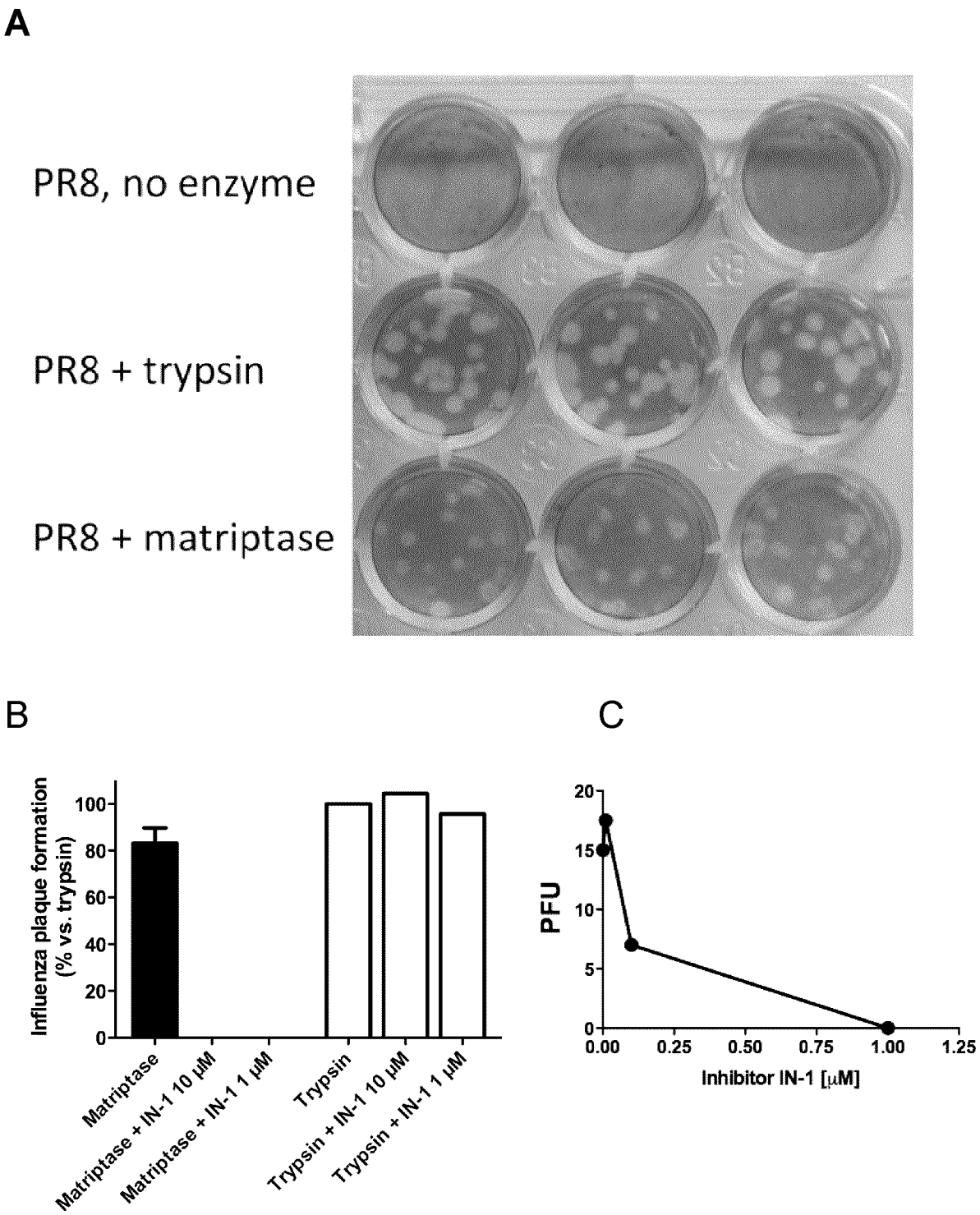
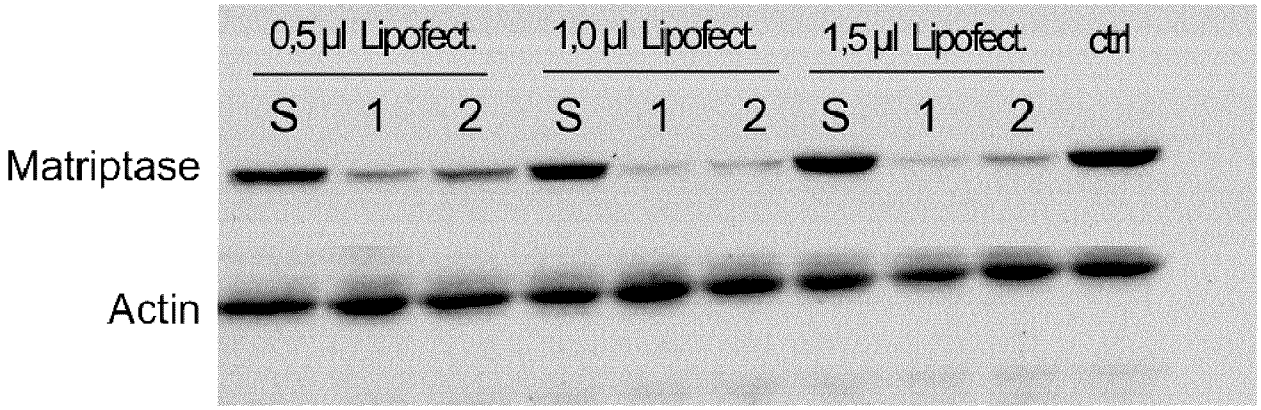

A
PR8, no enzyme
PR8 + trypsin
PR8 + matriptase
B
Influenza plaque formation (% vs. trypsin)
100
80
60
40
20
0
Matriptase
Matriptase + IN-1 10 µM
Matriptase + IN-1 1 µM
Trypsin
Trypsin + IN-1 10 µM
Trypsin + IN-1 1 µM
C
PFU
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
1.25
Inhibitor IN-1 [µM]

Figure 7

A


0,5 µl Lipofect.
1,0 µl Lipofect.
1,5 µl Lipofect.
ctrl
S 1 2 S 1 2 S 1 2
Matriptase
Actin

B

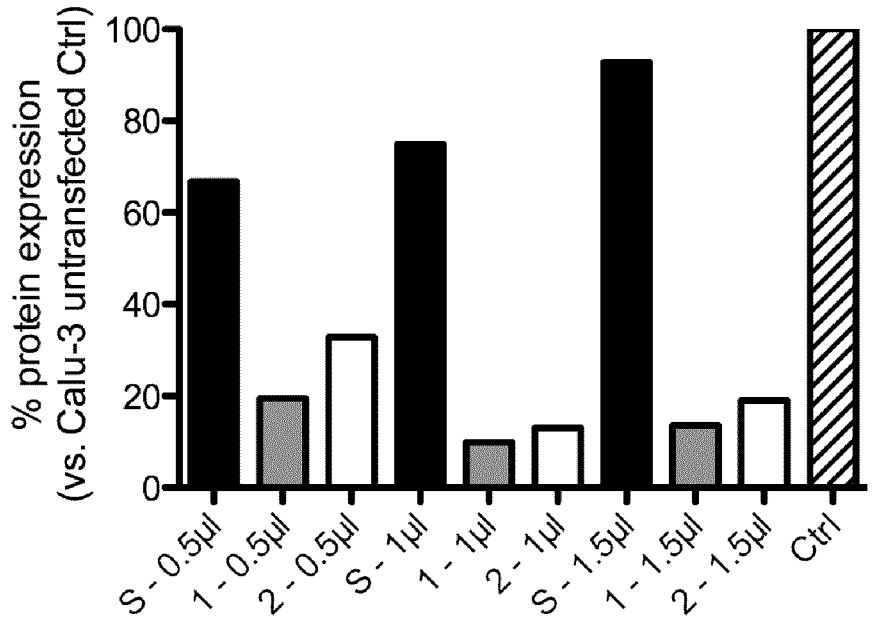

% protein expression
(vs. Calu-3 untransfected Ctrl)
100
80
60
40
20
0
S - 0.5µl
1 - 0.5µl
2 - 0.5µl
S - 1µl
1 - 1µl
2 - 1µl
S - 1.5µl
1 - 1.5µl
2 - 1.5µl
Ctrl

Figure 8

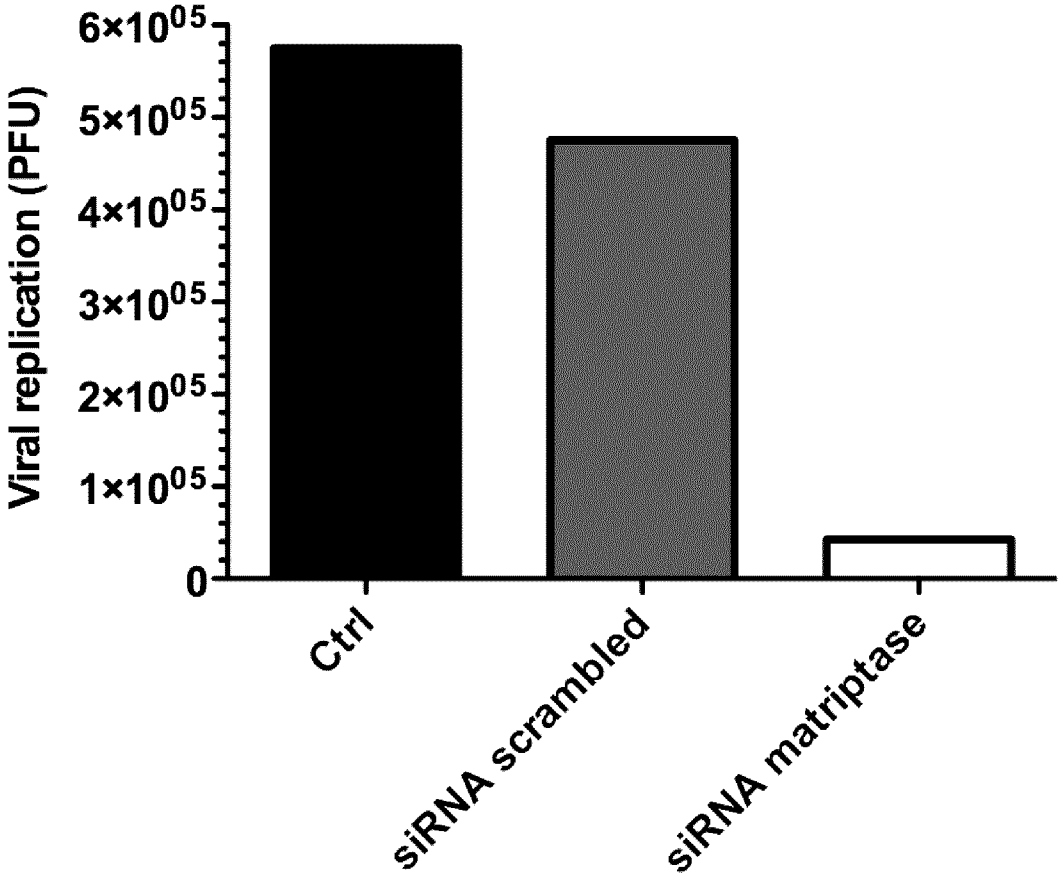

Viral replication (PFU)
6×10^05
5×10^05
4×10^05
3×10^05
2×10^05
1×10^05
0
Ctrl
siRNA scrambled
siRNA matriptase

Figure 9

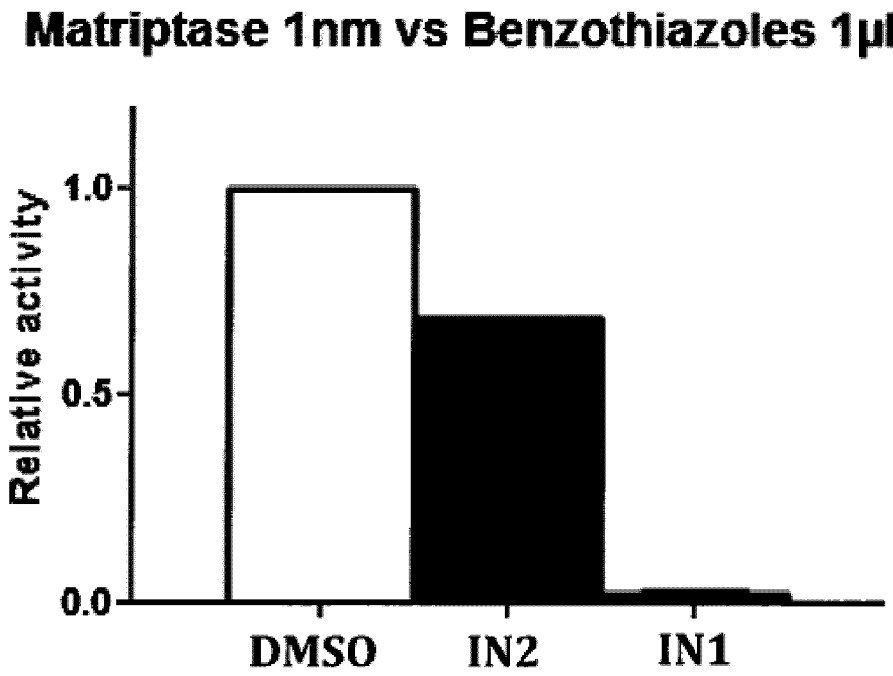


Figure 10

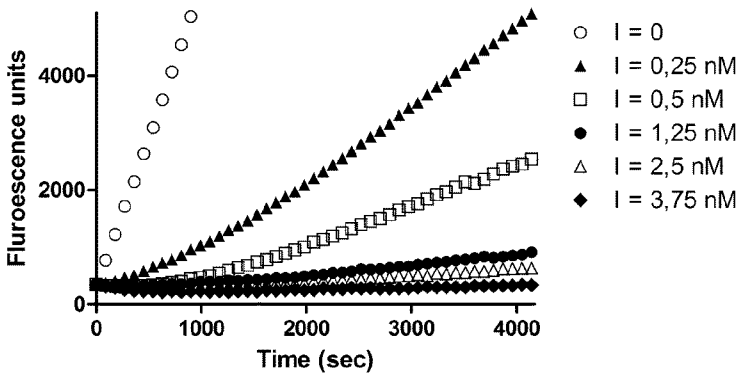


Figure 11A

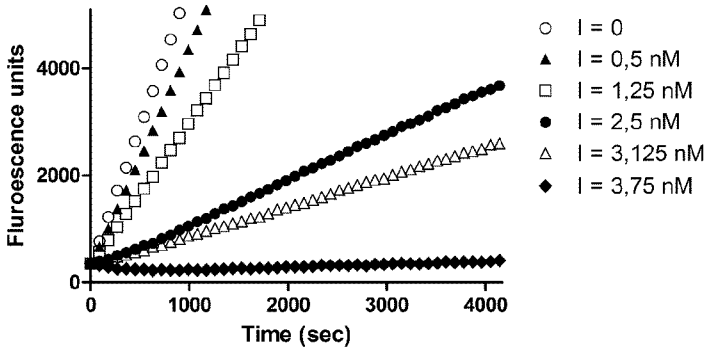


Figure 11B

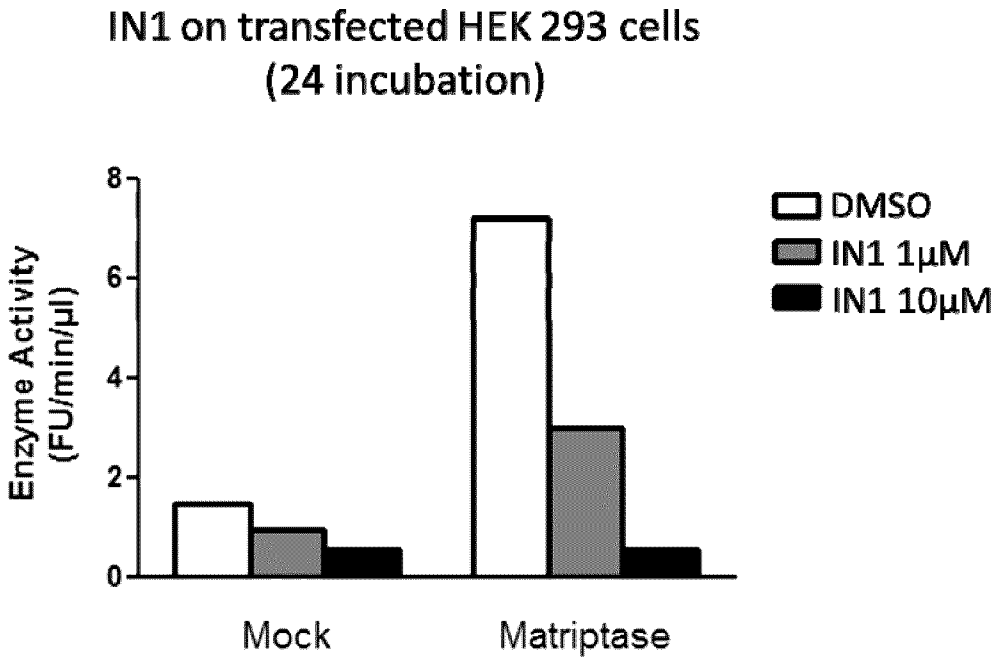


Figure 12

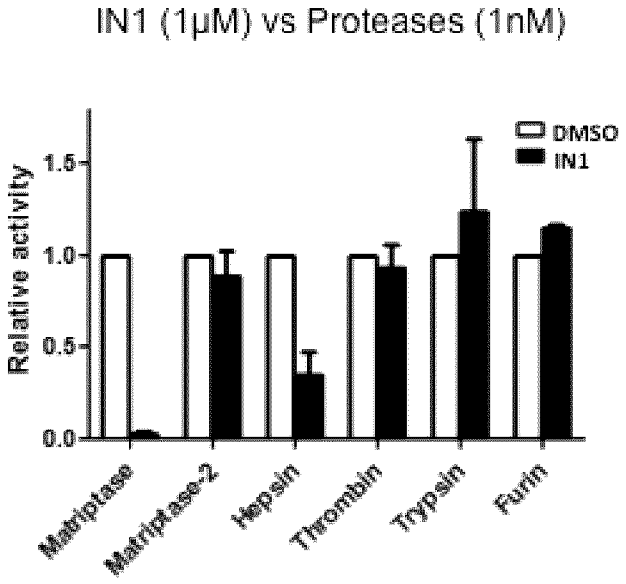


Figure 13A

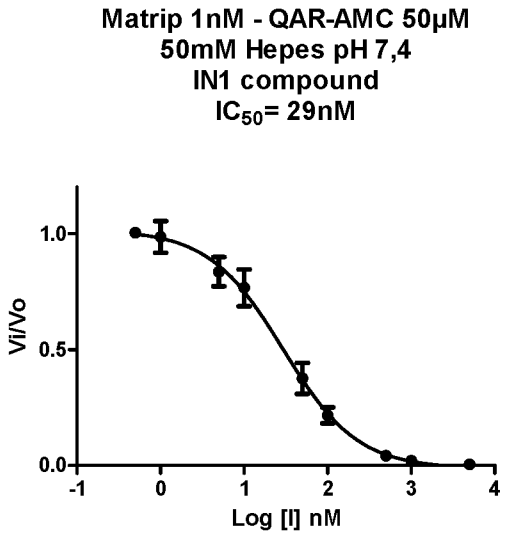


Figure 13B

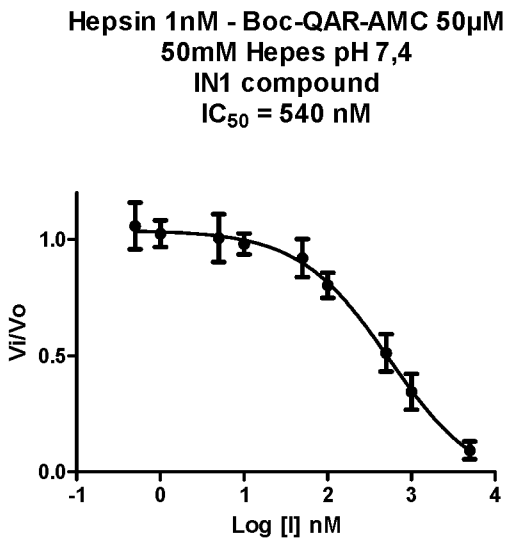


Figure 13C

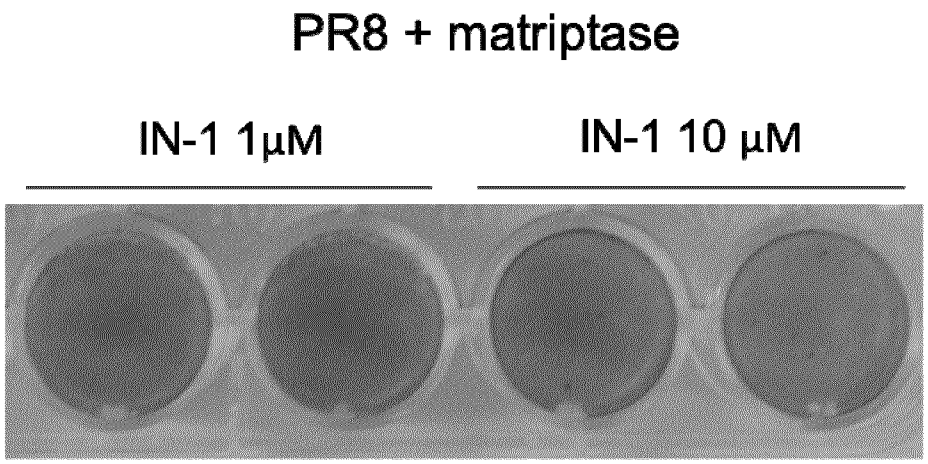


Figure 14

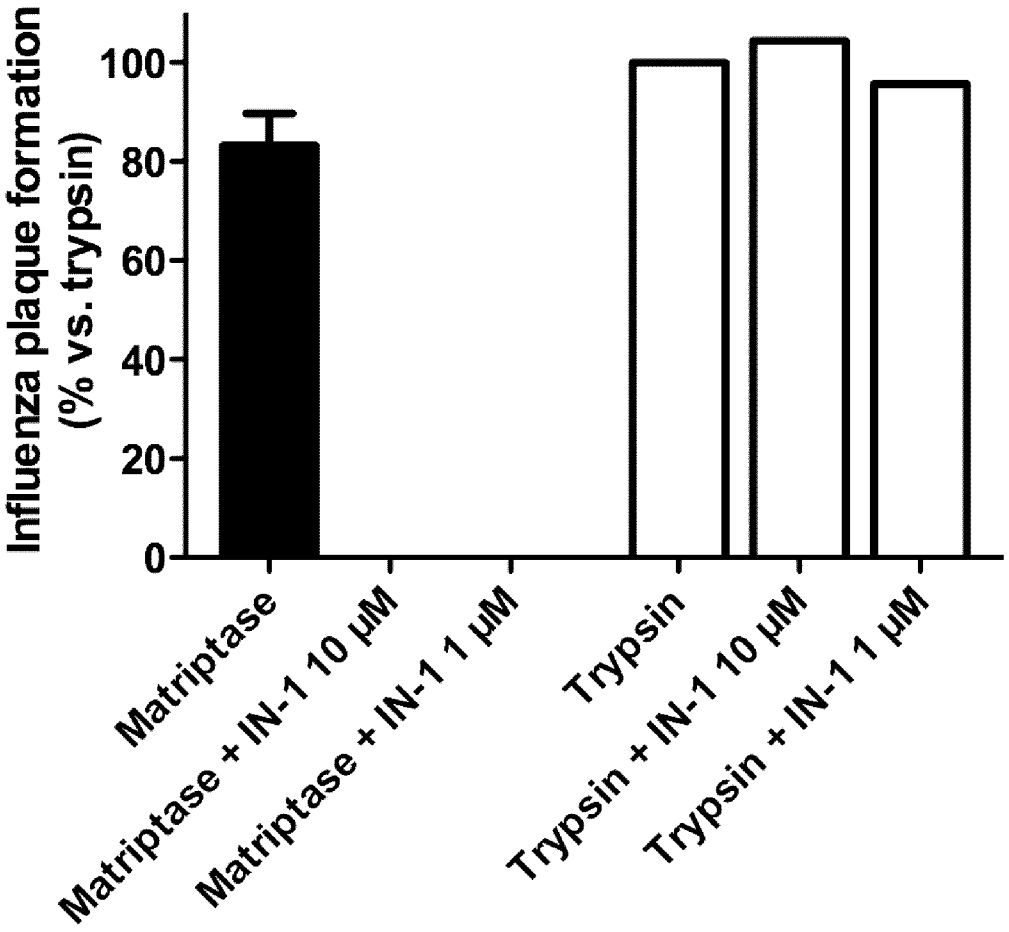

Influenza plaque formation
(% vs. trypsin)
100
80
60
40
20
0
Matriptase
Matriptase + IN-1 10 µM
Matriptase + IN-1 1 µM
Trypsin
Trypsin + IN-1 10 µM
Trypsin + IN-1 1 µM

Figure 15

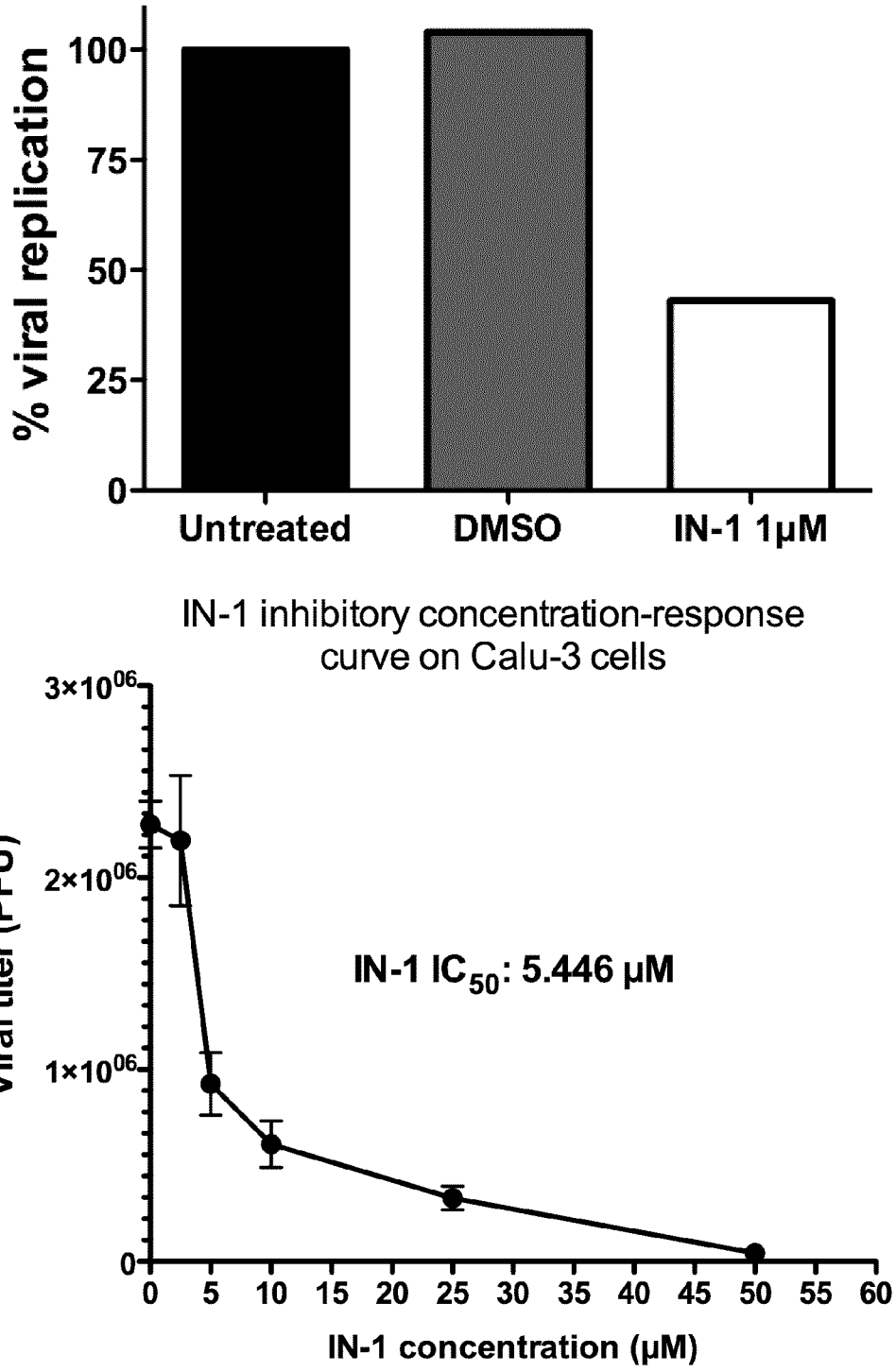

% viral replication
100
75
50
25
0
Untreated
DMSO
IN-1 1µM
IN-1 inhibitory concentration-response curve on Calu-3 cells
Viral titer (PFU)
3×10^06
2×10^06
1×10^06
0
IN-1 IC50: 5.446 µM
0 5 10 15 20 25 30 35 40 45 50 55 60
IN-1 concentration (µM)

Figure 16

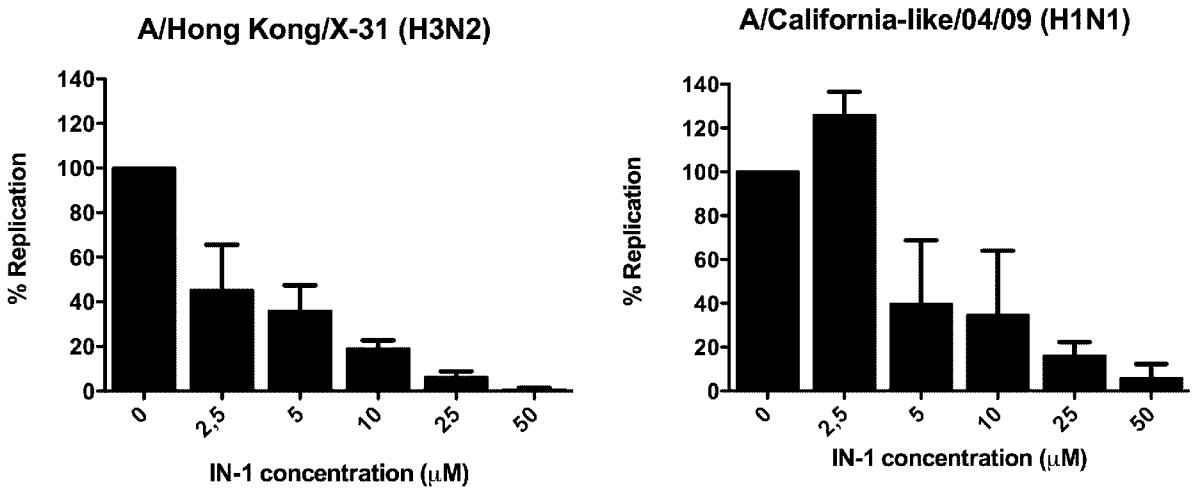


Figure 17

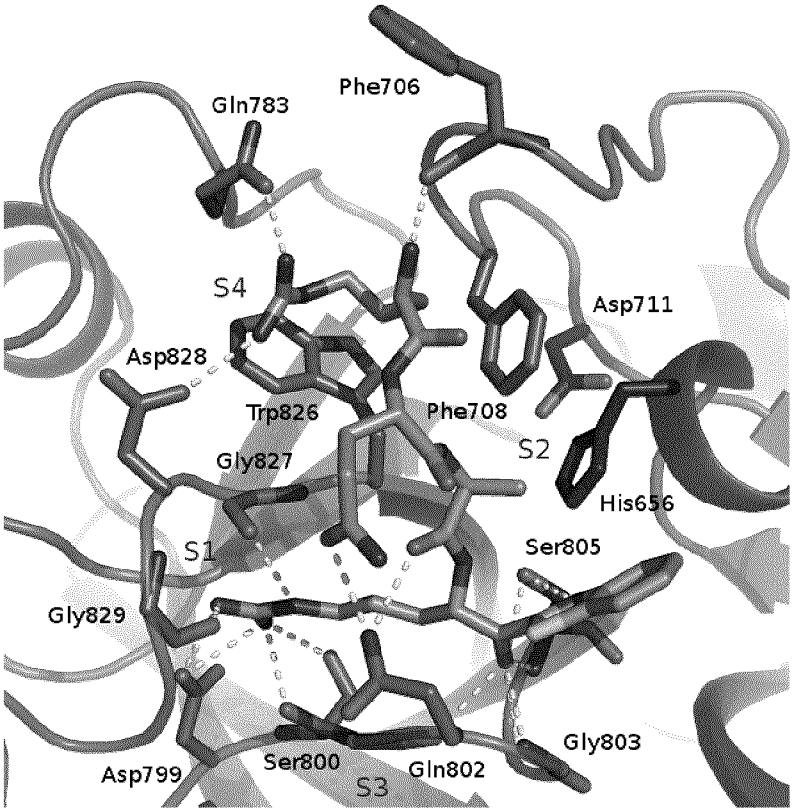
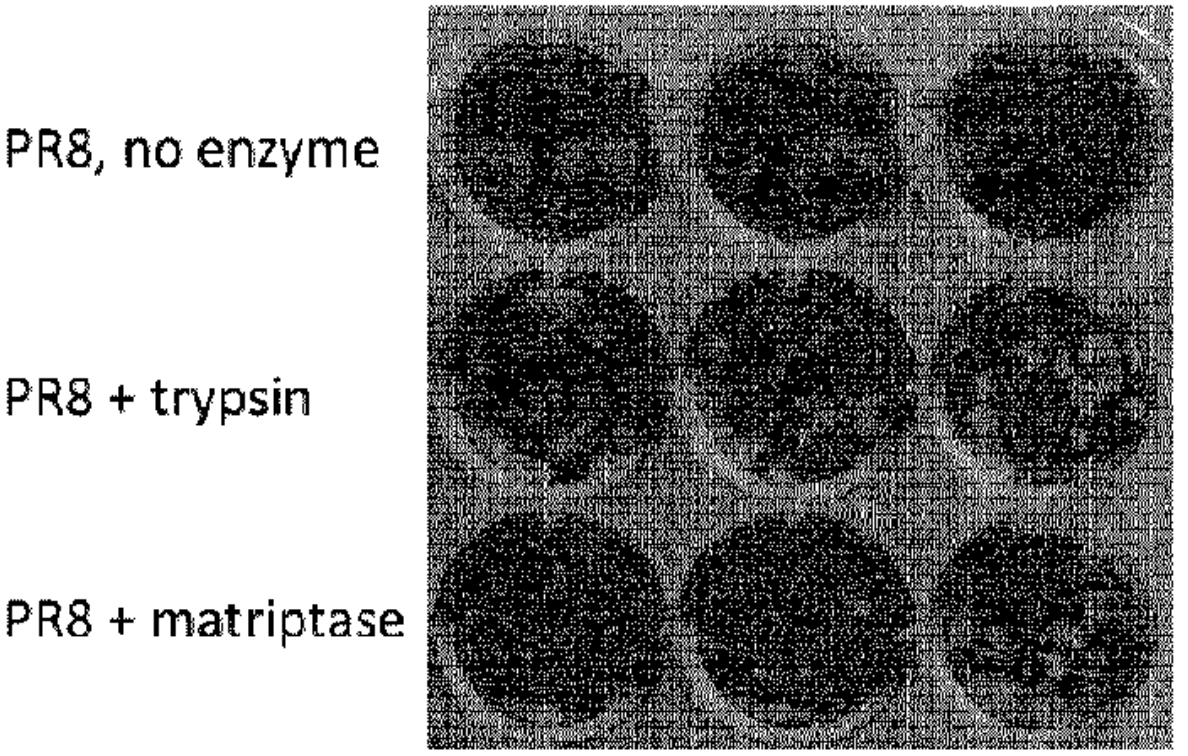
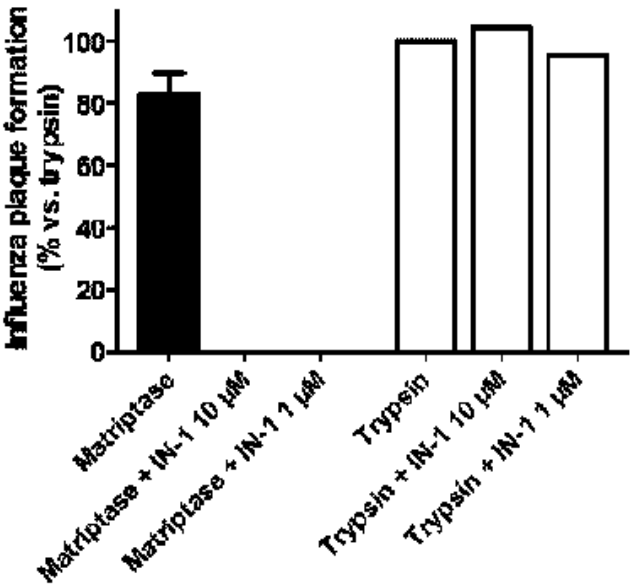


Figure 18

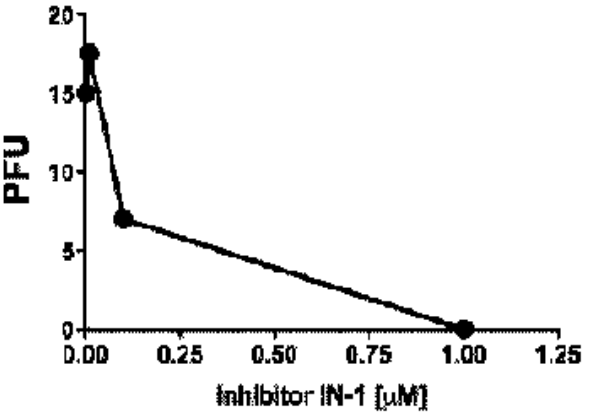
A


PR8, no enzyme
PR8 + trypsin
PR8 + matriptase

B


Influenza plaque formation (% vs. trypsin)
100
80
60
40
20
0
Matriptase
Matriptase + IN-1 10 µM
Matriptase + IN-1 1 µM
Trypsin
Trypsin + IN-1 10 µM
Trypsin + IN-1 1 µM

C


PFU
20
15
10
5
0
0.00
0.25
0.50
0.75
1.00
1.25
Inhibitor IN-1 [µM]