

Anti-viral methods and the use of an Anti-viral agent

Cross-Reference to Related Application

[0001] This application claims the benefit of priority of United States of America Provisional Patent Application No. 61/682,932 filed August 14, 2012, the contents of which being hereby incorporated by reference in its entirety for all purposes.

Field

[0002] The invention relates to methods of treating viral infection and compounds for use in treating viral infection.

Background

[0003] Viral infections have caused many disease outbreaks throughout history including smallpox, polio and yellow fever. In more recent times, viral infections have been responsible for pandemics such as the Spanish flu that killed up to 40 Million people and the HIV/AIDS epidemic that kills over 1 million each year. In addition there are often local outbreaks of diseases caused by coronaviruses, influenza viruses and a range of flaviviruses. Vaccines have been the most effective tool in reducing disease outbreaks caused by viral infections. Vaccines have drastically reduced outbreaks of polio, measles and smallpox but vaccines are very specific to a particular virus. Treatment of viral infections has proved even more challenging.

[0004] The incidence of dengue has increased 30-fold over the last 50 years infecting more than 50 million people annually, emerging as a pandemic-prone viral disease. As no approved vaccines are currently available, preventive and supportive measures are the primary tools to manage dengue. This increasing trend of dengue infection is a threat to the public health and requires controlling dengue to minimize the spread of dengue virus, prompting a search for anti-dengue therapeutics, either by targeting authentic viral proteins or host proteins.

[0005] Dengue is the most common human arboviral infection worldwide [1, 2]. It is endemic in more than 100 countries including South-East Asia, the Pacific, the Americas, Africa, and the Caribbean [3]. Dengue is transmitted by the bite of an *Aedes* mosquito infected with one of four dengue virus serotypes (DV1-4) [4]. Although most infections are generally mild, a complicated dengue virus infection can result in dengue haemorrhagic fever (DHF) and dengue shock syndrome [5]. The World Health Organization (WHO) estimates that 50-100 million infections occur annually, causing 500,000 DHF cases and 22,000 deaths, mostly among children. As currently no approved vaccines are available for dengue, prevention is the primary tool used such as; controlling the mosquito habitat and limiting exposure to bites. Treatment of acute dengue is mainly done via supportive measure using either oral or intravenous rehydration or blood transfusion [6]. Over the years, the increased incidence of dengue infection and its resulting complications prompts an urgent development of vaccine as a primary weapon to combat dengue. While attempts are being made to develop a dengue vaccine, in the meantime the on-going efforts to develop anti-dengue therapeutics will be also important in treating attacks of dengue fever and preventing dengue-related severe complications [7].

[0006] Dengue virus is an enveloped positive-single stranded RNA virus that belongs to the Flaviviridae family. Other than retroviruses RNA viruses replicate in the cytoplasm and positive-single stranded RNA virus are directly accessed by the host ribosomes to immediately form proteins. The dengue virus genomic RNA codes for a single polypeptide, which is further processed to contain three structural proteins (the capsid protein C, membrane protein M, and the envelope protein E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [7]. In recent years, numerous functional and structural studies provide insights into assembly mechanism of dengue virus particle and also molecular characteristics of the key dengue virus proteins for the life cycle of dengue virus [7]. There are several plausible approaches to develop anti-dengue viral drugs. The first approach might target proteins involved

in viral replication such as NS5, the viral RNA-dependent RNA polymerase, which copies the viral genetic material. Secondly, it may be possible to develop specific inhibitors of NS3, the viral protease, which is required for viral protein processing [8]. Thirdly, it may be possible to target dengue virus-receptor interaction and develop strategy to antagonize viral entry to host cells [3].

[0007] Mature dengue virus is roughly spherical being about 500 Å in diameter containing three structural proteins [9]. The major envelop protein E consists of three distinct structural domains [9, 10]. The crystal structure of the dengue virus -E protein revealed that it consists of three domains: domain I is structurally positioned between domain II, the homodimerization domain, and the immunoglobulin-like domain III [10]. The dengue virus -E protein is shown with N-linked glycosylated at Asn67 and Asn153. Glycosylation to viral proteins play important roles in viral virulence [11, 12]. The glycosylation at Asn153 has been suggested to play a role in stabilizing dimer formation [3, 13].

[0008] Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a C-type lectin receptor found on the surface of macrophages and DC. The receptor has a carbohydrate recognition domain that recognises high mannose-type oligosaccharides in glycoproteins. The envelope proteins of many viruses are glycosylated and DC-SIGN has been shown to recognise the envelop of several viruses.

[0009] It was shown that monocytes, macrophages, dendritic cells (DCs), including the skin-resident Langerhans cells, are primary targets for dengue virus infection [14, 15]. Heparin sulfate [16], Hsp90 [17], CD14 [18], GRP78 [19], and DC-SIGN [5, 20] have been identified as potential dengue virus receptors or viral entry-facilitating factors on mammalian cells. Interestingly, it was shown that dengue virus infection can be inhibited by soluble extracellular domain of DC-SIGN and by antibodies against DC-SIGN [21], indicating that DC-SIGN plays a key role in the dengue virus infection of immature DCs [22]. DC-SIGN is composed of a cytoplasmic domain, a

transmembrane domain, an extracellular neck domain of eight tandem 23-amino acid residue repeats, and a carbohydrate recognition domain (CRD). The cryo-electron microscopy structural studies of dengue virus in complex with DC-SIGN CRD suggest that the glycosylation at Asn67 might be important in mediating the binding of the virus particle to DC-SIGN/CD209 on DCs [22]. It is unclear if the protein-protein interactions or protein-carbohydrate interactions are more important for dengue virus E protein-host receptor interaction.

[0010] The underlying molecular mechanism between dengue virus and DC-SIGN prompted us to search for novel biological macromolecular agents that could specifically inhibit the key recognition events during dengue virus infection. Designing or discovering small molecules modulating protein-protein or protein-carbohydrate interactions are challenging and also time-consuming due to large binding interface in protein-protein interactions and flexibility of sugar binding sites.

[0011] Mistletoes are obligate semi-parasitic plants mostly from the order Santalales, which grow on branches of trees and shrubs like oak and fig trees all over the world. Extracts from European mistletoe (*Viscum album*) have been used as an adjuvant in cancer therapy, as they exhibit cytotoxic properties on cancer cells [23]. Lectins within the mistletoe extracts from *Viscum album* were found to be the major active constituent responsible for the cytotoxicity [23-25]. Mistletoe lectins (MLs) have been sub-classified into different isoforms according to their sugar binding specificities. European mistletoe lectin (EML) has three isoforms, ML-I specific to galactose, ML-II specific to galactose and N-acetyl galactosamine, and ML-III specific to N-acetyl galactosamine [26]. Korean mistletoe lectin (KML), isolated from Korean mistletoe (*Viscum album* var. *coloratum* [Kom.] Ohwi) has two isoforms, KML-IIU and KML-IIL. Both isoforms are specific to galactose and N-acetyl galactosamine [27].

[0012] MLs are heterodimeric glycoproteins consisting of two subunits, namely A and B chains that are linked by a disulfide bond with molecular sizes of about 29 and 34 kDa,

respectively[26, 28]. The B chain binds to appropriate receptors, preferentially CD75 gangliosides expressed on the target cell surface which lead to the internalization of lectins [29-32], while the A chain mediates the cytotoxic effects on cells by inhibiting protein synthesis via inhibition of 60S ribosomal subunit [33]. Because of this unique property, MLs are also classified as type-II ribosome-inactivating protein family [34]. MLs have also been reported to induce apoptosis in cancer cells by binding to death receptors, and activating relevant downstream caspases and mitochondria-apoptosome-mediated pathway [29, 35]. Other than mediating killing through apoptosis, the B chain of mistletoe lectin has been shown to possess immuno-modulatory properties as well. Administration of EML has been shown to increase the production tumour necrosis factor- α (TNF- α), interleukin-1 (IL-1) and IL-6 in patients suffering from breast cancer [36]. Another study demonstrated that the administration of KML into mice resulted in induction of TNF- α production from the macrophages, reduction in primary tumor growth, as well as suppression of tumor metastasis through inhibition of tumor angiogenesis [37]. Cancer is a major health problem and there is always a need for alternative treatments. A recombinant EML, is commercially produced under trade name of Aviscumine, which is currently undergoing phases I/II clinical trials [30]. Cancer is a major health problem and there is always a need for alternative treatments.

Summary

[0013] A first aspect of the invention includes a method for the treatment or prevention of a viral infection in a subject, the method comprising administering to said subject a mistletoe lectin in a therapeutically or prophylactically efficient amount.

[0014] Another aspect of the invention relates to a mistletoe lectin isolated and purified from a *Viscum* species other than *Viscum album* preferably from *Viscum ovalifolium*.

[0015] Another aspect of the invention relates to a pharmaceutical composition comprising a mistletoe lectin isolated and purified from a *Viscum* species other than *Viscum album* preferably from *Viscum ovalifolium* as defined above and a carrier or diluent.

[0016] Another aspect of the invention relates to Use of a mistletoe lectin for the preparation of a medicament to treat a viral infection.

[0017] Other aspects of the invention would be apparent to a person skilled in the art with reference to the following drawings and discription of various non-limiting embodiments.

Brief description of the drawings

[0018] The drawings are not necessarily drawn to scale, emphasis instead generally being placed upon illustrating the principles of various embodiments. In the following description, various embodiments of the invention are described with reference to the following drawings.

[0019] **Figure 1.** Purification of SML. SML was purified from Singapore mistletoes by affinity chromatography and subsequently analyzed on 12% SDS-PAGE in absence and presence of 2-mercapthanol. Lane 1, molecular weight markers (MW); Lane 2, KML (reduced); Lane 3, KML (non-reduced); Lane 4, SML (reduced), Lane 5, SML (non-reduced).

[0020] **Figure 2.** Effects of SML on cell proliferation and protein synthesis. A) Determination the effect of SML on different cell lines. SML is cytotoxic against various cancer cell lines (HCT116, A549, MCF7(caspase3+) and HeLa) but not normal cells (WI38). B) The ribosome-inactivating activity of SML on HCT116 colon cancer cells. 5×10^3 cells were seeded into each well and incubated with various concentrations of SML for 24 hours in 96-well plates. Ribosome-inactivating activity of SML was determined by the degree of

inhibition of luciferase translation in a rabbit reticulocyte system. The luciferase activity without SML was normalized to 100% translational activity. IC₅₀ for SML is 1 ng/ml.

[0021] Figure 3. SML induces apoptosis in HeLa cells. A significantly increased apoptosis was observed in SML-treated cells (B) which is compared with untreated cells (A) and KML-treated cells (C), respectively. Cells were treated with either 10 ng/ml SML or 10 ng/ml KML for 12 hours and stained with annexin V and propidium iodide and then examined for apoptosis by flow cytometry. All comparisons made are statistically significant with $P < 0.001$ (chi-square test).

[0022] Figure 4. Inhibition of DV replication by KML. Top 3 rows: DV1, 3 and 4 were pre-incubated with KML at the concentration of 0, 15 and 25 nM, respectively, for 30 min, and were used to infect C6/36 cells. At 48h post-infection, cells were fixed and subjected to immunofluorescence (IF) with anti-NS3. Bottom row: C6/36 cells were pre-incubated with KML, infected with DV1 for 48h, followed by IF.

[0023] Figure 5. Inhibition of DV replication by SML. DV1, 2, 3 and 4 were pre-incubated with SML at the concentration of 0, 15 and 25 nM, respectively, for 30 min, and were used to infect C6/36 cells. At 48h post-infection, cells were fixed and subjected to immunofluorescence detection with anti-NS3.

[0024] Figure 6. Immuno-coprecipitation of SML and Dengue envelope protein (E). The co-precipitated protein was detected by anti-E antibodies (molecular weight is 48,000). Anti-beta actin was used as a loading control (molecular weight is 43,000).

Detailed Description

[0025] In this study, we have isolated a new lectin from locally grown mistletoe *Viscum ovalifolium*, termed as Singapore mistletoe lectin (SML), and also cloned the gene encoding SML. The structural feature of SML and its unique sugar-binding characteristics led us not only

to test its ability to kill cancer cells but also examine its other potential biological efficacy such as anti-viral activity. To our surprise, our data demonstrated that SML and other mistletoe lectins exhibits an inhibitory activity during viral infection and might aid designing a novel anti-viral agent derived from plant lectin.

[0026] In our study, we isolated new mistletoe lectins from one of the local mistletoe species *Viscum ovalifolium* and presented its molecular properties and effects against cancer cells and dengue infection in cell culture model. Previously, no mistletoe lectins have been isolated from *Viscum* species other than *Viscum album*. Even in the herbal market only *Viscum album* plant species are mentioned as potential treatments. It is very unexpected that a mistletoe lectin can be isolated from *Viscum* species other than *Viscum album*. Further, we demonstrated that these new mistletoe lectins not only have similar structure and function to known mistletoe lectins (ML) isolated from *Viscum album* varieties but also that this new ML and known ML's have antiviral activity.

[0027] Accordingly, a first aspect of the invention includes a method for the treatment or prevention of a viral infection in a subject, the method comprising administering to said subject a mistletoe lectin in a therapeutically or prophylactically efficient amount.

[0028] "Treatment" and "treating" and synonyms thereof refer to therapeutic treatment wherein the object is to stop or reduce viral infection of a host cell. Preferably to at least affect viral infection of a host cell will stop or halt the viral infection or reduce (decrease) the symptoms. Preferably the subject is a mammal, preferably a human. Those in need of such treatment include an individual or patient that has been diagnosed with, a viral infection. A patient or an individual refers to an animal, such as a mammal preferably a human. In various embodiments the mistletoe lectin may be administered to a subject such as a patient or individual in need of treatment.

[0029] "Prevent" and "Prevention" and synonyms thereof refer to profalactic treatment wherein the object is to inhibit viral infection of a host cell. Preferably to at least affect viral infection of a host cell will prevent the viral infection or reduce (decrease) the occurrence of infection or at least the occurrence of symptoms. Preferably the subject is a mammal, preferably a human. Those in need of such treatment include an individual or patient that is suspected of being at risk of a viral infection. In various embodiments an individual or animal that has an arthropod bite, for example a mosquito bite, and/or presents with a fever may be suspected of being at risk of a viral infection. A patient or an individual refers to an animal, such as a mammal preferably a human. In various embodiments the cell is in vitro. In various other embodiments the cell is in vivo and the mistletoe lectin may be administered to a subject such as a patient or individual in need of treatment.

[0030] The mistletoe lectin preferably has a type II ribosome inactivating activity that inactivates eukaryotic ribosomes' by preventing the 60S subunit from binding to elongation factor 2. The mistletoe lectin preferably possesses cytotoxicity towards various types of tumor cells but not normal cells. The mistletoe lectin preferably is a glycoprotein having a heterodimeric structure comprising or consisting of two subunits, namely A and B chains that are linked by a disulfide bond. Preferably subunit A has a molecular size of about 29 to about 30 kilo Daltons (kDa) and subunit B has a molecular size of about 32 to about 34 kDa,. The B chain binds to appropriate receptors and virus envelope proteins, while the A chain mediates the inhibition of protein synthesis via inhibition of 60S ribosomal subunit. Preferably the mistletoe lectin is a *Viscum* species lectin.

[0031] Preferably the mistletoe lectin is isolated and or purified from a *Viscum* species. The *Viscum* species may include; *V. album*, *V. articulatum*, *V. bancroftii*, *V. capense*, *V. colortum*, *V. combreticola*, *V. cruciatum*, *V. diospyrosicola*, *V. fargesii*, *V. liquidambaricola*, *V.*

loranthi, *V. minimum*, *V. monoicum*, *V. multinerve*, *V. nudum*, *V. orientate*, *V. orientate*, *V. ovalifolium*, *V. rotundifolium*, *V. triflorum*, *V. whitei*, or *V. yunnanense*. Preferably the *Viscum* species is selected from the group comprising *Viscum album*, *Viscum album* var. *coloratum* and *Viscum ovalifolium*. In various embodiments the *Viscum* species is *Viscum ovalifolium*.

[0032] In various embodiments the mistletoe lectin is derived from a lectin isolated and purified from a *Viscum* species. A mistletoe lectin is derived from a lectin isolated and purified from a *Viscum* species would be a variant an example of a mistletoe lectin derived from a lectin isolated and purified from a *Viscum* species is the recombinant lectin Aviscumine derived from *Viscum album*. Methods of modifying nucleic acids to express recombinant polypeptide mistletoe lectins are known in the art. Such methods may be in vitro or comprise using a host cell having an expression system for production of the recombinant polypeptide. The host cell may be a prokaryotic cell, such a bacterial cell or a eukaryotic cell such as a yeast cell, a plant cell or a mammalian cell. In one embodiment the host cell is an E.coli cell. Such methods include cultivating the host cell under conditions that allow the expression of the lectin including the steps involved for glycosylation of the mistletoe lectin.

[0033] In various embodiments the polypeptides of the mistletoe lectin have about 520 to 590 amino acids, encoding a lectin. In various embodiments, the mistletoe lectin comprises, consists essentially of or consists of; (a) an amino acid sequence set forth in SEQ ID NO: 2; or SEQ ID NO. 4 or (b) a variant of the amino acid sequence or (a) that shares at least 80%, preferably at least 90%, more preferably at least 95%, at least 97%, at least 98% or at least 99 % sequence identity or sequence homology with the amino acid sequence set forth in SEQ ID NO:2 or SEQ ID NO. 4 over its entire length; or (c) an amino acid sequence that is a

functional fragment of the amino acid set forth in SEQ ID NO: 2 or SEQ ID NO. 4 in (a) or a variant defined in (b). Preferably the mistletoe lectin is a polypeptide or therapeutically active fragment thereof. Preferably the therapeutically active fragment comprises the N terminal A chain or the C terminal B chain region of the polypeptide or a functional fragment thereof.

[0034] In the context of the present invention a variant is an amino acid sequence that differs from the amino acid sequence set forth in SEQ ID NO: 2; or SEQ ID NO. 4 by one or more amino acids but retains that same function and/or structure. It is preferred to define "sequence identity" in terms of "substantial homology" or "substantial identity", when referring to polypeptides. Hence, a percentage of sequence identity is the extent to which two amino acid sequences are invariant. Whereby high sequence identity may suggest a structural and functional similarity. There are cases though where there is a low sequence identity but a structural and functional similarity. There are many Algorithms' and matrices' known in the art of bioinformatics to calculate sequence identity taking into account conservative modifications, insertions, deletions, or gaps either over a global alignment or a local alignment. A person skilled in the art will know which of these is most suitable for calculating the % sequence identity between a variant amino acid and the amino acid sequence set forth in SEQ ID NO: 2; or SEQ ID NO. 4. In various embodiments where the amino acid exhibits one or more of the functions of a mistletoe lectin then the % sequence identity may be at least about 70% identity with an entire naturally-occurring protein of the amino acid sequence set forth in SEQ ID NO: 2; or SEQ ID NO. 4 or a portion thereof, usually at least about 80% identity, and preferably at least about 90 or 95% identity with the amino acid sequence set forth in SEQ ID NO: 2; or SEQ ID NO. 4.

[0035] The term "polypeptide" refers to a polymer of amino acids and its equivalent and does not refer to a specific length of the product; thus, peptides, oligopeptides and proteins

are included within the definition of a polypeptide. This term also does not refer to, or exclude modifications of the polypeptide, for example, glycosylates, acetylations, phosphorylations, and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, natural amino acids, etc.), polypeptides with substituted linkages as well as other modifications known in the art, both naturally and non-naturally occurring.

[0036] Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

[0037] Percentage (%) homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

[0038] Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

[0039] However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

[0040] Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package and others known in the art. Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching.

[0041] Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is

preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

[0042] Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

[0043] The polypeptide homologues include those having the amino acid sequences, wherein one or more of the amino acids is/are substituted with another amino acid which substitutions do not substantially alter the biological activity of the molecule.

[0044] A “functional fragment” may refer to the functional domains of mistletoe lectins such as the N terminal A chain domain or the C terminal B chain domain comprises or consists of amino acids listed herein or allelic variants, homologues or fragments, thereof. In various embodiments any A chain domain from any mistletoe lectin would be a suitable functional fragment. Similarly any B chain domain from any mistletoe lectin would be a suitable functional fragment.

[0045] In various embodiments where the mistletoe lectin is SML the fragment comprises: (i) the N terminal A chain amino acids 1 to 254 of the sequence set forth in SEQ ID NO:2; or (ii) the the C terminal B chain amino acids 270 to 532 of the sequence set forth in SEQ ID NO:2 over its entire length; or (iii) a variant of the A and B chains having an amino acid sequence having at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% percent sequence homology with the amino acid sequence of the A and B chains of SEQ ID NO: 2 as defined above. Preferably the therapeutically active fragment of the N terminal A chain that inhibits viral protein synthesis via inhibition of 60S ribosomal subunit. Preferably the therapeutically active fragment of the

C terminal B chain binds to a virus envelope protein. It is postulated that the mistletoe lectin has a carbohydrate recognition domain within the B chain.

[0046] In various embodiments where the mistletoe lectin is KML the fragment comprises: (i) the N terminal A chain amino acids 34 to 287 of the sequence set forth in SEQ ID NO:4; or (ii) the the C terminal B chain amino acids 302 to 564 of the sequence set forth in SEQ ID NO:4 over its entire length; or (iii) a variant of the A and B chains having an amino acid sequence having at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99% percent sequence homology with the amino acid sequence of the A and B chains of SEQ ID NO: 4 as defined above. Preferably the therapeutically active fragment of the N terminal A chain that inhibits viral protein synthesis via inhibition of 60S ribosomal subunit. Preferably the therapeutically active fragment of the C terminal B chain binds to a virus envelope protein.

[0047] the method may include the mistletoe lectin as described herein being encoded or expressed by a nucleic acid.

[0048] The term " nucleic acid" as used herein refers to any nucleic acid molecule in any possible configuration, such as single stranded, double stranded or a combination thereof. Isolated nucleic acids include for instance DNA molecules, RNA molecules, analogues of the DNA or RNA generated using nucleotide analogues or using nucleic acid chemistry, locked nucleic acid molecules (LNA), peptide nucleic acid molecules (PNA) and tecto-RNA molecules. DNA or RNA may be of genomic or synthetic origin and may be single or double stranded. Such nucleic acid can be e.g. mRNA, cRNA, synthetic RNA, genomic DNA, cDNA, synthetic DNA, a copolymer of DNA and RNA, oligonucleotides, etc. In one embodiment the nucleic acid is a constructed cDNA determined from the isolated ML.

[0049] The nucleic acid molecule may be comprised in a vector such as a plasmid or any other vectors or expression systems known in the art.

[0050] The nucleic acid in a preferred embodiment comprising or consisting of (a) a nucleic acid sequence set forth in SEQ ID NO: 1 or 3. where by the nucleic acid sequence set forth in SEQ ID NO: 1 preferably encodes for and expresses the amino acid sequence set forth in SEQ ID NO: 2 isolated and purified from *Viscum ovalifolium* or an amino acid sequence having at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99 percent sequence homology with the amino acid sequence set forth in SEQ ID NO: 2 over its entire length; the nucleic acid sequence set forth in SEQ ID NO: 3 preferably encodes for and expresses the amino acid sequence set forth in SEQ ID NO: 4 isolated and purified from *Viscum album colaratum* or an amino acid sequence having at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99 percent sequence identity or sequence homology with the amino acid sequence set forth in SEQ ID NO: 4 over its entire length; or (b) a variant or derivative nucleic acid sequence of (a) that shares at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99 percent sequence identity or sequence homology with the expression product of a nucleic acid sequence of (a) and encodes a mistletoe lectin that shares at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98% or at least 99 percent sequence identity or sequence homology with the product encoded by the nucleic acid sequence of (a) over its entire length; or (c) a fragment of the nucleic acid sequence of (a) or (b) that encodes a functional mistletoe lectin fragment.

[0051] Preferably, the viral infection is caused by the virus comprising a virus having an envelope protein. Many viruses have envelope proteins and these are often glycosylated.

Without being limited to any theory we believe the interaction between the virus envelope

protein and the mistletoe lectin inhibits the interaction between the virus envelope protein and the host cell receptor molecule reducing the entry of the virus into the host cell thereby inhibiting or slowing down virus infection of the host cells of the subject. Preferably the C terminal B chain binds to or interacts with the virus envelope protein. All classes of viruses are known to include viruses with an envelope protein. Examples of DNA viruses with envelope proteins include herpesvirus or poxvirus. Examples of RNA viruses with envelope proteins include flavivirus, togavirus, corona virus orthomyxovirus, paramyxovirus, rabdovirus, bunyavirus, or filovirus. Examples of Retroviruses viruses with envelope proteins include HIV or Hepadnavirus.

[0052] In various embodiments the virus is an RNA virus. Without being limited to any theory it is noted that RNA viruses move into the cytoplasm to start replicating using the host ribosomes to translate the virus proteins. Unlike other classes of viruses, RNA viruses are not limited to the nucleus or dependant on the host cell cycle for replication. As the N terminal A chain of mistletoe lectins inhibits protein synthesis via inhibition of 60S ribosomal subunit this effect would be seen soon after entry of an RNA virus as RNA viruses can initiate replication more or less immediately upon entry into the host cell. In this way the effect of mistletoe lectin on the 60S ribosome will reduce replication of the virus inhibiting or slowing down the infection to other cells in the subject. In this embodiment preferably the RNA virus is a single stranded positive RNA virus preferably an arbovirus that are transmitted by arthropod vectors. Preferably a flavivirus known to cause diseases including yellow fever, dengue fever, Japanese encephalitis, hepatitis C, West Nile fever. Most preferably the virus is a dengue virus including any of the 4 serotypes.

[0053] Preferably the subject is a mammal, preferably a human.

[0054] A “therapeutically efficient amount” Is an amount that is able to stop or reduce viral infection of a host cell. Preferably to at least affect viral infection of a host cell will stop or halt the viral infection or reduce (decrease) the symptoms without being toxic to the host. Preferably the host is a mammal, preferably a human. A “prophylactically efficient amount” Is an amount that is able to inhibit viral infection of a host cell. Preferably to at least affect viral infection of a host cell will prevent the viral infection or reduce (decrease) the occurrence of infection or at least the occurrence of symptoms in the host without being toxic to the host. Dosages and administration of a mistletoe lectin may be determined by one of ordinary skill in the art of clinical pharmacology or pharmacokinetics. An effective amount of the antibody to be employed therapeutically, for example a mistletoe lectin as described herein, will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the mammal. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 10 ng to up to 100 mg per day, preferably about 10 ng to 10 mg per day. Doses may include a mistletoe lectin amount any where in the range of 10 to 100 ng or more preferably 25, 50, or 75 ng per day. In one embodiment the therapeutically or prophylactically efficient amount mistletoe lectin is between 15nM and 25nM.

[0055] Another aspect of the invention relates to a mistletoe lectin isolated and purified from a *Viscum* species other than *Viscum album* preferably from *Viscum ovalifolium*.

[0056] In various embodiments the mistletoe lectin from *Viscum ovalifolium* comprises, consists essentially of or consists of: (a) the amino acid sequence set forth in any one of SEQ ID Nos. 2; or (b) a variant amino acid sequence that shares at least 80 %, preferably at least 90 %, more preferably 95% sequence identity or sequence homologous to the amino acid

sequence of (a) over its entire length; or (c) an amino acid sequence that is a functional fragment of the amino acid sequence of (a) or (b).

[0057] Another aspect of the invention relates to a pharmaceutical composition comprising a mistletoe lectin isolated and purified from a *Viscum* species other than *Viscum album* preferably from *Viscum ovalifolium* as defined above and a carrier or diluent.

[0058] Mistletoe lectin produced according to the invention, can be administered for the treatment of viral infection in the form of pharmaceutical compositions. Alternatively, mistletoe lectin isolated and purified from a *Viscum* species other than *Viscum album* can be administered for the treatment of cancer.

[0059] Thus, the present invention also relates to compositions including pharmaceutical compositions comprising a therapeutically effective amount of the mistletoe lectin and a carrier. As used herein a compound will be therapeutically effective if it is able to inhibit viral entry into a host cell or inhibit viral protein synthesis via inhibition of 60S ribosomal subunit. A “therapeutically or prophylactically efficient amount” of a composition is as described above for a “therapeutically efficient amount” of a mistletoe lectin.

[0060] Pharmaceutical forms of the invention suitable for sterile injectable solutions for application may be prepared by incorporating the active ML compounds in the required amount in the appropriate solvent with several of the other ingredients enumerated herein, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above.

[0061] The carrier can be a solvent or dispersion medium containing, for example, water, ethariol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The carrier may be a serum acceptable

buffer. The proper fluidity can be maintained, for example, by the maintenance of dispersion and or by the use of surfactants. Preventing the action of microorganisms in the compositions of the invention is achieved by adding antibacterial and/or antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0062] The active ingredient may be held within a matrix which controls the release of the active agent. Preferably, the matrix comprises a substance selected from the group consisting of lipid, polyvinyl alcohol, polyvinyl acetate, polycaprolactone, poly(glycolic)acid, poly(lactic)acid, polycaprolactone, polylactic acid, polyanhydrides, polylactide-co-glycolides, polyamino acids, polyethylene oxide, acrylic terminated polyethylene oxide, polyamides, polyethylenes, polyacrylonitriles, polyphosphazenes, poly(ortho esters), sucrose acetate isobutyrate (S AIB), and combinations thereof and other polymers. Preferably, the matrix sustainably releases the agonist. The matrix may be a liposome.

[0063] Pharmaceutically acceptable carriers and/or diluents may also include any and all solvents, dispersion media, coatings, antibacterials and/or antifungals, isotonic and absorption delaying agents and the like. The carrier and/or diluents may be a serum acceptable buffer. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated.

[0064] "Treatment" and "treating" and synonyms thereof refer to therapeutic treatment wherein the object is to stop or reduce viral infection of a host cell. Preferably to at least affect viral infection of a host cell will stop or halt the viral invective or reduce (decrease) the

symptoms. Preferably the subject is a mammal, preferably a human. Those in need of such treatment include an individual or patient that has been diagnosed with, a viral infection. A patient or an individual refers to an animal, such as a mammal preferably a human. In various embodiments the cell is in vitro. In various other embodiments the cell is in vivo and the mistletoe lectin may be administered to a subject such as a patient or individual in need of treatment.

[0065] Preferably the mistletoe lectin pharmaceutical composition is administered to a subject is in a therapeutically effective amount. A therapeutically effective amount would be able to block viral entry or replication in a host cell in culture or in vivo. As used herein a "therapeutically effective amount" of the mistletoe lectin will be an amount that is capable of stopping or slowing viral infection of a host cell that may result in stopping or halting or reducing (decreasing) viral infection of a host cell. It may also refer to the prevention.

Dosages and administration of a compound of the invention in a pharmaceutical composition may be determined by one of ordinary skill in the art of clinical pharmacology or pharmacokinetics. An effective amount of the compound to be employed therapeutically, for example a polypeptide, will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the mammal. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 10 ng to up to 100 mg per infection or more per day, preferably about 1 μ g to 10 mg per infection. Doses may include protein amount any where in the range of 10 to 100 μ g or more preferably 25, 50, or 75 μ g per patient.

[0066] Another aspect of the invention relates to Use of a mistletoe lectin for the preparation of a medicament to treat a viral infection.

[0067] Preferably, the mistletoe lectins are as described herein.

[0068] The preparation of a medicament to treat a viral infection is preferably the preparation of a therapeutically or prophylactically efficient amount of a mistletoe lectin as described herein .

[0069] The present invention is further illustrated by the following examples. However, it should be understood, that the invention is not limited to the exemplified embodiments.

Examples of preferred embodiments

[0070] Isolation and purification of lectin from Singapore mistletoe *Viscum ovalifolium*

[0071] Plant material was collected from *Viscum ovalifolium* growing on *Ficus carica* as the host plant. Total protein was extracted from local mistletoe (*Viscum ovalifolium*) leaves and lectins were isolated and purified on a sugar affinity as previously described [27]. Briefly, mistletoe leaves were ground to a powder and mixed in 20 volumes of extraction buffer (100 mM potassium phosphate, 1 mM EDTA, 1% triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was homogenized and stirred at 4 °C overnight. After centrifugation at 10,000g for 20 minutes, the supernatant was subjected to two rounds of filtration; first with 11 μ m filter paper and subsequently with 0.2 μ m membrane filter. Ammonium sulphate was added to the filtrate to 70% saturation, and stirred at 4 °C overnight. The precipitate was dissolved in 1 x phosphate-buffered saline (PBS) and dialysed against the same buffer. Finally, the sample was applied to α -lactose agarose column (Sigma, St. L) and lectins were eluted with 0.1 M α -lactose. The purified lectins (SML) were stored at -80 °C until further use.

[0072] Identification of SML

[0073] To detect the two SML sub-units linked by disulfide bonds, two samples of purified SML were heated at 90 °C for 10 min in reducing (β -mercaptoethanol) and non-reducing gel loading buffers respectively. The protein samples were then resolved by SDS-PAGE, fixed and stained by Coomassie Brilliant Blue (Sigma Aldrich).

[0074] Approximately 0.22 mg of lectin was obtained from 20 g of frozen leaves. Analysis of the purified lectin on 10% SDS-PAGE with and without reducing agent confirmed that a single band migrates at 60 kDa under non-reducing conditions and two bands migrate at 32 kDa and 30 kDa under reducing conditions (Figure 1). The appearance of additional protein bands of smaller molecular sizes under reducing condition implies that the lectin consist of peptide chains that are linked by disulfide bonds. We named the purified lectin as Singapore Mistletoe Lectin (SML) and compared it with Korean Mistletoe Lectin (KML), which is a lectin isolated from *Viscum album colaratum*. Upon reduction, SML showed only two bands whereas KML showed four bands, suggesting that unlike KML, SML has only one type of isoform containing two peptide chains linked by disulfide bond(s).

[0075] The protein bands were excised from the gel and subjected to in-gel digestion overnight using trypsin. Part of the extracted tryptic peptides were analyzed using a 4800 MALDI-TOF/TOF-MS system (Applied Biosystems, Foster City, USA). The MS and MS/MS spectra were used to identify the (homolog) protein using Mascot by searching the spectra against SwissProt database.

[0076] To recover more amino acid sequence for the novel SML protein, the tryptic peptides from in-gel digestion were analyzed by a LTQ-FT Ultra LC-MS/MS system (Thermo Fisher, Bremen, Germany) as previously described (28). Briefly, peptides were separated and analyzed on a liquid chromatography (Dionex UltiMate 3000 Nano - LC Systems, Dionex, Singapore) at a 300 nl/min flow rate coupled to a LTQ-FT Ultra in a 60

min gradient. Peptides were then analyzed on LTQ-FT with an ADVANCE™ CaptiveSpray™ Source (Michrom BioResources) at an electrospray potential of 1.5 kV. A gas flow of 2, ion transfer tube temperature of 180 °C and collision gas pressure of 0.85 mTorr were used. The LTQ-FT was set to perform data acquisition in the positive ion mode in the m/z range of 350-1600 in the full MS scan. MS/MS spectrum was recorded in the ion trap with the default target value and a maximum accumulation time limit of 500ms.

[0077] The MS/MS spectra in the raw data were first extracted into the dta format using the extract_msn (version 4.0) in Bioworks Browser (version 3.3, Thermo Fisher Scientific, Inc.), and then the dta files were converted into Mascot generic file (mgf) format using an in-house program. Intensity values and fragment ion m/z ratios were not manipulated. The mgf file was searched against NCBI nr database using Mascot (version 2.2.07). The protein sequences of the identified homolog SML proteins were extracted from the NCBI nr database. Subsequently, the mgf file was re-analyzed using ProteinPilot v3 (Applied Biosystems, Foster City, USA) against the homolog SML proteins. In the ProteinPilot search, the amino acid substitutions was activated to allow searching a set of single amino acid substitutions using the BLOSUM 62 matrix. The Paragon Algorithm used in ProteinPilot can identify up to two substitutions per tryptic peptide. All identified peptides with >99% confidence were shortlisted for further analysis.

[0078] cDNA isolation encoding SML

[0079] Mistletoes living on oak trees were collected during Christmas time in Singapore farms and immediately frozen under liquid nitrogen and stored at -80 °C for long-term use. Total RNA of the mistletoe was isolated and purified by using the RNeasy Plant Mini Kit (QIAGEN, USA,). Reverse transcription of first-strand cDNA synthesis was carried out with 1 µg total RNA using the ImProm-IITM Reverse transcription system (Promega, USA). The

ImProm-IITM Reverse Transcriptase with Oligo (dT) 15 primer from Promega was used was used for RT-PCR (denaturation 70°C, 5 min; annealing 25°C, 5min; cDNA synthesis 42 °C, 60 min; termination 70°C, 5 min). The second-strand cDNA was amplified using a primer pair according to the conserved amino acid sequences from N-terminal A-chain and C-terminal B-chain of European mistletoe lectin, 5'- TAC GAG AGG CTA AGA CTC AG- 3' (from +154 to +173 of the European lectin) and 5'- TGG CAC GGG AAG CCA CAT TT- 3' (complimentary from +1727 to +1746 of the European lectin) (Nucleotide sequence data for European mistletoe lectin are available in the GenBank databases under the accession number A58957). The PCR amplification was run for 25 cycles with the same condition (denaturation at 94°C for 1 min, annealing at 65 °C for 1 min, extension at 72°C for 2 min). The amplified fragment was purified by Gel Extraction kit (QIAGEN, USA, Catalog number 28706). Once the PCR product was confirmed by sequencing and comparison with the amino acid sequence of the European mistletoe lectin (EML), the amplified cDNA was ligated into pUC18 vector (STRATAGENE, USA) and used for sub-cloning into other expression systems. cDNA was confirmed by DNA sequencing. Nucleotide sequence data for Singapore mistletoe lectin was deposited to GenBank with accession number JN085961.

[0080] cDNA translation and protein sequence alignment

[0081] To determine the percent homology between SML and other Ribosome inactivating proteins (RIPs), cDNA sequence of SML was translated into protein sequence using translate tool from Expasy Server (<http://au.expasy.org/tools/dna.html>). Multiple alignment analysis of protein sequence was made by using Vector NTI package and ClustalW. N-glycosylation sites in SML protein and other RIPs were predicted by using NetNGlyc 1.0 Server from Center for Biological Sequence Analysis, Technical University of Denmark.

[0082] De novo sequencing of SML

[0083] To further confirm the identity of SML, we performed mass spectrometric analysis on the purified protein. The MS results showed a significant hit with 74 matched peptides (95%) on β -galactoside specific lectin 3 from *Viscum album* (sp|P82683|ML3_VISAL). To determine the degree of homology between SML and EML, de novo sequencing of SML by MS was employed. Ten peptide fragments from SML were successfully sequenced. Comparison of SML N-terminal fragments from both A- and B-chain with their counterparts from EML revealed 80% amino acid sequence identity between N-termini of SML and EML. Based on the protein sequencing results, we have designed primers to clone cDNA coding for SML. The cDNA of SML confirmed that SML is highly homologous to KML and EML (Table 1), which confirmed the MS sequencing results as well. Given this high degree of sequence homology, structurally and functionally important sites are likely to be conserved as well. Thus, it is likely that SML has homologous receptors on cancer cells as EML. In addition, all the previously identified cysteines involved in intra- and inter-chain disulfide bonds are conserved and expected to form three intra-molecular and one inter-molecular disulfide bonds. Therefore, it is likely that SML, EML and KML share similar three-dimensional structural folds.

[0084] **Table 1.** Sequence comparison of SML-A and SMB-B subunits with other MLs

A-chain				
	SML-A	EML-A	KML-1A	HML-A
SML-A	100.00%	80.70%	82.30%	72.80%
EML-A	80.70%	100.00%	87.80%	85.80%
KML-A1	82.30%	87.80%	100.00%	79.50%
HML-A	72.80%	85.80%	79.50%	100%
B-chain				
	SMLB	EMLB	KML-1B	HMLB
SML-B	100.00%	80.20%	69.90%	74.50%
EML-B	80.20%	100%	71.80%	91.30%
KML-B1	69.90%	71.80%	100.00%	68.00%
HML-B	74.50%	91.30%	68.00%	100.00%

[0085] SML shows molecular characteristics similar to other MLs

[0086] Previous studies showed that lectins isolated from European and Korean mistletoe possess cytotoxicity towards various types of tumor cells but not normal cells [23, 27]. To investigate whether SML possess similar cytotoxic profiles in cancer cells, we treated several human cancer cell lines and one human non-cancerous cell line with increasing concentration of SML for 24 hours (Figure 2A) .

[0087] Cell lines

[0088] HeLa (cervical cancer), HCT116 (colon cancer), MCF-7/caspase-3 (+) (breast cancer), A549 (lung cancer), and WI38 (normal lung fibroblast) were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA) supplemented with 10% (v/v)

fetal bovine serum (Hyclone, USA), 2 mM L-glutamine (Invitrogen, USA) and 100 units/ml penicillin and streptomycin (Invitrogen, USA) at 37 °C under a humidified 5% CO₂ atmosphere.

[0089] Cell viability assay

[0090] Cells were seeded into 96-well plate at a density of 5 x 10³ cells per well. Various concentrations of SML ranging from 1 ng/ml to 1000 ng/ml were added after 24 hours of growth, and incubated for another 24 hours. After incubation, 20 µl of MTS solution (CellTiter96® Aqueous One Solution Cell Proliferation Assay from Promega, USA) was added, followed by 20 minutes incubation. Absorbance was measured at 490 nm using a microplate reader (BioRad, USA).

[0091] SML appeared to be cytotoxic towards all four cancer cell lines but not the normal cell line (Figure 2A). Although all of the four cancer cell lines were susceptible to SML, they showed varying degrees of sensitivity towards SML, with HeLa, a cervical cancer cell line, being the most sensitive towards SML.

[0092] SML induces apoptosis in tumor cells but not in normal cells

[0093] In our preceding experiments, we have showed that SML is cytotoxic against various tumor cells. So, we questioned whether cell death occurs as a result of apoptosis or necrosis.

[0094] HeLa cells were seeded into 6-well plate at a cell density of 2 x 10⁵ per well. On the subsequent day, they were treated with 10 ng/ml of SML and incubated for 12 hours. Cells were harvested by trypsinization, and stained with Annexin V-FLUOS and propidium iodide according to the manufacturer's instructions (Roche, Penzberg, Germany). Analysis was done by flow cytometer (BD Bioscience, San Jose, CA) using 488nm excitation, a 515

nm band pass filter for fluorescein detection, and a >600 nm band pass filter for propidium iodide detection.

[0095] When cancer cells are treated with 10 ng/ml of SML for 12 hours, the tumor cells undergo apoptotic cell death (Figure 3). On the other hand, we did not observe such morphological changes in WI38 when treated with the same concentration of SML, thus providing additional evidence that normal cells are not susceptible to SML (data not shown). This suggests that SML has selective cytotoxicity toward cancer cells.

[0096] Western blotting

[0097] Cells were lysed with lysis buffer (2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.002% bromophenol blue and 62.6 mM Tris-HCl, pH 6.8) by sonication followed by centrifugation at 10,000g at 4 °C for 15 minutes. The proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Proteins were probed with anti-caspase-3 (Cell Signalling Technology, USA) diluted at 1:1000 dilution in 1% skim milk and 0.5% Tween-20 in PBS, anti- β -actin at 1:10000 in 1% skim milk and 0.5% Tween-20 in PBS. Following primary antibody incubation, the membrane was washed three times with PBS containing 0.5% Tween-20 and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, USA) at room temperature for 1 hour followed by three washes with PBS, and detection was made with SUPREX Western blotting detection kit (Neuronex, Korea).

[0098] Ribosome-inactivating activity assay

[0099] Lectins are also known as type 2 ribosome-inactivating proteins as they are inactivate eukaryotic ribosomes' by preventing the 60S subunit from binding to elongation factor 2 [38]. In support of this, studies have shown that both EML and KML possess ribosome-inactivating activity [25, 39]. In our preliminary study, to assess the ribosome-

inactivating activity of SML, we used a cell-free rabbit reticulocyte lysate assay. A cell-free, non-radioactive assay for ribosome-inactivating activity using rabbit reticulocyte lysate in vitro translation system (Flexi rabbit reticulocyte lysate system, Promega, USA) was performed according to the manufacturer's instruction. Briefly, different concentrations of SML was added to a reaction mixture and incubated at 30 °C for 90 minutes. The reaction mixture was mixed with luciferase assay reagent (LAR) and luminescence intensity was measured with a luminometer (Tecan, Switzerland).

[00100] We demonstrated that SML inhibits translation in dose-dependent manner with an IC₅₀ of 1 ng/ml (Figure 2B), suggesting that SML shares a similar ribosome-inactivating activity with other MLs and ultimately contributes to the cytotoxic effects on the cancer cells (Figure 4B).

[00101] MLs Inhibit DV infection

[00102] While ML-mediated anticancer effects have been extensively investigated, currently, to our knowledge, the antiviral effects of ML have not examined. In this study, the inhibitory effects of the purified KML and SML on DV replication in C6/36 cells were tested.

[00103] Dengue virus replication assay

[00104] DV1, DV3 and DV4 stocks (10⁷ plaque-forming units per ml) were mixed with SML or KML at the final concentrations of 15 μM and 20 μM, respectively, and incubated room temperature for 30 min. The pre-treated viruses were then used to infect C6/36 cells grown on 96-well plates. The infection was performed at 37 °C for the first 2 h to allow adsorption of viruses to the cells, followed by incubation at 28 °C for 48 h. The cells were then fixed and subjected to immunofluorescent staining with anti-DV NS3 antibodies,

[00105] As shown in Figure 4, in cells without pre-treatment with KML, positive staining was observed in almost all cells, indicating efficient infection of cells by the three serotypes

of DV tested. In cells pretreated with 15 and 25 μ M of KML, positive staining was also observed in a very small proportion of cells, suggesting strongly inhibition of DV infection by this protein.

[00106] To exclude the possibility that KML may directly regulate cell metabolism and render an indirect effect on viral replication, C6/36 cells were pretreated with KML at the final concentration of 15 and 20 nM, respectively, for 16 h, then infected with DV1. The results showed there was no significant difference between the treated and non-treated cells for the viral protein production (Figure 4). It was noted that 20 nM of KML may have minor cytotoxicity to cells, as slightly reduced cell growth and viability of the pre-treated cells were observed (Figure 4).

[00107] Similarly, the inhibitory effect of SML on DV replication was investigated, showing strong inhibition of all four serotypes of DV replication in C6/36 cells. Comparing to KML, however, it appears that SML showed slightly weaker inhibitory effects. As can be seen in Figure 5, pre-treatment of virus stocks with 15 and 25 nM of SML reduced the viral replication to approximately 25 and 10 %, respectively, for all four serotypes of DV.

[00108] Immuno-coprecipitation experiments

[00109] 1 mg/ml of purified SML was labeled in Phosphate buffer saline pH7.4 by NHS-LC- Biotin purchased from Thermo Scientific. The biotinylation process was followed by company instruction manual (EZ-link Sulfo-NHS-LC Biotinylation kit, Catalog Number 21435, Thermo Scientific). The biotinylated SML at 100 nM concentration were incubated with 500 μ l Dengue-infected C6/36 cell lysate after 72 hours post-propagation. The mixture was incubated at room temperature for 5 hours and followed the binding step with 30 μ l of Streptavidin agarose (Novagen, USA). 500 μ l of non-infected C6/36 cell lysate as a negative control was followed the same procedure for pull-down assay with the biotinylated SML and

mixing with 30 μ l of Streptavidin agarose. The protein-protein complex was precipitated by centrifuging at 13,000 $\times$ g for 5 min. The unbound proteins were washed out with three times PBS buffer pH 7.4. The final proteins with the beads were eluted with 20 μ l of 1 $\times$ SDS Loading dye. The E protein band was detected by Western blotting and probed with Rabbit anti-E protein (1 to 10,000) dilution. HRP-conjugated anti-rabbit in 5000 dilution was used as a secondary antibody.

[00110] Binding of SML to Dengue serotype 2 envelope protein (E)

[00111] Among the 10 confirmed DV-encoded proteins, E protein plays a key role in the initial stage of DV infection by mediating fusion of the membrane of the endocytosed DV particles with the endosomal membrane, following binding of the virus to cellular receptor(s) on the cell surface and viral entry into cells. In addition, E is the major antigen for induction of neutralizing antibodies. The protein is known to be posttranslationally modified by glycosylation at two putative positions (Asn 67 and Asn 153). Because the specific nature of SML B-chain (lectin domain) can bind to sugar on the target cell surface receptors, it is assumed that this lectin B-chain might be relevant to the interactions with Dengue E protein and capable to block the virus attachment process via binding the N-linked glycans on virus protein. In order to prove this hypothesis, initial pull-down assay was conducted by immunoprecipitating SML protein with Dengue envelope from the mosquito-derived virus. Due to the high efficiency and available, we prefer to use Dengue virus serotype 2 for our further interest study. Dengue virus serotype 2 was propagated in mosquito C6/36 cell line within 72 hours in order to recognize the typical infectious phenotype. Insect cell lysate containing virus particles was incubated with biotinylated-SML protein at 100 nM concentration. The protein complex was precipitated by streptavidin agarose which have high affinity to the vitamin biotin. Following SDS-PAGE and Western blotting, the precipitated proteins were

detected by using anti-E antibodies. The E protein-recognizing band was detected in cell lysate pre-incubated with 100 nM SML, but not in either infected or non-infected cell lysate without prior incubation with SML (Figure 6). It is more reasonable to see no detectable E band with molecular weight about 48,000 in the sample loading only the biotinylated SML-bound streptavidine agarose (first lane from the left to right, Figure 6). The mosquito C6/36 cell lysate was loaded as positive control to detect Dengue envelope protein (last lane from left to right, Figure 6). Our preliminary result supports the idea that the mechanism for inhibition of Dengue viral infection might depend on the direct protein-protein interaction between SML with Dengue envelope protein.

[00112] By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[00113] By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

[00114] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional

features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00115] By "about" in relation to a given numerical value, such as for temperature and period of time, it is meant to include numerical values within 10% of the specified value.

[00116] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00117] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

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Claims

1. Method for the treatment or prevention of a viral infection in a subject, the method comprising administering to said subject a mistletoe lectin in a therapeutically or prophylactically efficient amount.
2. The method of claim 1, wherein the mistletoe lectin is a *Viscum* species lectin.
3. The method of claim 1 wherein the mistletoe lectin is isolated and purified from a *Viscum* species.
4. The method of claim 2 or 3, wherein the *Viscum* species is selected from *Viscum album*, *Viscum album* var. *coloratum* or *Viscum ovalifolium*.
5. The method of any one of claims 1 to 4, wherein the mistletoe lectin comprises, consists essentially of or consists of:
 - (a) an amino acid sequence set forth in any one of SEQ ID Nos. 2 or 4; or
 - (b) a variant of the amino acid sequence of (a) that shares at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity or sequence homology with the amino acid sequence of (a) over its entire length; or
 - (c) an amino acid sequence that is a functional fragment of the amino acid sequence of (a) or (b).
6. The method of any one of claims 1 to 5, wherein the mistletoe lectin is encoded by
 - (a) a nucleic acid sequence set forth in SEQ ID NO: 1 or 3; or
 - (b) a variant of the nucleic acid sequence of (a) that shares at least 80 %, preferably at least 90 %, more preferably at least 95% sequence identity or sequence homology with the expression product of a nucleic acid sequence of (a) and encodes a mistletoe lectin that shares at least 80 %, preferably at

least 90 %, more preferably at least 95 % sequence identity or sequence homology with the product encoded by the nucleic acid sequence of (a) over its entire length; or

(c) a fragment of the nucleic acid sequence of (a) or (b) that encodes a functional mistletoe lectin fragment.

7. The method of any one of claims 1 to 6, wherein the virus is an RNA virus.
8. The method of any one of claims 1 to 7, wherein the virus is a single stranded positive RNA virus.
9. The method of any one of claims 1 to 8, wherein the virus is a flavivirus.
10. The method of any one of claims 1 to 9, wherein the virus is an arbovirus.
11. The method of any one of claims 1 to 10, wherein the virus is a dengue virus.
12. The method of any one of claims 1 to 11, wherein the subject is a mammal, preferably a human.
13. A mistletoe lectin isolated and purified from a *Viscum* species other than *Viscum album* preferably from *Viscum ovalifolium* comprising, consisting essentially of or consisting of:
 - (a) an amino acid sequence set forth in any one of SEQ ID Nos. 2; or
 - (b) a variant amino acid sequence that shares at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity or sequence homology with the amino acid sequence of (a) over its entire length; or
 - (c) an amino acid sequence that is a functional fragment of the amino acid sequence of (a) or (b).
14. A pharmaceutical composition comprising a mistletoe lectin according to claim 14 and a carrier or diluent.