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(54) **PEPTIDE CHIMERIC MOLECULES HAVING ANTIVIRAL PROPERTIES AGAINST VIRUSES OF THE FLAVIVIRIDAE FAMILY**

(57) The present invention is relative to chimerical peptides, whose primary structure holds at least one segment which inhibits the activation of the NS3 protease of a virus from the *Flaviviridae* family, they also contain a cell penetrating segment and they are capable of inhibiting or attenuate the viral infection. This invention is also relative to pharmaceutical compounds which contain these chimerical peptides for the prevention and/or treatment of the infection caused by a virus of the *Flaviviridae* family.

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Description

FIELD OF THE INVENTION

[0001] The present invention is relative to the pharmaceutical industry, more specifically to chimerical peptides, whose primary structure holds at least a segment which inhibits the NS3 protease of a virus from the *Flaviviridae* family, they also includes a cell penetrating segment and they are capable to inhibit or attenuate the viral infection. The invention is also relative to pharmaceutical compounds which contain the chimerical peptides for the prevention and/or treatment of the infection caused by viruses from the *Flaviviridae* family.

DESCRIPTIVE MEMORY

[0002] The *Flaviviridae* family is constituted by enveloped positive single-stranded RNA viruses, which belong to one of three genera: *Flavivirus*, *Hepacivirus* and *Pestivirus.* The *Flavivirus* genus includes more than 70 virus, many of them cause relevant diseases in humans and in other species. Members of this genus are the Yellow Fever Virus (YFV), Dengue Virus (DV), Japanese Encephalitis Virus (JEV), Tick-borne Encephalitis Virus (TBE), West-Nile Virus (WNV), St. Louis Encephalitis Virus (SLEV) and others. The Hepatitis C virus (HCV) is the prototype of the *Hepacivirus.* As members of the genus *Pestivirus* we find the Bovine Viral Diarrhea virus (BVDV), the Classical Swine Fever virus (CSFV), Border Disease virus (BDV) and others.

[0003] The viruses belonging to different genera of the *Flaviviridae* family, do not display antigenic cross-reactivity and show diverse biological properties, however they show evident similarities in aspects such as virion morphology, genome organization and the replication strategy (Leyssen, P., De Clercq, E., Neyts, J. 2000 Perspectives for the treatment of infections with Flaviviridae. Clin Microbiol Rev., 3: 67-82; Rice, C. M. 1996. Flaviviridae: the viruses and their replication, p. 931-960. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa.; Westaway, E. G. 1987. Flavivirus replication strategy. Adv. Virus Res.33:45-90)

[0004] The HCV constitutes an important health problem worldwide. According to the WHO about 3% of the world population has been infected by the virus, indicating that more than 170 millions of chronic carriers are in risk of developing cirrhosis and/or liver cancer (Consensus Panel. EASL International Consensus Conference on Hepatitis C, Paris, 26-28 February 1999, Consensus Statement. J. Hepatol., 1999, 30, 956). Every year about 3-4 millions new infections by HCV arise worldwide (Tan, S. L., Pause, A., Shi, Y. & Sonenberg, N. (2002) Nat. Rev. Drug Discov. 1,867-881). At least 85% of the infected patients evolves to a chronic infection (Alter, M. J., E. E. Mast, L. A. Moyer, and H. S. Margolis. 1998. Hepatitis C. Infect. Dis. Clin. North Am. 12:13-26). The chronic Hepatitis C frequently ends in cirrhosis and/or cancer, whether it is symptomatic or asymptomatic. Follow-up studies carried out for 10-20 years show development of cirrhosis in 20-30 % of the patients, and 1-5% of these patients could develop cancer in the following 10 years (Dutta, U., J. Kench, K. Byth, M. H. Khan, R. Lin, C. Liddle, and G. C.Farrell. 1998. Hepatocellular proliferation and development of hepatocellular carcinoma: a case-control study in chronic hepatitis C. Hum. Pathol. 29:1279-1284; Pontisso, P., C. Belluco, R. Bertorelle, L. De Moliner, L. Chieco Bianchi, D. Nitti, M. Lise, and A. Alberti. 1998. Hepatitis C virus infection associated with human hepatocellular carcinoma: lack of correlation with p53 abnormalities in Caucasian patients. Cancer 83:1489-1494). It is estimated that the number of deaths per year caused by HCV in the United States can reach 35000 by the year 2008 (Dutta, U., J. Kench, K. Byth, M. H. Khan, R. Lin, C. Liddle, and G. C.Farrell. 1998. Hepatocellular proliferation and development of hepatocellular carcinoma: a case-control study in chronic hepatitis C. Hum. Pathol. 29: 1279-1284; Pontisso, P., C. Belluco, R. Bertorelle, L. De Moliner, L. Chieco Bianchi, D. Nitti, M. Lise, and A. Alberti. 1998. Hepatitis C virus infection associated with human hepatocellular carcinoma: lack of correlation with p53 abnormalities in Caucasian patients. Cancer 83:1489-1494.).

[0005] Currently, the anti-HCV treatments approved by the FDA are the interferon monotherapy and the interferon-ribavirin combined therapy (Dymock, B. W. Emerging Drugs 2001, 6(1), 13 *and references within).* Recently, the use of pegylated interferon variants have been approved, which increase the therapeutic efficacy of these treatments, but they are still far from being ideals. Because of the seriousness of this disease, new and more effective treatments are needed.

[0006] The *Flavivirus* infecting human beings are transmitted by arthropods such as ticks and mosquitoes, which makes these diseases so difficult to eradicate (Monath, T. P., and F. X. Heinz. 1996. Flaviviruses, p. 961-1034. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa.). The Yellow Fever is still an important cause of hemorrhagic fever, with mortality rates as high as 50%, although a vaccine is already available.

[0007] The Dengue Virus is pandemic in tropical areas and its re-emergence is an increasing public health problem worldwide. It is estimated that annually occur approximately 100 million infections by dengue virus and 2.5 milliard persons live in endemic areas (Gubler, D.J. 1998. Clin. Microbiol. Rev. 11, 480-496.; Monath, T.P. (1994) Proc. Natl. Acad. Sci USA 91, 2395-2400.) During the period 1990-1998, the average number of Dengue Hemorrhagic Fever (DHF) cases reported to the WHO was 514 139 per year, including 15 000 deaths, although it is considered that the real burden

of the disease is some times higher. However, neither vaccines nor specific antiviral treatments are commercially available. The dengue virus complex is conformed by four distinct viruses or serotypes (VD1-VD4), which are related genetically and antigenically. The DV is transmitted to humans by the mosquitoes, mainly by the *Aedes aegypti.* The infection causes diverse clinical manifestations varying from asymptomatic and benign to an undifferentiated febrile illness or more severe manifestations like the DHF and the potentially lethal Dengue Shock Syndrome (DSS). The most severe clinical manifestations are frequently associated to sequential infections with two different serotypes *(*Halstead, S.B. Neutralization and antibody-dependent enhancement of dengue viruses. Adv. Virus Res. 60:421-67., 421-467, 2003. Hammon WMc. New haemorragic fever in children in the Philippines and Thailand. Trans Assoc Physicians 1960; 73: 140-155*).* Epidemiological studies have been carried out showing evidences of sequential infection with different serotypes as a risk factor for severe disease *(*Halstead, S.B. Neutralization and antibody-dependent enhancement of dengue viruses. Adv. Virus Res. 60:421-67., 421-467, 2003. Hammon WMc. New haemorragic fever in children in the Philippines and Thailand. Trans Assoc Physicians 1960; 73: 140-155*).* This phenomena has been explained through the theory of the "antibody dependent enhancement (ADE)", which postulates that an increase in infectivity is associated with a more efficient cell entry of the virus mediated by FC receptor of the infected cells *(*Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science 1988; 239: 476-481*).*

[0008] Other *Flavivirus* is the JEV, which is the main cause of viral encephalitis worldwide. About 50000 cases occur annually in Asia with a high mortality rate of 30% and causing long lasting neurological disorders in 30% of cases (Kalita, J., and U. K. Misra. 1998. EEG in Japanese encephalitis: a clinicoradiological correlation. Electroencephalogr. Clin. Neurophysiol. 106:238-243*.;* Kaluzova, M., E. Eleckova, E. Zuffova, J. Pastorek, S. Kaluz, O. Kozuch, and M. Labuda. 1994. Reverted virulence of attenuated tick-borne encephalitis virus mutant is not accompanied with the changes in deduced viral envelope protein amino acid sequence. Acta Virol. 38:133-140*).*

[0009] Severe encephalitis is also caused by other *Flaviviruses* like the TBEV, being two subtypes of this virus: the eastern type with an associated mortality of 20% and the western type with 1-2% (Heinz, F. X., and C. W. Mandl. 1993. The molecular biology of tick-borne encephalitis virus. APMIS 101:735-745.); the Murray Valley Encephalitis (MVE) in Australia (Mackenzie, J. S., and A. K. Broom. 1995. Australian X disease, Murray Valley encephalitis and the French connection. Vet. Microbiol. 46:79-90); the SLEV in the western United States and the WNV, which is endemic in Africa, Middle East and the Mediterranean and has also caused recent outbreaks in the United States. Since it appeared in the United States in 1999, it has expanded very fast, infecting about 15 000 persons and leading to more than 600 deaths. However, currently there are not available vaccines or drugs which protect against the WNV (van der Meulen, K. M., Pensaert, M. B. and Nauwynck, H. J. (2005) West Nile virus in the vertebrate world. Arch. Virol. 150, 637-657).

[0010] Hemorrhagic manifestations are caused by other *Flaviviruses* like the Omsk Hemorrhagic Fever Virus (OHFV) in Russia, with a lethality rate between 0.5-3% and the Kyasanur Forest Disease Virus (KFDV) in India (Monath, T. P., and F. X. Heinz. 1996. Flaviviruses, p. 961-1034. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa*.).*

[0011] Other *Flavivirus,* the Louping ill virus (LIV) infects mainly sheep, although occasional human infections have also been reported (Davidson, M. M., H. Williams, and J. A. Macleod. 1991. Louping ill in man: a forgotten disease. J. Infect. 23:241-249).

[0012] The *Pestivirus* BVDV, CSFV and BDV cause important diseases in animals. In their respective hosts the cause severe affections which usually lead to death, although these viruses can cross species causing a milder disease in other hosts. Frequently, infections occur by oronasal or transplacental route. The latter is responsible of persistent infections which are a threat for the rest of the livestock (Edwards, S., P. M. Roehe, and G. Ibata. 1995. Comparative studies of border disease and closely related virus infections in experimental pigs and sheep. Br. Vet. J. 151:181-187*.).*

[0013] It is presumed that the members of the *Flaviviridae* family share a similar replication strategy. The viral replication cycle begins with the adhesion of the virus to the host cell surface. In the case of Dengue virus, it has been shown that the virus binds to glycosaminoglycans, which could be the initial site of interaction with the cells. It has been also shown that the virus binds to DC-SIGN, although it is likely that the role of these molecules is related to the viral concentration on the cell surface or in the spread of the virus to secondary replication sites in vivo. After the initial binding, the virus interacts with high affinity receptors and/or co-receptors, which mediate the virus entry into the cell by endocytosis. In the case of the WNV, it has been postulated that the $\alpha_v\beta_3$ integrin could serve to these means (Chu, J. J-H., and Ng, M.-L., 2004. Interaction of West Nile Virus with αvβ3 Integrin Mediates Virus Entry into Cells. J. Biol. Chem 279, 54533-54541). It has also been shown that the HCV binds to the cellular receptor CD81 *(*Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani. 1998. Binding of hepatitis C virus to CD81. Science 282:938-941*).* Once the virus is localized in the endocytic compartments, a drop in the compartment pH induces the fusion process between the viral and the cell membrane, and this process is mediated by structural changes of the fusion protein of the virus envelope. This process leads to the discharge of the virus capsid into the cytoplasm, where the viral RNA is later released.

[0014] In the cytoplasm the genomic RNA of the virus, interacts through its non-coding 5' region (5'UTR) with the ribosome, leading to the translation of the virus unique open reading frame. This way, the precursor viral polyprotein is

synthesized, which in the case of *Flaviviruses* includes three structural proteins (C, preM and E) and five non structural proteins (NS1-5). This polyprotein is then modified co- and post-translationally giving rise to the individual mature functional proteins of the virus. The RNA-dependent RNA-polymerase of the virus with associated cofactors produces copies of negative-single stranded RNA, which are later used as templates for the synthesis of the genomic positive-single stranded viral RNA. The viral proteins participating in the replication are associated to membranous structures apparently related to the endoplasmic reticulum (ER).

[0015] After the replication is completed, the genomic RNA associates with the nucleocapsid, the immature virions bud into the lumen of the ER (budding occurs at the membrane of the ER or related membranous structures induced by the virus), covered by a lipid envelope containing viral proteins. Passing through the exocytic pathway, the envelope proteins are glycosilated and become mature, leading to the final release of the mature virions to the extracellular space.

[0016] The replication of the *Flaviviriae* requires the NS3pro protease (localized approximately in the first 180 residues of the non structural protein NS3) for the correct processing of the precursor polyprotein, this way constituting an attractive potential target for the design of antiviral drugs (Chappell, K. J., Nall, T. A., Stoermer, M. J., Fang, N. X., Tyndall, J. D., Fairlie, D. P. and Young, P. R. (2005) Site-directed mutagenesis and kinetic studies of the West Nile Virus NS3 protease identify key enzyme-substrate interactions. J. Biol. Chem. 280, 2896-2903. SHIRYAEV, S.A., RATNIKOV, B.I., CHEKANOV, A.V., SIKORA, S., ROZANOV, D. V., GODZIK,A., WHANG, J., SMITH, J.W., HUANG, Z., LINDBERG, I., SAMUEL, M.A., DIAMOND, M.S. and Alex Y. STRONGIN, A.Y., 2006. Cleavage targets and the D-arginine-based inhibitors of the West Nile virus NS3 processing proteinase. Biochem. J. 393, 503-511. Kolykhalov, A. A.; Mihalik, K.; Feinstone, S. M.; Rice, C. M. J. Virol. 2000, 74, 2046; Bartenschlager,R.; Lohmann, V. J. Gen. Virol. 2000, 81, 1631. Matusan, A. E., Kelley, P. G., Pryor, M. J., Whisstock, J. C., Davidson, A. D. and Wright, P. J. (2001) J. Gen. Virol. 82, 1647-1656).

[0017] In *Flavivirus* this protease is responsible for the proteolytic cleavage at the junctions NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4N/NS5, as well as the internal cleavage in C, NS3 and NS4A (Chambers, T. J., Nestorowicz, A., Amberg, S. M. and Rice, C. M. (1993) Mutagenesis of the yellow fever virus NS2B protein: effects on proteolytic processing, NS2B-NS3 complex formation, and viral replication. J. Virol. 67, 6797-6807. Jan, L. R., Yang, C. S., Trent, D. W., Falgout, B. and Lai, C. J. (1995) Processing of Japanese encephalitis virus non-structural proteins: NS2B-NS3 complex and heterologous proteases. J. Gen. Virol. 76, 573-580. Lobigs, M. (1993) Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. Proc. Natl. Acad. Sci. U.S.A. 90, 6218-6222. Yamshchikov, V. F. and Compans, R. W. (1994) Processing of the intracellular form of the west Nile virus capsid protein by the viral NS2B-NS3 protease: an in vitro study. J. Virol. 68, 5765-5771).

[0018] In HCV, NS3pro mediates the proteolytic processing of the viral polyprotein in the segment comprised between the proteins NS2-NS5B (R. Bartenschlager, 1999, The NS3/4A proteinase of the hepatitis C virus: unravelling structure and function of an unusual enzyme and a prime target for antiviral therapy. J. Viral Hepat. 6, 165-).

[0019] Besides the central role played by the NS3pro protease in the viral replication cycle processing the virus proteins, this protein can also process cellular substrates and hence it could be involved in various mechanisms of cellular damage and pathogenesis (Shiryaev, S. A., Ratnikov, B. I., Chekanov, A. V., Sikora, S., Rozanov, D. V., Godzik, A., Wang, J., Smith, J. W, Huang, Z., Lindberg, I., Samuel, M. A., Diamond, M. S. and Strongin, A. Y. (2005) The cleavage targets and the (D)-arginine-based inhibitors of the West Nile virus NS3 processing proteinase. Biochem. J. 393, 503-511).

[0020] Thus, it has been shown that the NS3 protease from WNV produces proteolytic cleavage in neuronal myelin basic protein (MBP). Regarding DV and WNV, it has been suggested that NS3 is involved in the induction of virus mediated apoptosis (Ramanathan, M. P., Chambers, J. A., Pankhong, P., Chattergoon, M., Attatippaholkun, W., Dang, K., Shah, N. and Weiner, D. B. (2005) Virology *doi:10/1016/j. virol. 2005.08.043)*

[0021] For its optimal function, the NS3 protease needs to interact with other viral protein or cofactor, the NS2B protein in *Flavivirus* and NS4A in *Hepacivirus* and *Pestivirus.* In DV the presence of NS2B induces an increase in the proteolytic activity of NS3 between 3300 and 6600 times (Yusof, R., Clum, S., Wetzel, M., Murthy, H.M. & Padmanabhan, R., 2000. J. Biol. Chem. 275, 9963-9969).

[0022] In HCV, NS3 binding to NS4A is required for the proteolytic cleavage at NS3/4A, NS4A/B and NS4B/5A and it increases the efficiency of the processing at the junction NS5A/B (Bartenschlager R, Ahlborn LL, Mous J, Jacobsen H. Kinetic and structural analyses of hepatitis C virus polyprotein processing. J Virol 1994; 6: 5045-5055. Failla C, Tomei L, De Francesco R. Both NS3 and NS4A are required for proteolytic processing of hepatitis c virus nonstructural proteins. J Virol 1994; 6: 3753-3760. Lin C, Pragai BM, Grakoui A, Xu J, Rice CM. Hepatitis C virus NS3 serine proteinase: trans-cleavage requirements and processing kinetics. J Virol 1994; 6: 8147-8157. Tanji Y, Hijikata M, Satoh S, Kaneko T, Shimotohno K. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J Virol 1995; 6: 1575-1581). The addition of a NS4A fragment to NS3pro in a 10 times molar excess increases the catalytic efficiency coefficient K_{cat}/K_m in aproximately 40 times (SHIMIZU, Y., YAMAJI, K., MASUHO, Y., YOKOTA, T., INOUE, H., SUDO, K., SATOH, S. y SHIMOTOHNO, K. 1996. Identification of the Sequence on NS4A Required for Enhanced Cleavage of the NS5A/5B Site by Hepatitis C Virus NS3 Protease. J. Virol 70, 127-132).

[0023] The crystal structures of NS3pro and the NS3pro-NS2B complex from DV and the complex formed by NS3pro-NS2B from WNV with a peptide inhibitor have been experimentally determined (Murthy, H.M., Clum, S. & Padmanabhan,

R., 1999. J. Biol. Chem. 274, 5573-5580. Murthy, H.M., Judge, K., DeLucas, L. & Padmanabhan, R., 2000. J. Mol. Biol. 301, 759-767. Erbel P, Schiering N, D'Arcy A, Renatus M, Kroemer M, Lim SP, Yin Z, Keller TH, Vasudevan SG, Hommel U., 2006. Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. Nat. Struct Mol. Biol.).

[0024] Similarly, the crystal structures of NS3pro and the complex NS3pro/NS4A from HCV have also been determined (Love, R.A., Parge, H.E., Wickersham, J.A., Hostomsky, Z., Habuka, N., Moomaw, E,W., Adachi, T., Hostomska, Z., 1996. The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. Cell. 87, 331-342. Kim, J.L., Morgenstern, K.A., Lin, C., Fox, T., Dwyer, M.D., Landro, J.A., Chambers, S.P., Markland, W., Lepre, C.A., O'Malley, E.T., Harbeson, S.L., Rice, C.M., Murcko, M.A., Caron, P.R., Thomson, J.A., 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell. 87, 343-535. Erratum in: Cell, 89:159, 1997).

[0025] The NS3pro protease adopts a chymotrypsin-like fold, which comprises two beta barrels and the His51-Asp75-Ser135 catalytic triad being localized in a crevice created between these domains. The binding of NS2B protein induces large changes in the tridimensional structure of NS3pro, affecting both the N- and C-terminal domains and comprising changes in the location and extend of the secondary structural segments.

[0026] The structure of the complex formed by the NS3pro-NS2B active protease with a peptide inhibitor shows that NS2B forms a belt around NS3pro, adopting a mainly extended structure and including five beta strands.

[0027] The first three strand are associated to beta strands from NS3 protein: the strand Trp53-Ala58 (WNV numbering) runs antiparallelly to the NS3 beta strand Gly21-Met26 corresponding to the N-terminal beta barrel and the beta strands Glu67-Ile68 and Arg74-Asp76 are parallel to the beta strands B2a y B2b of the NS3 C-terminal beta barrel.

[0028] The strands 4 and 5 form a beta hairpin, which interacts with the substrate binding site contacting the E1b-F1 loop from the N-terminal beta barrel. The folding of NS2B below the E2b-F2 beta hairpin of the C-terminal barrel induces a conformational change in this region of NS3 which leads to the arrangement of residues important for substrate recognition (Gly151, Gly153 y Tyr161). The residue Tyr161 makes pi-cation interactions with the arginine at P1 position. The negative electrostatic potential associated to the main chain carbonyl groups of residues Asp82-Gly83 and the atom Od1 of Asn84 from NS2B makes it favorable the interaction with the positive charge of the guanidinium group from the arginine at position P2. This way, they contribute to the conformation of the S2 site. Thus, the NS2B binding to NS3 completes essential elements of the enzyme active site and it also contributes to the thermodynamic stability of protein folding. These facts offer a structural basis for understanding the activation process of this protease.

[0029] In the case of HCV, NS3 activation is mediated by the binding of the beta strand Thr20-Leu31 from NS4A, which is structurally equivalent to strand 1 of NS2B from *Flavivirus (*SHIMIZU, Y., YAMAJI, K., MASUHO, Y., YOKOTA, T., INOUE, H., SUDO, K., SATOH, S. y SHIMOTOHNO, K. 1996. Identification of the Sequence on NS4A Required for Enhanced Cleavage of the NS5A/5B Site by Hepatitis C Virus NS3 Protease. J. Virol 70, 127-132*).*

[0030] Among the current approaches being carried out to obtain antiviral molecules against *Flaviviridae,* those based on NS3 inhibition are focused mainly in developing inhibitors targeting the active site. These approaches seem to be very promising, which is supported by recent results achieved in the development of drugs against HCV. However, these experiences have also shown clearly the difficulties inherent to these approaches. One of the more prominent is the generation of escape mutants. The polymerases of RNA viruses have relatively low fidelity and in the case of HCV it introduces a mutant per copy of the virus genome. It results in the fact that molecules developed by this means although they are very potent they could have a limited useful lifetime. It has lead to the introduction of therapeutic interventions based on cocktails of drugs as a need for antiviral treatments. It has also been observed that escape mutants raised against one drug can frequently escape the antiviral activity of other drugs targeting the same active site.

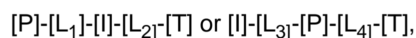
[0031] The present invention describes novel methods aimed to the design of antiviral agents against *Flaviviridae,* which are based in the concept of inhibition of the NS3 protease activation process. The key approach of this concept is the design of peptidic molecules and/or drugs capable of blocking the interaction between NS3 and its cofactor (NS2B or NS4A), and hence being able to interfere with the correct folding of the active NS3 protease. Such molecules are capable to bind to regions of the NS3 protease which are involved in the interaction with the cofactor, and compete with it and/or stabilize the structure of the inactive protease.

[0032] An advantage of this invention is that the probability of generation of escape mutants against these molecules is expected to be lower compared to those inhibitors of the active protease which compete with the substrate for the active site. The molecules of the present invention bind to binding sites in NS3 which are involved in protein-protein interactions essential for the viral replication cycle, therefore mutations generated in these regions of NS3 should have additional compensatory mutations in the cofactor.

[0033] Other advantage is the high specificity of the inhibitory activity displayed by these molecules. It is due to the fact that its binding sites on NS3 are essentially specific for the viral protease and they are not present on the host serine proteases. Furthermore, the host serine proteases have active sites showing specificities very similar to NS3 and hence they could be potential targets for toxicity of active site blocking drugs.

[0034] In the present invention we describe chimerical peptidic molecules which inhibit infections by *Flaviviridae,* and

whose primary structure can be described according the following formula:



$$[P]-[L_1]-[I]-[L_{2]}-[T] \text{ or } [I]-[L_{3]}-[P]-[L_{4]}-[T],$$

where, [P] is the amino acid sequence of a "cell penetrating peptide", typically of 10-30 amino acids, which have the capacity to allow the internalization of the whole peptidic molecule into the cell cytoplasm and to get access to the contiguity of the rough endoplasmic reticulum (RER); [L1, L2, L3, L4], are linker sequences of 0-6 residues; [I], is a NS3pro activation inhibitor sequence, containing residues which make contacts with at least one amino acid from the beta strands B2a and B2b of the C-terminal beta barrel, or from the beta strand A1 of the N-terminal beta barrel of the NS3pro protein from *Flavivirus* (or the corresponding structurally equivalent regions of *Pestivirus* or *Hepacivirus*) in its active or inactive conformation; [T], amino acid sequence between 0 and 10 residues, which is typically one or two signals of retention in the ER (like the sequences KDEL, KKXX and LRRRRL), or the sequence XRR with the capability to bind the P1 and P2 substrate binding sites of the NS3pro protease of *Flavivirus.*

[0035] More specifically, we have shown that peptides which have been designed according to the present invention are capable of inhibit the viral infection by DV.

Cationic cell penetrating peptides

[0036] The present invention describes the design of chimerical peptides which are capable to inhibit the viral infection of viruses from the *Flaviviridae* family. The designed peptides contain an [I] segment, which inhibit the activation of the viral NS3pro protease. However, in this invention we show that synthetic peptides with amino acid sequences corresponding to the segment [I] are not capable to penetrate the target cells and hence they do not inhibit the viral infection in cell lines and *in vivo.* Inhibition of the viral infection is achieved combining the [I] segment with a cell penetrating [P] segment.

[0037] A number of peptides derived from certain proteins have the capability to penetrate into the cells and get access into the cytoplasm and nucleus. These peptides are known as cell penetrating peptides or protein transduction domains (PTD) (Joliot, A., and Prochiantz, A. (2004) Transduction peptides: from technology to physiology. Nat. Cell Biol. 6, 189-96. Snyder, E. L., and Dowdy, S. F. (2004) Cell penetrating peptides in drug delivery. Pharm. Res. 21, 389-93. Deshayes, S., Morris, M. C., Divita, G., and Heitz, F. (2005) Cellpenetrating peptides: tools for intracellular delivery of therapeutics. Cell. Mol. Life Sci. 62, 1839-49). The most studied PTDs are the cationic peptides derived from proteins such as the HIV transcription factor TAT, the homeobox antennapedia (penetratin) from *Drosophila melanogaster* and the protein VP22 from the Herpes simplex virus. These peptides have raised great interest as potential carriers for the introduction of cargo molecules into the cells in order to enhance their biological activity, being these cargoes very diverse in nature like small drug-like molecules or genes and proteins. The potential of the PTDs as vectors for molecules with therapeutic interest have been shown in cell systems and also in animal models (Beerens, A. M., Al Hadithy, A. F., Rots, M. G., and Haisma, H. J. (2003) Protein transduction domains and their utility in gene therapy. Curr. Gene Ther. 3, 486-94. Wadia, J. S., and Dowdy, S. F. (2003) Modulation of cellular function by TAT mediated transduction of full length proteins. Curr. Protein Pept. Sci. 4, 97-104.)Wadia, J. S., and Dowdy, S. F. (2005) Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. AdV. Drug DeliVery ReV. 57, 579-96. Rudolph, C., Schillinger, U., Ortiz, A., Tabatt, K., Plank, C., Muller, R. H., and Rosenecker, J. (2004) Application of novel solid lipid nanoparticle (SLN)-gene vector formulations based on a dimeric HIV-1 TAT-peptide in vitro and in vivo. Pharm. Res. 21, 1662-9).

[0038] A significant amount of research have been carried out in order to elucidate the mechanisms by which these peptides can get access into the cytoplasm and the nucleus passing through biological barriers formed by the cellular membrane systems such as the plasma membrane, the membranes of the endocytic compartments and the nucleus. Recently, it has been shown that a number of previously documented observations in culture cells regarding the cellular localization and cell entry of the PTDs at low and physiological temperature were due to artifacts caused by the fixation procedues and unspecific binding of peptides to the plasma membrane (Richard, J. P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M. J., Chernomordik, L. V., and Lebleu, B. (2003) Cellpenetrating peptides. A reevaluation of the mechanism of cellular uptake. J. Biol. Chem. 278, 585-90. Vives, E., Richard, J. P., Rispal, C., and Lebleu, B. (2003) TAT peptide internalization: seeking the mechanism of entry. Curr. Protein Pept. Sci. 4, 125-32).

[0039] The most recent results suggest that endocytosis plays an essential role in the entry of PTDs into the cells. However, a detailed and generally accepted description of the intracellular traffic of these peptides has not emerged yet.

[0040] It was first reported that TAT peptide fusion proteins entry into the cells passing to neutral caveosomes via plasma membrane caveolae, but more recent studies have shown that caveolae are not required and TAT peptide cell entry occurs by macropinocytosis (Ferrari, A., Pellegrini, V., Arcangeli, C., Fittipaldi, A., Giacca, M., and Beltram, F. (2003) Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. Mol. Ther. 8, 284-94. Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape

of TAT-fusion proteins after lipid raft macropinocytosis. Nat. Med. 10, 310-5). Consistently with the postulated cell entry mediated by endocytosis, the PTDs have been observed in early and recycling endosomes. However, the biological activity shown by the molecules associated to the PTDs indicates that these peptides should escape at least partially from the endocytic compartments by a still unknown mechanism getting access into the cytosol. Colocalization of internalized TAT peptide with the Golgi marker BODIPY-ceramide has been reported, consistently with its lacks of visualization in later endosomes and lysosomes labeled with Lysotracker (Fischer, R., Kohler, K., Fotin-Mleczek, M., and Brock, R. 2004. A stepwise dissection of the intracellular fate of cationic cellpenetrating peptides. J. Biol. Chem. 279, 12625-35*)*.

[0041] These data suggest that these peptides are capable to traffic to the Golgi directly from the early endosomes, which is consistent with a potential peptide entry into the cytosol from the ER preceded by retrograde transport of the peptides from the Golgi. However, other studies have reported colocalization of peptides in acidic late endocytic structures and in lysosomes. Such results have been reported for TAT peptide, octaarginine, TAT protein and conjugates of liposomes with TAT peptide (Al-Taei, S., Penning, N. A., Simpson, J. C., Futaki, S., Takeuchi, T., Nakase, I., and Jones, A. T. 2006. Intracellular Traffic and Fate of Protein Transduction Domains HIV-1 TAT Peptide and Octaarginine. Implications for Their Utilization as Drug Delivery Vectors. Bioconjugate Chem. 17, 90-100. Fretz, M. M., Koning, G. A., Mastrobattista, E., Jiskoot, W, and Storm, G. (2004) OVCAR-3 cells internalize TAT-peptide modified liposomes by endocytosis. Biochim. Biophys. Acta 1665, 48-56. Vendeville, A., Rayne, F., Bonhoure, A., Bettache, N., Montcourrier, P., and Beaumelle, B. (2004) HIV-1 Tat enters T cells using coated pits before translocating from acidified endosomes and eliciting biological responses. Mol. Biol. Cell 15, 2347-60).

[0042] However, it is possible that the PTDs could exploit various different mechanisms of cell entry and intracellular traffic, depending of several factors like cell type, nature of the PTD, temperature, cargo, etc.

Detailed description of the Invention

[0043] The present invention describes two topological variants of chimerical peptides which inhibit the viral infection by *Flaviviridae*:

$$[P]\text{-}[L_1]\text{-}[I]\text{-}[L_{2}]\text{-}[T] \text{ o } [I]\text{-}[L_{3}]\text{-}[P]\text{-}[L_{4}]\text{-}[T],$$

[0044] As [P] penetrating peptide are preferably selected, but not restricted to those, cationic peptides with the capability to carrier cargo molecules into the cells. As possible cationic peptides could be chosen penetratin, polyarginines of 7-10 residues such as R9 nonapeptide or R10 decapeptide or TAT peptide, although any other peptide sequence between 10-30 residues showing similar penetrating capability could be selected. These penetrating cationic peptides have the capability to penetrate into the cytoplasm of the cell via endocytosis, which could involve the traffic through the ER. This property is favorable for the biological activity of these peptides because it guarantees the peptide localization in the contiguity of the RER, the place where the precursor polyprotein synthesis and processing is carried out and constitutes the target of peptide antiviral activity.

[0045] Alternatively, other cell penetrating peptides could be also used as [P] segments like the cationic dendrimeric peptides or peptides comprising D-amino acids, which are very resistant to proteolytic degradation. The cationic peptides also guarantee a good biodistribution *in vivo* of the peptides from the present invention, allowing its favorable effective concentration in organs and tissues infected by *Flaviviridae,* to higher levels compared to larger molecules as the monoclonal antibodies. One example could be the use of peptides permeable to the blood-brain barrier (BBB) to treat *Flaviviridae* infections causing encephalitis like TBE, WNV, JEV, SLEV and KV. The molecular transport through the BBB is a formidable problem even for small drugs aimed for treatment of intraencephalic diseases (Temsamani, J. and Vidal, P. 2004. The use of Cell-penetrating peptides for drug delivery. Drug Discov. Today 9, 1012-1019).

[0046] The NS3pro protease inhibitory sequence [I], has the capability to inhibit or modify the interaction between the proteins NS3 and NS2B from *Flavivirus* (or between NS3 and NS4A from *Hepacivirus* and *Pestivirus*), and this way it affects the correct folding of NS3pro which is necessary for the protease activation process. In one embodiment of this invention, [I] consists in the sequence Asp50-Glu62 of the protein NS2B from DV2, or its homologous sequences from other *Flavivirus.* This sequence contains the residues corresponding to the beta strand 1 of the protein NS2B, which makes contacts with residues located at the N-terminal beta barrel of the active NS3pro protein. Thus, peptides according to the topologies described in this invention, compete with the native sequence of the cofactor NS2B protein during the folding of the NS3pro protein to the adoption of its active conformation. It leads to the formation of inactive NS3pro-peptide complexes because the full activation requires structural rearrangements not only in the N-terminal domain, but also in the C-terminal beta barrel two. Protease activation would need an additional binding of the region Glu66-Ile86 of the protein NS2B to the C-terminal domain of NS3pro. In addition, the binding of the segment [I] serves as an anchor of the peptides of the present invention to the protein NS3, in such a way that the N- or C-terminal extensions of these peptides could alter the surface topography of NS3 and interfere with its interactions with viral and/or host proteins. Such interactions include the substrate recognition and/or other interactions related to the conformation and/or functioning of

the viral replication complex. Thus, in one embodiment of the present invention the [P] segment corresponding to the first topological variant are poly-D arginine, which besides having the cell penetrating property are also inhibitors of the *Flavivirus* NS3pro protease (SHIRYAEV, S.A., RATNIKOV, B.I., CHEKANOV, A. V., SIKORA, S., ROZANOV, D. V., GODZIK,A., WANG, J., SMITH, J.W., HUANG, Z., LINDBERG, I., SAMUEL, M.A., DIAMOND, M.S. and Alex Y. STRONGIN, A.Y., 2006. Cleavage targets and the D-arginine-based inhibitors of the West Nile virus NS3 processing proteinase. Biochem. J. 393, 503-511). Thus, the binding of the [I] segment/anchor to NS3pro facilitates the fit of the polyArg peptides in the substrate binding site of the protease, well corresponding to the same chain anchored by the peptide (cis-inhibition) or a different chain (trans-inhibition). In an analog way, *Hepacivirus* and *Pestivirus* inhibitory peptides incorporate as [I] sequence the segment corresponding to the region Thr20-Leu31 of the NS4A protein (numbering of HCV), which is structurally equivalent to the beta strand 1 of the NS2B protein from *Flavivirus.*

[0047] In a second embodiment, which applies to *Flavivirus,* the [I] segment does not relate to any specific segment of the NS2B sequence, but consists in a peptide sequence with the capability to bind to NS3pro protein and stabilize the N-terminal barrel in its inactive conformation. In this case the peptide sequence makes contacts with the segment corresponding to Tyr23-Tyr33 of the NS3pro protein from DV2, or a homologous region corresponding to other *Flavivirus.* In addition, the [I] segment also makes stabilizing structural contacts with the residues from the segments Ala1-Gly14 and Ala56-Met59 of the protein NS3pro. Therefore, these peptides promote their inhibitory effect by interfering with the native folding of the protein NS3, inducing a folding pathway leading to the inactive conformation of the protease.

[0048] Such [I] sequences could be obtained by theoretical methods and/or experimental methods which make use of combinatorial libraries. In case of design by theoretical methods, the invention implies the use of one or various methods of computational molecular modeling and the use of three dimensional structural models of the protein NS3pro in its inactive conformation. Making use of the method(s) of computational modeling and the spatial coordinates of the 3D structural model of the inactive NS3pro protein, it is possible to model a polypeptide main chain in an extended conformation, which forms an antiparallel beta strand with the segment corresponding to the beta strand A1 of the N-terminal beta barrel. In addition, it is also possible to model the side chains of the polypeptide chain, in such a way that the chemical identity of this side chains and its conformers imply energetically favorable atomic contacts. This invention involves the combined exploring by computational means of the peptide sequence and conformational space, the side chain rotamer space of the peptide and also of the protease and the selection of the most favorable peptide variants according to an energy scoring of the obtained models, which indicate a potential higher affinity of the peptide-protein interaction.

[0049] The coordinates corresponding to the inactive NS3pro structural models could originate from experimental data obtained by the methods of x-ray diffraction and/or NMR or by the use of models obtained by computational modeling methods. In the case of DV2, the coordinates could be obtained from the file 1 BEF of the Protein Data Bank (PDB). For other *Flavivirus,* it is possible to obtain 3D models by the method of homology modeling.

[0050] In the present invention we describe the [I] sequence: **QWPALPKIEAQDG,** which was designed according to this second embodiment of the present invention. The figure 1D shows a computational model of the tridimensional structure of the NS3pro-[I] complex corresponding to this embodiment. According to this model, the [I] segment adopt an extended beta strand structure associated to the segment Gly29-Y33 of NS3pro (DV2 numbering).

[0051] Additionally, combinatorial libraries of synthetic peptides or phage displayed peptides libraries could be used in order to obtain [I] sequences with similar properties to peptides described in the second embodiment of the present invention. In this case the recombinant NS3pro protein is used as target for ligand selection or biopanning.

[0052] In other embodiment of the present invention, the [I] segment consist in the sequence Ser70-Gly82 of the protein NS2B from DV2, or its homologous sequences from other *Flavivirus.* This sequence contains the beta strands 3 and 4 of the protein NS2B, which contact and form part of the active NS3pro protease. Thus, peptides according to the topologies described in this invention, compete with the corresponding segment of the cofactor NS2B protein during the folding of the NS3 protein to its active conformation and hamper the proteolytic processing at the junction NS2B-NS3. It leads to the formation of inactive NS3pro-peptide complexes, because these peptides interfere with the correct configuration of the substrate binding site, in particular at P2 site, which is essential for the enzyme catalytic activity.

[0053] In addition, the binding of the [I] segment corresponding to peptides of this invention serves as an anchor to the protein NS3, such that the N-terminal or C-terminal extension of the peptides could modify the surface topography of NS3 and interfere with the interaction of this protein with other viral or host proteins.

[0054] In an embodiment related to the previous one (peptide 10 in table1), the [I] segment consists in the sequence Ser70-Ile86 of the NS2B protein from DV2, or its homologous sequences from other *Flavivirus.* This region includes, besides the beta strands 3 and 4, also the beta strand 5 of NS2B. In this case, the peptides corresponding to the first topological variant include a C-terminal extension comprising a [L2] segment of 3 or 4 residues and a [T] segment consisting of the tripeptide XRR, with a C-terminal carboxylic group. The sequences of these peptides are consistent with their binding to the NS3 protein adopting its active conformation, the beta strand 5 and the loop between the strands 4 and 5 guarantee the correct formation of the P2 site.

[0055] Moreover, the binding of the segment [I] facilitates the structural changes in the C-terminal beta barrel which

are necessary for the activation, such as the change in orientation of the E2b-F2 beta hairpin, which allows the arrangement of important residues involved in the substrate recognition like Gly151, Gly153 and Tyr161. However, the formed complex is inactive, because the [L2] segment serves as stabilizing-linker allowing the additional binding of the [T] segment to the substrate binding site, with the dipeptide RR occupying the positions S1 and S2. Thus, the protease active site becomes blocked by the peptide.

[0056] The segments [L1], [L2], [L3] and [L4] of the present invention are linker sequences of 0-6 residues, which connect the segment [P], [I] and [T], depending on the topological variant. These linker segments contains mainly small and/or polar amino acids (Gly, Ser, beta-Ala), which provide flexibility. These linker segments could also consist of sequences capable to interact favorably with residues from the NS3pro protein, providing the peptides of the present invention with an additional stabilizing effect.

[0057] The [T] segments of the present invention are sequences between 0 and 10 amino acids, localized at the C-terminal ends of the peptides. In an embodiment, the [T] segment is an ER retention signal, like the KDEL sequence. The addition of this signal facilitates the traffic of peptides by retrograde transport to the ER. The increase in peptide concentration within the ER contributes to enhance the transport of the peptides to the cytosol. It results in an increase of the effective peptide concentration in the contiguity of the ER, where occurs the synthesis of the viral polyprotein and in particular the synthesis of the NS3pro.

[0058] The incorporation of the KDEL signal to the peptide sequence is compatible with the presence of cationic cell penetrating peptides as [P] segments, because the retrograde transport through the ER is a putative pathway of cationic peptide penetration into the cytosol. This way of penetration involved the traffic of peptides from early endosomes to the ER via the trans-Golgi network (TGN). The sequence KDEL interacts with the KDEL receptor present at the TGN which transport the peptide to the ER where it is discharged.

[0059] The peptide transport from the ER lumen into the cytosol is an efficient process, which occurs through channels present at the ER membrane formed by the Sec61 protein from the translocon complex. This mode of penetration into the cytosol is exploited by bacterial toxins like the cholera toxin, Ricin and the exotoxin A from *Pseudomonas,* etc.

Use of a FG hairpin based segment as cell penetrating peptide. Inhibitory effect of this segment on DV entry into the cell.

[0060] The antiviral activity displayed by peptides of the present invention is based primarily on the inhibition of NS3 protease activation process. The [I] segments or modules described in the present invention as inhibitors of the viral protease activation process, have the capability to bind to the NS3pro protein and to block the interaction between this protein with the viral protein NS2B from *Flavivirus* (NS4A in *Hepacivirus*), which is necessary for activation of the protease. However, the presence of this segment does not guarantee that peptides are capable to block the viral infection *in vitro* and *in vivo.* Thus, we show in the example 3 that the segment Ser70-Gly82 corresponding the protein NS2B from DV2 is capable of inhibiting the viral infection *in vitro* only if it is present in the same polypeptide chain together with a cell penetrating peptide. In order to inhibit the viral infection, the peptides of the present invention need to penetrate cells, get access into the cytosol and bind the NS3pro protein, whose folding take place at the cytosolic face of the ER membrane.

[0061] It is known that the LRP1 receptor interacts and internalize into the cells about 30 natural ligands, among them the pertussis exotoxin A (Herz J, Strickland DK. (2001) LRP: a multifunctional scavenger and signaling receptor. J Clin Invest. 108:779-84. Kounnas MZ, Morris RE, Thompson MR, FitzGerald DJ, Strickland DK, Saelinger CB, 1992. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. J Biol Chem 267:12420-12423). This receptor is expressed in the majority of cell types, tissues and organs. The DV has also the capacity to infect many cell lines and organs, therefore the use of peptides containing a cell penetrating peptide based on the sequence of the FG hairpin, is very favorable in order to achieve an affective internalization into the infection susceptible cells. LRP1 expression is high in the liver and the brain, which are main target organs of diseases caused by *Flaviviridae.* For example, the viruses from the TBE and JEV complexes cause encephalitis and the YFV is mainly viscerotropic and causes hepatitis. For the same reason, this segment would be also effective against HCV, as present in the anti-HCV peptides described in the present invention.

[0062] In the particular case of chimerical peptides described in the present invention as inhibitors of dengue infection, those modules based on the FG hairpin possess a bifunctional character. Besides the already described role as cell penetrating segment, this segment displays also anti-DV antiviral activity per se. Previously it has been shown that peptides based on the FG hairpin inhibit infection productive cell entry of DV by a mechanism which involves an step occurring after virus adhesion to the plasma membrane These peptides are highly efficient inhibiting viral infection when they are present in solution at the moment of virus entry into the cell. Furthermore, as shown in the example 2, the peptides of the present invention, which do not possess a cell penetrating segment based on the FG hairpin and whose antiviral effect is based only in their NS3 protease inhibitory modules, are less efficient if they are administered to the media at the same time as the virus. These peptides (lacking FG hairpin segment) show their maximum antiviral activity if they are preincubated with the cells before the virus is added, which is consistent with a mechanism of inhibition

requiring cell penetration and an effective intracellular localization in order to inhibit the NS3 protease activation.

[0063] Therefore, a novel element of the present invention consists in the combination of a cell entry inhibitor (which is also a cell penetrating peptide) and a segment inhibiting the viral protease activation.

[0064] Hence, these chimerical peptides possess a biological activity profile which is more favorable compared with those peptides based in only one of these segments, considering the relationship between the moments of peptide addition with respect to the beginning of the viral infection.

Cell penetration and intracellular fate. N-terminal lipidation and retention in the ER.

[0065] The present invention applies also for the lipidation of the previously described chimerical peptides. The herein mentioned lipidation typically consists on the myristoylation or the palmitoylation at the N-terminal ends of peptides. In this patent as myristoylation we mean the chemical modification of the peptides by covalent attachment of the myristic acid $CH_3(CH_2)_{12}CO_2H$ to the N-terminal group of the peptides by means of an amide bond, resulting in the chemical structure $CH_3(CH_2)_{12}CO_2$-NH-P, where P is the amino acid sequence of the myristoylated peptide. Similarly, the palmitoylation results in the addition of the $CH_3(CH_2)_{14}CO_2H$ palmitic group. As lipidation we also mean herein the covalent attachment of the lipid chain to the side chains of the amino acid residues SER and/or TYR, added as N-terminal extensions to the peptides. In order to exert their antiviral activity, which is based primarily on the inhibition of the NS3 protease activation, the peptides of the present invention need to cross various biological barriers, consisting of diverse membrane systems of the cell.

[0066] These peptides need to transit from the extracellular space to their final fate optimal for the antiviral effect, the cytosolic face of the ER. In general, lipidation increases peptide lipophilicity, which is a favorable property regarding the interaction with biological membranes. In this invention we have originally combined peptide lipidation with the addition of some traffic and cellular localization signals (sequences) which enhance the biological activity of peptides. Thus, this design is aimed to increase the efficiency of various steps involved in the manifestation of peptide antiviral activity on the cells: adsorption on the plasma membrane, cell penetration, intracellular traffic/retrograde transport, intracellular localization on the ER membrane and the interaction with NS3pro protein.

[0067] The choice of the chemical nature of the lipid(s) adequate for peptide lipidation is not trivial. One premise of the chimerical peptide design of the present invention, consists in selecting specific lipid(s) for its chemical conjugation to the peptides, in such a way that this chemical modification affect favorably the physicochemical and functional properties of the peptides concerning the different processes involved in their antiviral action: binding to the plasma membrane, cell penetration/endocytosis, intracellular traffic/retrograde transport, transport to cytosol and binding to NS3pro. During this process, the peptides should interact with membranes of different biophysical properties, and participate in the transport between different intracellular compartments. An optimal lipid regarding one individual step could be detrimental respect to other steps and therefore being not indicated for lipidation of antiviral peptides of the present invention. As an example, considering the interaction with the ER membrane, the monosaturated glycerolipids are potentially favorable. These lipids are common in this membrane (Keenan T.W. AND Morrea, D.J. Phospholipid class and fatty acid composition of Golgi apparatus isolated from rat liver and comparison with other cell fractions. Biochemistry 9: 19-25, 1970), which is characterized by its higher fluidity and smaller thickness compared to the plasma membrane rich in sphingolipids, sterols and disaturated phospholipids. Thus, an unsaturated lipid with relatively short chain which would be adequate for insertion in the ER membrane would not be favorable in the plasma membrane. This kind of lipids would localize preferably in the most fluid domains of the plasma membrane, segregated from domains rich in sphingolipids and cholesterol such as the lipid rafts, which are involved in endocytosis. Various previous analysis of the endocytic routing of lipid analogs differing in the nature of their hydrophobic tails have shown that short tailed unsaturated lipids after being endocytosed are efficiently recycled back to the plasma membrane via the endocytic recycling compartment (ERC) and saturated long tailed lipids are routed through the endocytic way to late endosomes and lysosomes (Mukherjee, S., Soe, T. T and Maxfield, F.R. 1999. J. Cell Biol., 144, 1271-1284; Koval, M., and R.E. Pagano, 1989. J. Cell Biol. 108:2169-2181; Mayor, S., J.F. Presley, and F.R. Maxfield, 1993. J. Cell Biol. 121:1257-1269; Sandhoff, K., and A. Klein., 1994. FEBS Lett. 346:103-107).

[0068] Previous studies have reported examples of peptide myristoylation facilitating cell penetration and the biological activity of peptides at their corresponding intracellular targets (P.J. Bergman, K.R. Gravitt, C.A. O'Brian, An N-myristoylated protein kinase C-alpha pseudosubstrate peptide that functions as a multidrug resistance reversal agent in human breast cancer cells is not a P-glycoprotein substrate, Cancer Chemother. Pharmacol. 40 (1997) 453-456. B.R. Kelemen, K. Hsiao, S.A. Goueli, Selective in vivo inhibition of mitogen-activated protein kinase activation using cell-permeable peptides, J. Biol. Chem. 277 (2002) 8741-8748. T. Eichholtz, D.B. de Bont, J. de Widt, R.M. Liskamp, H.L. Ploegh, A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor, J. Biol. Chem. 268 (1993) 1982-1986).

[0069] However, myristoylation per se does not guarantee peptide penetration into the cells. In fact, there are examples of myristoylated peptides which do not penetrate into the cells and it has been postulated that penetration depends also on the nature of the peptide, being favorable properties having a positive net charge and a homogeneous distribution

of basic residues between acid and hydrophobic residues (Carrigan, C.N., Imperiali, B. 2005, Anal. Biochem. 341 290-298).

[0070] The first steps in the interaction of peptides of the present invention with cells are the adhesion to the plasma membrane and/or the binding to molecules present on the membrane. The addition of myristoyl or palmitoyl groups increases the lipophilicity of peptides of this invention, facilitating the peptide binding to the plasma membrane. Besides the presence of the lipid, the peptides of this invention contain sequences of cell penetrating peptides, which interact with molecules present in the membrane. An example is the case of cationic cell penetrating peptides which interact with glycosaminoglycans, in particular with heparin-like heparan sulfates.

[0071] It has been shown that binding to heparan sulfates is essential for cell penetration by cationic peptides. They can also interact with other negatively charged molecules of the plasma membrane like anionic lipids and proteins. Similarly, other peptides which interact with endocytic cell receptors can function as carrier or cell penetrating peptides facilitating the entry of cargo molecules into the cells. Lipidation of peptides of the present invention therefore increases the binding affinity to the plasma membrane providing an additional anchoring site.

[0072] In general, myristoylated proteins containing a cluster of basic residues juxtaposed to a myristoylation signal interact favorably with membranes rich in cholesterol and sphingolipids *acylated* amino-term*inal domains in subcellular localization.* Mol. Biol. Cell 10, 3771-3786*.* Cholesterol, Sphingolipid-enriched Membranes But Not to Lipid Rafts/Caveolae. Mol. Biol. Cell 12, 3601-3617 *AND* GERRIT VAN MEER. 2001. The Organizing Potential of Sphingolipids in Intracellular Membrane Transport. Physiol. Rev. 81, 1689-1723*.* Simons, K. and Ikonen, E. 1997. Functional rafts in cell membranes. Nature, 387: 569-572*).* Therefore, the myristoylation is favorable for the capacity of peptides of the present invention to penetrate cells, in particular those peptides containing cationic cell penetrating peptides and/or polyarginines as signals for retention at the cytosolic face the ER membrane. In fact, it has been shown that lipidated peptides, containing certain positive charge can penetrate into the cells (Carrigan, C.N., Imperiali, B. 2005, Anal. Biochem. 341 290-298*).*

[0073] Detergent resistant specialized microdomains of the membrane (DRMs) rich in glycosphingolipids and cholesterol seem to be essential for internalization of various bacterial toxins into the cells (Cholera toxin, Ricin, Shiga toxin, etc) and molecules associated to these DRMs like the GM1 ganglioside and the sphingolipid Gb3 are receptors of some of these toxins which penetrate cells by endocytosis (Spangler, B. D. (1992) Microbiol. Rev. 56, 622-647*.* Fujinaga Y, Wolf AA, Rodighiero C, Wheeler TE, Tsai B, Allen L, Jobling MG, Rapoport TA, Holmes RK, Lencer WI. 2003. Gangliosides that associate with lipid rafts mediate transport of cholera and related toxins from the plasma membrane to endoplasmic reticulum. Mol Biol Cell 14: 4783-4793*.* Salamero J, Johannes L. 2001. Targeting of Shiga toxin B-subunit to retrograde transport route in association with detergent-resistant membranes. Mol Biol Cell 12: 2453-2468 These toxins exert their activities in the cytosol after passing through a retrograde transport process which involves the traffic from the endosomes to the ER, well directly or through the TGN Annu Rev Cell Dev Biol 18: 1-24). Then these toxins pass from the ER to the cytosol also by retrograde transport and apparently making use of the ER associated degradation mechanism (ERAD) (Lord, J. M., and Roberts, L. M. (1998) J. Cell Biol. 140, 733-736. Lord, J. M., Deeks, E., Marsden, C. J., Moore, K., Pateman, C., Smith, D. C., Spooner, R. A., Watson, P., and Roberts, L. M. (2003) Biochem. Soc. Trans.31, 1260-1262*.* AbuJarour, R.J., Dalal, S., Hanson, P.I. and Draper, R.K. 2005. J. Biol. Chem. 280, 15865-15871 *.*

[0074] Thus, the potential colocalization of the lipidated peptides of the present invention in membrane domains rich in sphingolipids would be consistent with their potential capacity to exploit a cell penetration mechanism based in the above mentioned lipid dependent retrograde transport used by bacterial toxins.

[0075] In other embodiment of the present invention the designed peptides use as cell penetrating peptide the FG hairpin from domain III of the envelope protein of DV3 or the homologous peptides of serotypes 1, 2 and 4. Previously, it has been shown that these peptides bind to the cellular receptor LRP1 Herz J, Strickland DK. (2001) LRP: a multifunctional scavenger and signaling receptor. J Clin Invest. 108:779-84. Kounnas MZ, Morris RE, Thompson MR, FitzGerald DJ, Strickland DK, Saelinger CB. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. J Biol Chem 1992;267:12420-12423).

[0076] In this invention we have shown that peptides corresponding to the FG hairpin are capable to mediate the cell entry of peptide cargoes. The lipidation (myristoylation or palmitoylation) of these FG hairpin containing peptides would increase the effective affinity for their cellular receptor by enhancing the partition of the peptides in the lipid membrane. This peptide lipidation is also consistent with an increase in the cell penetrating potential of peptides via endocytosis mediated by LRP1 receptor.

[0077] One possibility is that these peptides penetrate into the cells in a similar way to PTx. This toxin get access into the cytosol by retrograde transport from the endosomes passing successively through the TGN, the ER and then to the cytosol. PTx has the capacity to exploit at least two retrograde transport pathways mediated by LRP1 interaction: a) lipid dependent pathway and b) lipid independent pathway (Smith, D. C., Spooner, R. A., Watson, P. D., Murray, J. L., Hodge, T. W., Amessou, M., Johannes, L., Lord, J. M. and Roberts, L. M., 2006. Internalized Pseudomonas Exotoxin A can Exploit Multiple Pathways to Reach the Endoplasmic Reticulum. Traffic, 7: 379-393). The lipid dependent pathway seems to be related to the localization of a 20 % of LRP1 molecules in lipid rafts of the plasma membrane. Those peptides

of the present invention, which are lipidated and have the capability to interact with LPR1 can potentially exploit more efficiently the lipid dependent pathway, in particular those peptides including a basic cluster of polyarginines (added as an ER retention signal) have a favorable composition to localize in raft adjacent membrane domains rich in cholesterol and sphingolipids.

[0078] Other embodiment of the present invention consists in peptides having the KDEL signal at the C-terminal end. These peptides are synthesized with a carboxylic C-terminal end in order to make functional the KDEL signal for retention at the lumen of the ER (Teasdale, R. D. & Jackson, M. R., 1996. Annu. Rev. Cell Dev. Biol. 12, 27-54*).* The addition of this signal to the peptide sequences contributes favorably to their retrograde transport from the Golgi to ER and the later retention of these peptides in the lumen of the ER. Thus, this signal contributes to the penetration into the cytosol of those peptides which make use at least partially of the retrograde transport pathway. A higher efficiency of the transport leads to a higher cytosolic concentration of the peptides and hence a more potent blocking activity of NS3 protease activation. In the example 3 we have shown that the addition of the KDEL signal to peptides of the present invention can lead to an increase of the antiviral activity of the peptides.

[0079] The addition of the KDEL signal is valid for peptides of the present invention having or not lipids attached in their N-terminal ends. It is consistent with the fact that this signal is found both in soluble and in type II membrane proteins of the ER. Lipidated peptides of the present invention present in the lumen of the ER would adopt a topology similar to the type II membrane proteins.

[0080] In the case of peptides of the present invention having an FG hairpin related cell penetrating segment, the addition of the KDEL signal provides these peptides with the additional capacity of interfering with the anterograde transport of the receptor LRP1 and hence leading to a decrease of their receptor expression levels on the plasma membrane. Therefore, the combination of these sequence/signals has an indirect negative effect on the entry of the virus into the cells reducing the expression of the receptor at the plasma membrane, and this effect is additional to the above described direct effect of peptides based on the FG hairpin blocking the cell entry of the virus. Previous evidences indicate that peptides based on the FG hairpin favor the interaction of LRP1 with its chaperone receptor associated protein RAP affecting the LRP1 mediated cell entry of the virus.

[0081] A common property of peptides of the present invention is that they display antiviral activity based on inhibition of NS3 protease activation. The inhibition of protease activation is achieved by blocking specifically the interaction of the protein NS2B (NS4A in *Hepacivirus*) with the NS3pro domain, being this interaction a necessary condition for the correct folding and full activity of the protease.

[0082] The protein folding and activation of the protease NS3, as well as the folding and processing of the core protein and the rest of non-structural proteins, takes place at the cytosolic face of the ER membrane. Therefore, a way to enhance the antiviral activity of the peptides of the present invention consists in increasing their intracellular localization at the ER membrane. With this aim, the peptides could be chemically lipidated (myristoylated or palmitoylated) at the N-terminal end. The lipidated peptides have the capacity to interact favorably with lipid membranes. The better association of the lipidated peptides with the ER membrane (favored by the lipid moiety) increases the effective apparent affinity of the interaction between the peptides and the NS3pro protein, an effect related to the following factors: 1) increase of the local peptide concentration, 2) the bimolecular interaction occurs in two dimensions (the plane of the membrane) and 3) the fast lateral diffusion of lipidated peptides at the membrane. Furthermore, those peptides lipidated at their N-terminal end when associated to the cytosolic face of the ER membrane simulate topologically the type I membrane proteins, thus they acquires not only the correct localization but also an orientation respect to the membrane which is similar to the viral NS2B protein (NS4A in *Hepacivirus).*

[0083] In general, when there are not additional signals like palmitoylation and/or basic clusters, the myristoylation of cytosolic proteins induce a localization of these proteins mainly at the ER membrane *acylated amino-terminal domains in subcellular localization.* Mol. Biol. Cell 10, 3771-3786). The association of the myristate per se with the membrane is not strong enough and does not guarantee the total retention of peptides in the ER membrane. However, the ER membrane constitutes the 60% of the intracellular membranes which guarantee a significant effective concentration of the peptides with respect to the rest of membranes. When in addition to myristoylation basic clusters are also present, the cytosolic proteins localize mainly at the inner face of the plasma membrane and in endosomes.

[0084] Various peptides of the present invention contain cationic segments as cell penetrating peptides which guarantee also a favored interaction with negatively charged molecules located at the outer face of the plasma membrane. Some peptide of the present invention contains clusters of arginines as cationic segment which also constitute ER retention/redirection signals (Teasdale, R. D. & Jackson, M. R. (1996) Annu. Rev. Cell Dev. Biol. 12, 27-54. Zerangue, N., Schwappach, B., Jan, Y. N. & Jan, L. Y. (1999) Neuron 22, 537-548. Schutze, M. P., Peterson, P. A. & Jackson, M. R. (1994) EMBO J. 13, 1696-1705). These peptides have been designed with the aim to display simultaneously both properties: an efficient cell penetration and an intracellular localization mainly at the cytosolic face of the ER membrane. The arginines based traffic signals to the ER are highly efficient and play an important role in the mechanism of quality control of membrane proteins (Chang, X. B., Cui, L., Hou, Y. X., Jensen, T. J., Aleksandrov, A. A., Mengos, A. & Riordan, J. R. (1999) Mol. Cell 4, 137-142. Margeta-Mitrovic, M., Jan, Y. N.& Jan, L. Y. (2000) Neuron 27, 97-106).

[0085] Unlike the dilysine signal which is restricted to the C-terminal end of type I membrane protein, the arginines based signals are found in many positions of the sequence of membrane proteins, including the N- and C-terminal ends and also the internal loops located at the cytosolic face. The versatility of the retention signals based in arginines have been exploited in the present invention in order to design peptides which combine them with the C-terminal KDEL signal. Thus, some lipidated peptides of the present invention containing these signals, enter into the cells and transit to the cytosol by retrograde transport, being favored by the KDEL signal during their transit to the ER and later retained at the cytosolic face of the ER membrane supported by the arginines based signal.

[0086] In one embodiment of the present invention we have included the design of lipidated peptides whose sequence contain two successive putative retention signal for retention at the cytosolic face of the ER membrane. The resulting sequence is LRRRRLRRRRL, which corresponds to two consecutive LRRRRL sequence overlapped in a central Leu residue. The sequence of four consecutive arginine preceded by a hydrophobic residue is typical of

[0087] RE retention sequences (Zerangue, N., Malan, M.J., Fried, S.R., Dazin, P.F., Jan, Y.N., Jan, L.Y. and Schwappach, B. 2001. PNAS, 98: 2431-2436). In these regards, one of the novel aspects of the present invention is that the resulting sequence have the duality of being an efficient RE retention signal and also a cell penetrating peptide. The cell penetrating property of the resulting sequence is provided by the eight arginine residues which is similar to the polyarginine sequences, very efficient cationic PTDs.

DESCRIPTION OF THE FIGURES

[0088]

Figure 1: Design of peptide inhibitors of NS3pro protease activation. A: Multiple sequence alignment of NS2B protein sequences from *Flavivirus.* The herein described activation inhibitor segments are highlighted with double arrows, the light (dark) gray arrow corresponds to the segment bound to the N-terminal (C-terminal) beta barrel domain of NS3pro. B: Three dimensional structural model of the NS2B-NS3pro complex of *Flavivirus.* The segment D_{50}-E_{62} of NS2B from DV2 bound the N-terminal beta barrel domains of NS3pro and segment S_{70}-G_{82} of NS2B from DV2 bound to the C-terminal domain are highlighted. C: NS3pro protease activation inhibitory segments D_{50}-E_{62} and S_{70}-I_{86}-GGGGRR. The C-terminal extension of the latter peptide binds to the protease active site, blocking the interaction of the protease with its substrates. D: Model of the complex formed by NS3pro in its inactive conformation (structure of the protein without NS2B) and a computationally designed peptide of the present invention.

Figure 2: Assay of inhibition of infection by dengue 2 virus in Vero cells. A: Percentage of reduction in the number of plaques due to the presence of peptide NS2Bden2+TAT with and without preincubation before addition of the virus to the cells. B: Assay of the antiviral activity of peptides TAT and NS2Bden2+TAT at different concentrations, with (pre) and without preincubation (no pre).

Figure 3: Effect of incubation time on the antiviral activity of peptide NS2Bden2+TAT. PX1: not related negative control peptide (TAT peptide fusion to a not related sequence); P10: peptide NS2Bden2+TAT (TAT fusion to a peptide from NS2B of DV, peptide No.1 of table 1). The assayed preincubation times were 0, 30, 60 and 180 minutes.

Figure 4: Role of internalization on the antiviral activity of peptide NS2Bden2+TAT. A: After preincubation with peptides, they remain present in the media at the time of virus addition to the cells. B: The peptides are retired from the media, through various washing before virus addition to the cells. pNR+TAT: peptide No. 18 of table 1. The pNR+TAT peptide is a negative control of the experiment. Its primary structure is analog to peptide NS2Bden2+TAT, the [I] segment have an amino acid composition identical to peptide NS2Bden2+TAT but the sequence was permutated (peptide 18 of table 1).

Figure 5: Effect of penetrating peptide identity and ER retention signal on the antiviral activity of peptides. NS2Bden2+TAT: peptide 1 of table 1; NS2Bden2+pP2: peptide 2 of table 1, cell penetrating segment is penetratin; NS2Bden2+pRR: peptide 3 of table 1, decaarginine as cell penetrating peptide; NS2Bden2+TAT+KDEL: peptide 4 of table 1; pNR+TAT: peptide 18 of table 1, negative control; NS2Bden2: segment [I] of peptide NS2Bden2+TAT.

Figure 6: Antiviral activity of peptides against homologous and heterologous serotypes of DV. The antiviral activity of peptides was tested by reduction of the number of viral plaques in presence of VD1 (A), VD3 (B) and VD2 (C). Rosseta: peptide computationally designed to bind to the N-terminal domain of NS3pro of DV2 (peptide 5 of table 1); NS2Bden2+poliR: peptide 3 of table 1, decaarginine as cell penetrating peptide; NS2Bden2+TAT: peptide 1 of table 1; NS2Bden1+TAT: peptide 6 of table 1; NS2Bpermutado+TAT: peptide 18 of table 1, negative control of

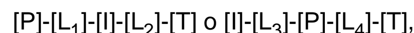
the experiment. The primary structure of NS2Bpermutado+TAT is analogous to peptide NS2Bden2+TAT, the [I] segment have an amino acid composition identical to NS2Bden2+TAT but the sequence was permutated.

EXAMPLES

Example 1

Design and synthesis of chimerical peptides inhibitor of the infection by *Flaviviridae*

[0089] The chimerical peptides inhibitor of the infection by *Flaviviridae* described in this invention have a primary structure according to the following topologies:



$$[P]\text{-}[L_1]\text{-}[I]\text{-}[L_2]\text{-}[T] \text{ o } [I]\text{-}[L_3]\text{-}[P]\text{-}[L_4]\text{-}[T],$$

where, [P] is the amino acid sequence of a cell penetrating peptide, typically of 10-30 residues, which have the capacity to facilitate the internalization of the whole peptide molecule into the cell cytoplasm and to get access to the contiguity of the RER; [L1, L2, L3, L4], are linker sequences of 0-6 residues; [I], is an amino acid sequence which blocks the activation of NS3pro protease, residues of this segment make contacts with at least one amino acid from the beta strands B2a and B2b of the C-terminal beta barrel domain, or the beta strand A1 of the N-terminal beta barrel domain of NS3pro protein from *Flavivirus* (or the structurally corresponding region in *Hepacivirus* or *Pestivirus*), being the NS3pro protein in its active or inactive conformation (figure 1); [T], sequence of 0 to 10 residues, typically is one or two signals of retention in the ER like the sequences KDEL and LRRRRL, or the sequence XRR which displays a capability to binding to the protease active site.

[0090] Tables 1 and 2 show sequences of chimerical peptides according to the topologies 1 and 2 respectively. The basic peptide design is based in the presence of a protease activation inhibitor segment [I] and a cell penetrating segment [P]. As [I] segments are included the sequences D_{50}-E_{62}, S_{70}-G_{82} and S_{70}-I_{86} of the NS2B protein from DV1-4. The corresponding sequences from WNV and HCV are also included. The segment D_{50}-E_{62} binds to the N-terminal domain of NS3pro and the segments S_{70}-G_{82} y S_{70}-I_{86} bind to the C-terminal domain (figure 1A-C).

Table1. Design of chimerical peptides according to the topology [P]-[L_1]-[I]-[L_2]-[T]

No	*[P]*	*[L1]*	*[I]*	*[L2]*	*[T]*	*Virus*	*Penetrating peptide*	*Target domain*
1	**YGRKKRRQRRRPPQ**	**GGG**	**SSPILSITISEDG**			dengue 2	TAT	C-terminal
2	**RQIKIWFQNRRMKWKK**	**GGG**	**SSPILSITISEDG**			dengue 2	penetratin	C-terminal
3	**RRRRRRRRRR**	**GGG**	**SSPILSITISEDG**			dengue 2	R10	C-terminal
4	**YGRKKRRQRRRPPQ**	**GGG**	**SSPILSITISEDG**	**GG**	**KDEL***	dengue 2	TAT	C-terminal
5	**YGRKKRRQRRRPPQ**	**GGG**	**QWPALPKIEAQDG**			diseño	TAT	N-terminal
6	**YGRKKRRQRRRPPQ**	**GGG**	**ASFINILVEVQDDG**			dengue1	TAT	C-terminal
7	**YGRKKRRQRRRPPQ**	**GGG**	**VSHNLMITVDDDG**			dengue3	TAT	C-terminal
8	**YGRKKRRQRRRPPQ**	**GGG**	**SSPIIEVKQDEDG**			dengue4	TAT	C-terminal
9	**YGRKKRRQRRRPPQ**	**GGG**	**SSERVDVRLDDDG**			WNV	TAT	C-terminal
10	**YGRKKRRQRRRPPQ**	**bA**	**SSPILSITISEDGSMSI**	**GGG**	**GRR***	dengue 2	TAT	C-terminal
11	**YGRKKRRQRRRPPQ**	**GGG**	**DLELERAADVKWE**			dengue 2	TAT	N-terminal
12	**RRRRRRRRRR**	**GGG**	**DLELERAADVKWE**			dengue 2	R10	N-terminal
13	**YGRKKRRQRRRPPQ**	**GGG**	**DLELERAADVKWE**	**GG**	**KDEL***	dengue 2	TAT	N-terminal
14	**YGRKKRRQRRRPPQ**	**GGG**	**DLSLEKAAEVSWE**			dengue1	TAT	N-terminal
15	**YGRKKRRQRRRPPQ**	**GGG**	**DLTVEKAADVTWE**			dengue3	TAT	N-terminal
16	**YGRKKRRQRRRPPQ**	**GGG**	**DLSLEKAANVQWD**			dengue4	TAT	N-terminal
17	**YGRKKRRQRRRPPQ**	**GGG**	**DMWIERTADITWE**			WNV	TAT	N-terminal
18	**YGRKKRRQRRRPPQ**	**GGG**	**LEGSDISPSTISI**			negative control	TAT	
19	**YGRKKRRQRRRPPQ**					negative control	TAT	
20	**YGRKKRRQRRRPPQ**	**GGG**	**TGSVVIVGRIIL**			HCV	TAT	N-terminal
21	**YGRKKRRQRRRPPQ**	**GGG**	**TGSVVIVGQIIL**			HCV	TAT	N-terminal
22	**CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**			dengue 2	FG-den3	C-terminal
23	**CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**			dengue 2	FG-den3	N-terminal
24	**CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDGSMSI**	**GGG**	**GRR***	dengue 2	FG-den3	C-terminal
25	**CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**	**GG**	**KDEL***	dengue 2	FG-den3	C-terminal
26	**CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**	**GG**	**KDEL***	dengue 2	FG-den3	N-terminal
27	**myr-bA-CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**	**bA**	**LRRRRL**	dengue 2	FG-den3	C-terminal
28	**myr-bA-CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**	**bA**	**LRRRRL**	dengue 2	FG-den3	N-terminal
29	**pal-bA-CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**	**bA**	**LRRRRL**	dengue 2	FG-den3	C-terminal

(continued)

No	[P]	[L1]	[I]	[L2]	[T]	Virus	Penetrating peptide	Target domain
30	**pal-bA-CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**	**bA**	**LRRRRL**	dengue 2	FG-den3	N-terminal
31	**myr-bA-CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**			dengue 2	FG-den3	C-terminal
32	**myr-bA-CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**			dengue 2	FG-den3	N-terminal
33	**pal-bA-CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**			dengue 2	FG-den3	C-terminal
34	**pal-bA-CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**			dengue 2	FG-den3	N-terminal
35	**myr-bA-CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**	**bA**	**LRRRRLKDEL***	dengue 2	FG-den3	C-terminal
36	**myr-bA-CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**	**bA**	**LRRRRLKDEL***	dengue 2	FG-den3	N-terminal
37	**pal-bA-CSNIVIGIGDKALKINWC**	**bA**	**SSPILSITISEDG**	**bA**	**LRRRRLKDEL***	dengue 2	FG-den3	C-terminal
38	**pal-bA-CSNIVIGIGDKALKINWC**	**bA**	**DLELERAADVKWE**	**bA**	**LRRRRLKDEL***	dengue 2	FG-den3	N-terminal
39	**RRRRRRRRRR**	**GGG**	**SSPILSITISEDG**	**GG**	**KDEL***	dengue 2	R10	C-terminal

*: carboxilic C-terminal end. Myr-: covalent attachment of a myristoyl group to the N-terminal end of the peptide. Pal-: covalent attachment of a palmitoyl group to the N-terminal end of the peptide. bA: beta-Alanine. FG-den3: sequence corresponding to the FG hairpin of domain III of the envelope protein from DV3, two cysteins bound by disulfide bridge are added at the N- and C-terminal ends of the segment.

Table 2. Design of chimerical peptides according to the topology [I]-[L_3]-[P]-[L_4]-[T]

No	*[I]*	*[L3]*	*[P]*	*[L4]*	*[T]*	*Virus*	*Penetrating Peptide*	*Target domain*
1	**SSPILSITISEDG**	**GGG**	**YGRKKRRQRRRPPQ**			dengue2	TAT	C-terminal
2	**SSPILSITISEDG**	**GGG**	**RRRRRRRRRR**			dengue2	R10	C-terminal
3	**SSPILSITISEDG**	**GGG**	**RRRRRRRRRR**	**GG**	**KDEL***	dengue2	R10	C-terminal
4	**SSPILSITISEDG**	**GGG**	**YGRKKRRQRRRPPQ**	**GG**	**KDEL***	dengue2	TAT	C-terminal
5	**ASHNILVEVQDDG**	**GGG**	**YGRKKRRQRRRPPQ**			dengue1	TAT	C-terminal
6	**VSHNLMITVDDDG**	**GGG**	**YGRKKRRQRRRPPQ**			dengue3	TAT	C-terminal
7	**SSPIIEVKQDEDG**	**GGG**	**YGRKKRRQRRRPPQ**			dengue4	TAT	C-terminal
8	**SSERVDVRLDDDG**	**GGG**	**YGRKKRRQRRRPPQ**			WNV	TAT	C-terminal
9	**DLELERAADVKWE**	**GGG**	**YGRKKRRQRRRPPQ**			dengue2	TAT	N-terminal
10	**DLELERAADVKWE**	**GGG**	**YGRKKRRQRRRPPQ**	**GG**	**KDEL***	dengue2	TAT	N-terminal
11	**DLELERAADVKWE**	**GGG**	**RRRRRRRRRR**			dengue2	R10	N-terminal
12	**DLELERAADVKWE**	**GGG**	**RRRRRRRRRR**	**GG**	**KDEL***	dengue2	R10	N-terminal
13	**DLSLEKAAEVSWE**	**GGG**	**RRRRRRRRRR**			Dengue	R10	N-terminal
14	**DLTVEKAADVTWE**	**GGG**	**RRRRRRRRRR**			Dengue3	R10	N-terminal
15	**DLSLEKAANVQWD**	**GGG**	**RRRRRRRRRR**			Dengue4	R10	N-terminal
16	**DMWIERTADITWE**	**GGG**	**RRRRRRRRRR**			WNV	R10	N-terminal
17	**SSPILSITISEDG**	**bA**	**LRRRRLbALRRRRL**	**bA**	**KDEL***	dengue2	2(LR4L)	C-terminal
18	**SSPILSITISEDG**	**bA**	**LRRRRLbALRRRRL**			dengue2	2(LR4L)	C-terminal
19	**SSPILSITISEDG**	**bA**	**LRRRRLRRRRL**			dengue2	2(LR4L)	C-terminal
20	**myr-SSPILSITISEDG**	**bA**	**LRRRRLRRRRL**			dengue2	2(LR4L)	C-terminal
21	**pal-SSPILSITISEDG**	**bA**	**LRRRRLRRRRL**			dengue2	2(LR4L)	C-terminal
22	**myr-SSPILSITISEDG**	**bA**	**LRRRRLbALRRRRL**	**bA**	**KDEL***	dengue2	2(LR4L)	C-terminal
23	**myr-SSPILSITISEDG**	**bA**	**LRRRRLbALRRRRL**			dengue2	2(LR4L)	C-terminal
24	**pal-SSPILSITISEDG**	**bA**	**LRRRRLbALRRRRL**	**bA**	**KDEL***	dengue2	2(LR4L)	C-terminal
25	**pal-SSPILSITISEDG**	**bA**	**LRRRRLbALRRRRL**			dengue2	2(LR4L)	C-terminal
26	**DLELERAADVKWE**	**bA**	**LRRRRLbALRRRRL**	**bA**	**KDEL***	dengue2	2(LR4L)	N-terminal
27	**myr-DLELERAADVKWE**	**bA**	**LRRRRLbALRRRRL**	**bA**	**KDEL***	dengue2	2(LR4L)	N-terminal
28	**pal-DLELERAADVKWE**	**bA**	**LRRRRLbALRRRRL**	**bA**	**KDEL***	dengue2	2(LR4L)	N-terminal
29	**DLELERAADVKWE**	**bA**	**LRRRRLRRRRL**			dengue2	2(LR4L)	N-terminal
30	**myr-DLELERAADVKWE**	**bA**	**LRRRRLRRRRL**			dengue2	2(LR4L)	N-terminal
31	**pal-DLELERAADVKWE**	**bA**	**LRRRRLRRRRL**			dengue2	2(LR4L)	N-terminal
32	**DLSLEKAAEVSWE**	**bA**	**LRRRRLRRRRL**			Dengue1	2(LR4L)	N-terminal

(continued)

No	[I]	[L3]	[P]	[L4]	[T]	Virus	Penetrating Peptide	Target domain
33	**DLTVEKAADVTWE**	**bA**	**LRRRRLRRRRL**			Dengue3	2(LR4L)	N-terminal
34	**DLSLEKAANVQWD**	**bA**	**LRRRRLRRRRL**			Dengue4	2(LR4L)	N-terminal
35	**DLELERAADVKWE**	**bA**	**CSNIVIGIGDKALKINWC**			dengue2	FG-den3	N-terminal
36	**myr-DLELERAADVKWE**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRL**	dengue2	FG-den3	N-terminal
37	**pal-DLELERAADVKWE**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRL**	dengue2	FG-den3	N-terminal
38	**myr-DLELERAADVKWE**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRLKDEL***	dengue2	FG-den3	N-terminal
39	**pal-DLELERAADVKWE**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRLKDEL***	dengue2	FG-den3	N-terminal
40	**SSPILSITISEDG**	**bA**	**CSNIVIGIGDKALKINWC**			dengue2	FG-den3	C-terminal
41	**myr- SSPILSITISEDG**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRL**	dengue2	FG-den3	C-terminal
42	**pal- SSPILSITISEDG**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRL**	dengue2	FG-den3	C-terminal
43	**myr- SSPILSITISEDG**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRLKDEL***	dengue2	FG-den3	C-terminal
44	**pal-SSPILSITISEDG**	**bA**	**CSNIVIGIGDKALKINWC**	**bA**	**LRRRRLKDEL***	dengue2	FG-den3	C-terminal

*: carboxilic C-terminal end. Myr-: covalent attachment of a myristoyl group to the N-terminal end of the peptide. Pal-: covalent attachment of a palmitoyl group to the N-terminal end of the peptide. bA: beta-Alanine. FG-den3: sequence corresponding to the FG hairpin of domain III of the envelope protein from DV3, two cysteins bound by disulfide bridge are added at the N- and C-terminal ends of the segment.

[0091] The present invention concerns also the design of antiviral chimerical peptides against the other members of the *Flaviviridae* family. Peptide inhibitors against other *Flaviviridae* include as [I] segments, the analogous segments from the corresponding NS2B protein sequence (in *Flavivirus*) or NS4A (in *hapacivirus*). In the list of sequences of the present invention we include additional chimerical peptides analogous to those shown on tables 1 and 2, whose [I] segment corresponds to other *Flavivirus* (YFV, JEV, TBE, WNV) and the *Hepacivirus* HCV.

[0092] As [P] segments we consider the TAT peptide, R10, penetratin, the cationic sequences LRRRRLRRRRL and LRRRRL-bAla-RRRRL and the segment S376-W391 of the envelope protein of DV3 (loop FG of domain III). The later segment includes cysteines at its N- and C-terminal ends, which form a disulfide bridge and stabilize the beta hairpin conformation observed in the three dimensional structure of the envelope protein.

[0093] As terminal [T] segments we include the ER retention signals LRRRRL, KDEL and their combination LRRRRLK-DEL. The presence of these signals enhances the effective localization of peptides in the ER, which affect favorably their antiviral activity. We also include as [T] segment the sequence GRR, linked by the tripeptide GGG to the [I] segment of sequence S_{70}-I_{86}. As shown in figure 1C, peptides with this primary structure bind to the C-terminal domain of NS3pro protein and the GRR segment localizes at the protease active site, blocking its interaction with substrates. As linker segments we include in table 1 and 2 the tripeptide GGG, the dipeptide GG and the amino acid beta-Alanine.

[0094] Peptides myristoylated and palmitoylated at the N-terminal end are also included. The lipidation of these peptides increases the efficiency of the adhesion to the plasma membrane, cell entry and the final localization in the RE membrane. Lipidation is carried out by chemical methods. In the table 1 and 2, the lipids are attached directly to the N-terminal ends or to an N-terminal beta-Alanine residue.

[0095] Various peptides segments included in table 1 and 2 display more than one single function. The segments LRRRRLRRRRL and LRRRRL-bAla-RRRRL besides being cell penetrating peptides comprise two consecutive ER retention signals.

[0096] The [P] segment corresponding to the sequence of the region S376-W391 of the envelope protein from DV3, besides being a cell penetrating peptide, is also an inhibitor of the virus entry into the cells. Therefore the use of this segment in peptides of the present invention increases the inhibitory effect of these peptides.

[0097] The peptides of the present invention could be obtained by chemical synthesis o by recombinant DNA technology, alone or as part of fusion proteins. Expression as fusion proteins can increase the expression levels and stability of peptides against degradation by host proteases. These peptide sequences could be joined to the fusion proteins through linkers corresponding to substrate sequences of specific proteases, and thus the peptides can be isolated by successive proteolysis and purification.

Peptide synthesis

[0098] Solid phase peptide synthesis was performed on an Fmoc-AM-MBHA resin, using the Fmoc/tBu strategy (Barany, G. and Merrifield, R. B. J Am Chem Soc. 99 (1977) 7363-7365). The synthesis was carried out manually in 10 ml syringes equipped with porous frit and all reactive and solvents were discarded by vacuum filtration. The amino acids were coupled by activation with DIC/HOBt, monitoring the completion of the coupling reaction by the ninhydrin assay (Kaiser, E., Colescott, R. L., Bossinger, C. D., Cook, P. I. Anal Biochem. 34 (1970) 595-598).

[0099] The synthesized peptides were detached from the resin by treatment with a solution of TFA/EDT/H2O/TIS (94%/2.5%/2.5%/1%), precipitated with ether, and lyophilized during 72 h. Peptide cyclization by forming a disulphide bridge was achieved by oxidation with DMSO (Andreu, D., Albericio, F., Sol, N. A., Munson, M. C., Ferrer, M. and Barany, G., Pennington, M. W. and Dunn, B. M. (Eds), Peptide Synthesis Protocols, Methods in Molecular Biology, Totowa, NJ, 1994, pp. 91-169). In all cases, the peptides were purified by RP-HPLC and the collected fractions were analyzed again by analytical RP-HPLC. The final preparation of each peptide was obtained by pooling the fractions with a chromatographic purity equal to or higher than 99%. The mass of the peptide on each final preparation was verified by ESI-MS mass spectrometry.

[0100] The mass spectra were acquired with a hybrid mass spectrometer with octagonal geometry QTOF-2TM (Micromass, UK), equipped with a Z-spray electronebulization ionization source.

[0101] The software used for the acquisition and processing of the spectra was MassLinx, ver. 3.5 (Waters, USA).

Example 2

Inhibition of viral infection in Vero cells

[0102] In order to prove the antiviral activity *in vitro* of chimerical peptides described on the present invention, the peptides were tested in plaque reduction neutralization assay in Vero cells (PRNT). Vero cells were grown in 24-well plates to approximately 90% confluence, and washed twice with MEM medium without FCS. Peptide dilutions were added according to the particular assay and incubated typically during 1 h at 37 °C. After the incubation, the virus was

added at a multiplicity of infection of 0.1, followed by a subsequent incubation for 1 hour at 37 C. In certain experiments the peptides were added simultaneously with the virus (without preincubation) or the peptide preincubation time was modified. At the end of the second incubation, the unbound virus was eliminated by washing, and the cells were incubated for 5 days at 37 C in high density medium (MEM supplemented with non essential amino acids, 1% FCS, 1% carboxymethylcellulose) in order to propitiate the appearance of lytic plaques. The plaques were visualized by staining with 0.1% Naphtol Blue Black in 0.15 Mol/L sodium acetate. Two replicates were used per experimental point in each assay, and three independent determinations were performed. The inhibition percentage was calculated according to the expression:

$$100x\left[1-\frac{No.plaques}{No.Plaques.virus.control.}\right]$$

[0103] The figure 2 shows that peptide NS2Bden2+TAT (peptide 1 of the table 1) inhibit the infection by DV2, in a dose dependent manner, with a IC50 of approximately 50-60 μM. The peptides showed no signs of toxicity on the cells at the assayed conditions. The sequence of peptide NS2Bden2+TAT contains two essential modules: the cell penetrating segment TAT and the protease NS3pro activation inhibitor segment, which targets the C-terminal domain of the protease. The TAT peptide did not show antiviral activity and caused an increase in the number of plaques (figure 2B). This result is consistent with the peptide design: the antiviral activity residing on the segment specifically related to the VD2 (strain S16803). The observed enhancement of the virus infection in presence of the TAT peptide could be related to an increase in the entry of the virus into the cells facilitated by the PTD property of this peptide. Increasing cell penetration of viruses mediated by PTD has been previously observed in other systems (Gratton JP, Yu J, Griffith JW, et al. Cell-permeable peptides improve cellular uptake and therapeutic gene delivery of replication-deficient viruses in cells and in vivo. Nat Med 2003; 9: 357-63).

[0104] The presence of the TAT peptide segment in the sequence of peptide NS2Bden2+TAT is necessary for its antiviral activity, because the NS3pro protease activation inhibitor segment [I] per se does not show antiviral activity *in vitro* (figure 5).

[0105] The figure 2 shows that the antiviral activity of the chimerical peptide NS2Bden2+TAT increases if the peptide is preincubated with cells 1 h before the addition of the virus. This result is consistent with the fact that the target for the antiviral activity of the peptide is an intracellular event, and preincubation allows a higher amount of peptide to penetrate into the cells and localize at the ER membrane, previously to the beginning of the virus replication.

[0106] In order to characterize the effect of preincubation on the antiviral activity of peptide NS2Bden2+TAT we studied the relationship between plaque reduction neutralization, preincubation time and peptide dose. As negative control we used a non related chimerical peptide displaying an structure similar to NS2Bden2+TAT. This peptide contains at the N-terminal end the sequence of the TAT peptide and at its C-terminal end a sequence which have been shown to bind to the protein E7 from human papilloma virus. The figure 3 shows that for peptide concentrations less than 100 μM, preincubation is necessary for the antiviral activity and this activity increases with the time of preincubation between 0 and 1 hour. This result is consistent with the intracellular localization of the target for the antiviral effect and the need for peptide transport from the extracellular space to the cytosol.

[0107] However, between 1 and 3 hours of preincubation, we do not observe more differences. One possible explanation could be that at these times equilibrium is reached between the kinetics of accumulation of peptide in the cytosol and the intracellular degradation of the peptide.

[0108] The negative control peptide does not show antiviral activity at any of the assayed conditions indicating that the antiviral effect of the peptide NS2Bden2+TAT is due specifically to the sequence of the segment corresponding to the NS2B protein.

[0109] Figure 4 shows additional evidence indicating that the antiviral activity of the peptide NS2Bden2+TAT is related to an intracellular effect. In this case, besides the previously described usual assay conditions (figure 4A), the antiviral activity of the peptide was also determined when the peptide was retired from the media by successive washing of cells previous to the addition of the virus (figure 4B). In both conditions of the assay, the antiviral activity of the peptide was very similar, indicating that the antiviral effect depends on previously internalized peptide. In these assays, the peptide 18 of table 1 was used as negative control. This peptide has a design similar to the peptide NS2Bden2+TAT, but the C-terminal segment consists of a sequence of the same length and amino acid composition as the NS3pro protease activation inhibitor [I] segment of NS2Bden2+TAT, but the original sequence was randomized. This peptide did not show antiviral activity in any condition, indicating that the antiviral activity of NS2Bden2+TAT depends on the selected sequence fragment of NS2B.

Example 3

Effect of the nature of the cell penetrating peptide and the ER retention signal on the antiviral activity of peptides

[0110] In order to determine the role of the cell penetrating peptide and the ER retention signal on the antiviral activity of peptides of the present invention we tested peptides No 1, 2, 3 and 4 of table 1 for inhibition of the viral infection by VD2 in Vero cells, using the assay described in the example 2.

[0111] The peptides 2 and 3 have a primary structure similar to the peptide NS2Bden2+TAT (péptido 1), but displaying penetratin and decarginine respectively as cell penetrating segments. The peptide 4 consists on the addition of the KDEL signal at the C-terminal end of peptide NS2Bden2+TAT. The C-terminal group of peptide 4 is carboxylic in order to make functional the ER retention signal.

[0112] The figure 5 and the table 3 show that the peptide NS2Bden2-pRR (peptide 3 on table 1) displays the higher antiviral activity, almost an order more potent than the peptide NS2Bden2+TAT. One possible explanation is that decaarginine peptide is more resistant to proteolysis in the intracellular environment of the cell ((Fischer, R., Kohler, K., Fotin-Mleczek, M., and Brock, R. 2004. A stepwise dissection of the intracellular fate of cationic cell-penetrating peptides. J. Biol. Chem. 279, 12625-35). The peptide NS2Bden2-pP2 (peptide 2) shows an antiviral activity similar to NS2Bden2+TAT, however it displays significant cytotoxicity. The addition of the KDEL signal increase slightly the antiviral activity of the peptide, suggesting that the peptide NS2Bden2+TAT uses at least partially the retrograde transport to get access into the cytosol.

[0113] The peptide NS2Bden2 which lacks the cell penetrating segment does not inhibit the antiviral infection, showing that the inclusion of this kind of segment is required in the peptides of the present invention.

Table 3. PRNT50 and cytotoxicity (CTE) of peptides in Vero cells

Peptide	PRNT50	CTE
NS2Bden2+TAT	60 µM	150 µM
NS2Bden2+TAT+KDEL	40 µM	>150 µM
NS2Bden2-pRR	<10 µM	>50 µM
NS2Bden2-pP2	75 µM	50 µM
NS2Bden2	--	--

CTE: cytotoxic effect, the values indicate peptide concentrations causing damage to 50% of the monolayer.

Example 4

Antiviral activity of peptides against homologous and heterologous virus

[0114] An expected property of antiviral agents is to possess a wide spectrum of antiviral activity, at least against the related most similar viruses. This is also the case in the development of antiviral molecules against dengue virus: 1) dengue is actually a complex of four different viruses, 2) there are difficulties for an early specific diagnosis and 3) in the affected countries, dengue is frequently endemic, occurring the cocirculation of more that one serotype.

[0115] The four dengue serotypes are related viruses with similar amino acid sequences (70-80% identity) of their structural and non structural proteins. Therefore, it is reasonable that differences in the amino acid sequences of NS2B and/or NS3pro could affect the infection inhibitory capacity of peptides of the present invention against the heterologous viruses.

[0116] In order to evaluate the cross-reactivity or serotype specificity of the antiviral activity of peptides of the present invention, we tested peptides 1, 3 and 6 of the table 1 for inhibition of the viral infection by DV1-3 in Vero cells, using the assay described in the example 2. The tested viral strains were West Pac 74 of DV1, S16803 of DV2 and CH53489 of DV3. The peptide 6 (NS2Bden1+TAT) has a primary structure similar to NS2Bden2+TAT, but it has a NS3 activation inhibitor segment corresponding the protein NS2B from DV1. We also included in the analysis the peptide 5, designed by computational methods.

[0117] The figure 6 shows that the peptide NS2Bden2+pRR (peptide 3 on table 1) is equally potent against the three serotypes. The peptide NS2Bden2+TAT also inhibits the serotypes 1-3 although with a lower antiviral activity. The peptide NS2Bden1+TAT (peptide 6) however shows only partial inhibition against serotypes 1 and 3. This result is consistent with the fact that serotypes 1 and 3 are phylogenetically closer to each other and their proteins are more similar.

Claims

1. Chimerical peptides, **characterized by** having a primary structure comprising at least one segment inhibitor of the activation of the NS3pro protease from a virus of the *Flaviviridae* family and a cell penetrating segment and these peptides are able to inhibit or attenuate the infection by the virus.

2. Chimerical peptides, according to the claim 1, **characterized by** containing a segment inhibitor of the activation of the NS3pro protease, this segment is a peptide able to bind to NS3pro and to block or interfere the interaction of NS2B and NS3pro from a *Flavivirus* or the protein NS4A from a *Hepacivirus,* and this blocking or interference results in the formation of a NS3 protein displaying a diminished proteolytic activity or without proteolytic activity.

3. Chimerical peptides according to the claim 1, **characterized by** containing a segment inhibitor of the activation of the NS3pro protease, this segment is a peptide able to bind to NS3pro and contact at least one residue comprised in the region Gly21 - Lys28 of the N-terminal domain of the NS3pro protein from DV2 or structurally equivalent residues of the NS3pro protein from other *Flavivirus.*

4. Chimerical peptides according to the claim 1, **characterized by** containing a segment inhibitor of the activation of the NS3pro protease, this segment is a peptide able to bind to NS3pro and contact at least one residue comprised in the region Gly114-Thr118, Ser127 and Val162 of the C-terminal domain of the NS3pro protein from DV2 or structurally equivalent residues of the NS3pro protein from other *Flavivirus.*

5. Chimerical peptides according to the claim 1, where the mentioned segment inhibitor of the activation of the NS3pro protease contact at least one residue comprised in the region Glu32-Thr38 of the N-terminal domain of the NS3pro protein from HCV or structurally equivalent residues of the NS3pro protein from other *Hepacivirus.*

6. Chimerical peptides according to the claim 1, **characterized by** containing a segment inhibitor of the activation of the NS3pro protease which comprise the region Asp50-Glu62 of the NS2B protein from DV2 or the structurally equivalent segment of the protein NS2B from other *Flavivirus*, or an amino acid sequence showing a sequence similarity higher of equal to the 80% identity.

7. Chimerical peptides according to the claim 1, **characterized by** containing a segment inhibitor of the activation of the NS3pro protease which comprise the region Ser70-Gly82 of the NS2B protein from DV2 or the structurally equivalent segment of the protein NS2B from other *Flavivirus,* or an amino acid sequence showing a sequence similarity higher of equal to the 80% identity.

8. Chimerical peptides according to the claim 1, **characterized by** containing a segment inhibitor of the activation of the NS3pro protease which comprise the region Thr20-Leu31 of the NS4A protein from HCV or the structurally equivalent segment of the protein NS4A from other *Hepacivirus,* or an amino acid sequence showing a sequence similarity higher of equal to the 80% identity.

9. Chimerical peptides according to the claim 1, where the mentioned virus from the *Flaviviridae* family is one of the following *Flavivirus*: West Nile virus, St Louis Encephalitis virus, DV1, DV2, DV3, DV4, Japanese Encephalitis virus, Yellow Fever virus, Kunjin virus, Kyasanur Forest Disease virus, Tick-borne Encephalitis virus, Murray Valley virus, LANGAT virus, Louping ill virus, Powassan virus.

10. Chimerical peptides according to the claim 1, where the mentioned virus from the *Flaviviridae* family is a *Hepacivirus* and specifically HCV.

11. Chimerical peptides according to the claim 1, **characterized by** containing a cell penetrating segment which is one of the following cationic PTDs: TAT peptide, heptaarginine, octaarginine, nonaarginine, decaarginine, or a peptide of sequence LRRRRLRRRRL or LRRRRL-bAla-LRRRRL.

12. Chimerical peptides according to the claim 1, **characterized by** containing a cell penetrating segment which comprises the region Ser376-Trp391 of the envelope protein from DV2 or a structurally equivalent segment from other serotype of dengue virus.

13. Chimerical peptides according to the claim 1, **characterized by** containing signals of retention and/or localization in the endoplasmic reticulum and the presence of this signal increases the antiviral activity of the peptide.

14. Chimerical peptides according to the claim 1, **characterized by** being lipidated and the lipidation increases the antiviral activity of the peptide.

15. Chimerical peptides according to the claim 1, **characterized by** having a primary structure included in the sequence listing.

16. A pharmaceutical composition **characterized by** containing one or more peptides according to the claim 1, and this pharmaceutical composition is efficacious for the prophylactic and/or therapeutic treatment against the infection by one or more viruses from the *Flaviviridae* family.

Figure 1A.

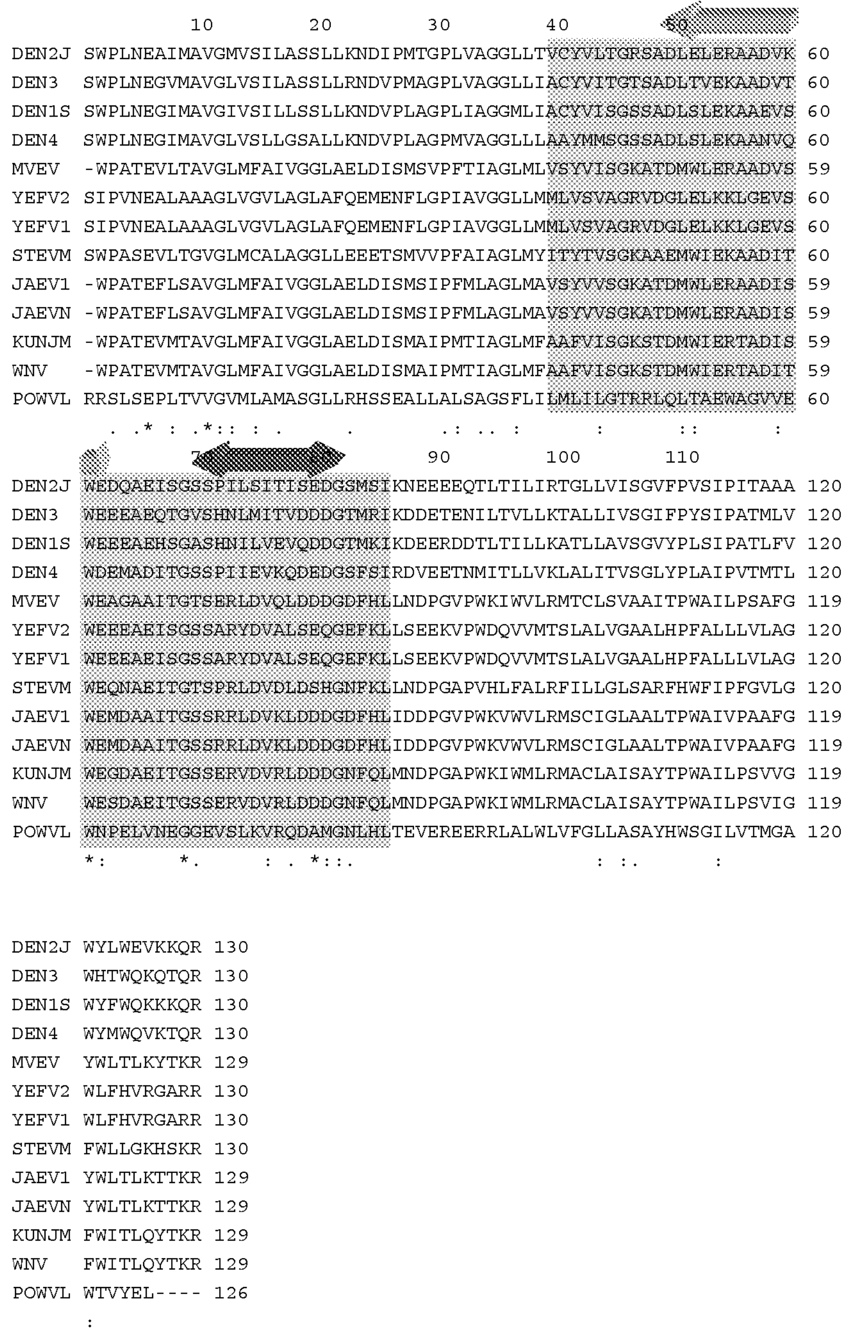


Figure 1.

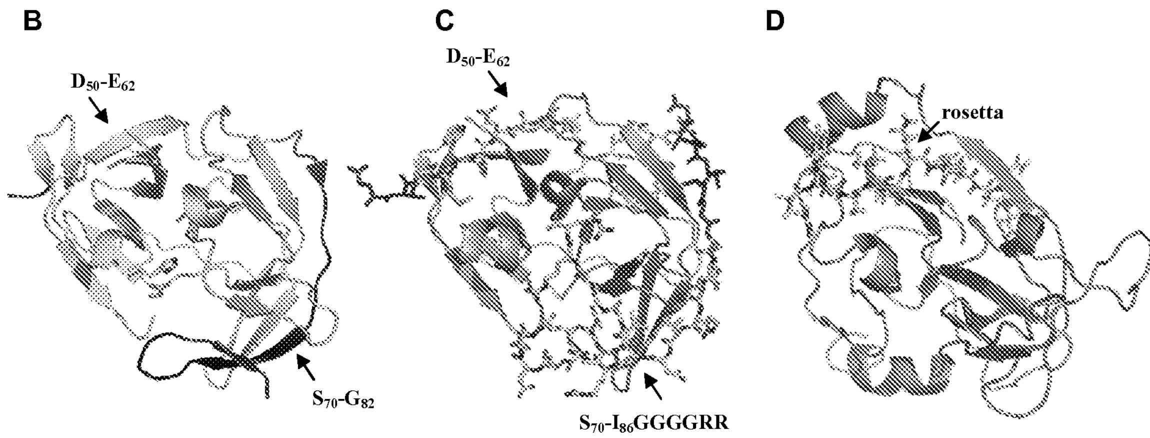


Figure 2.

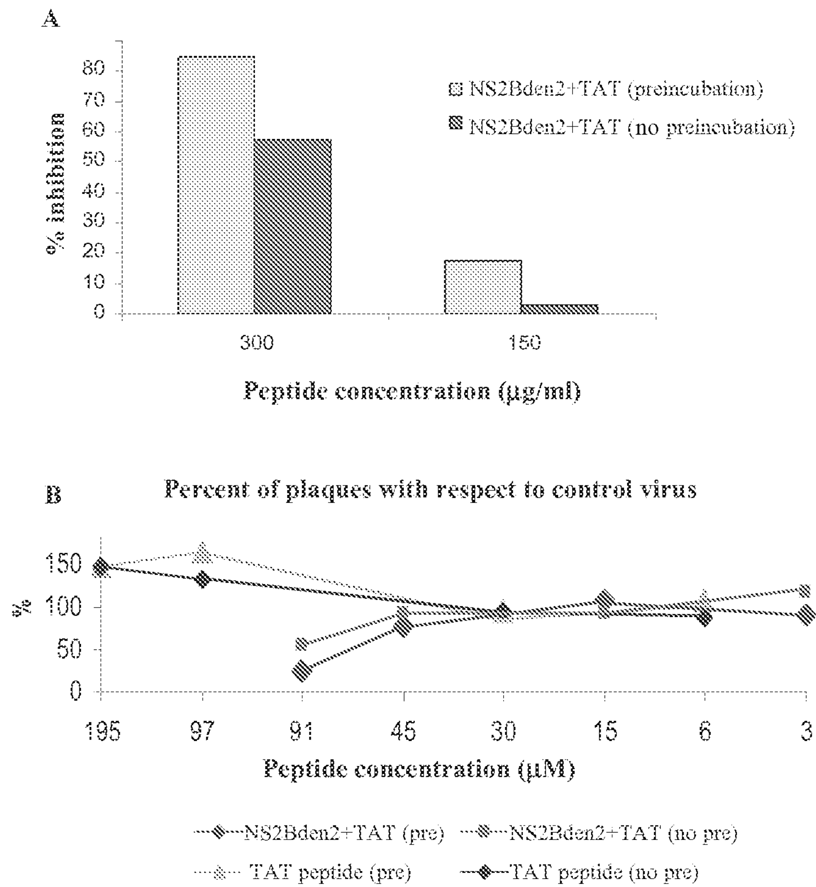
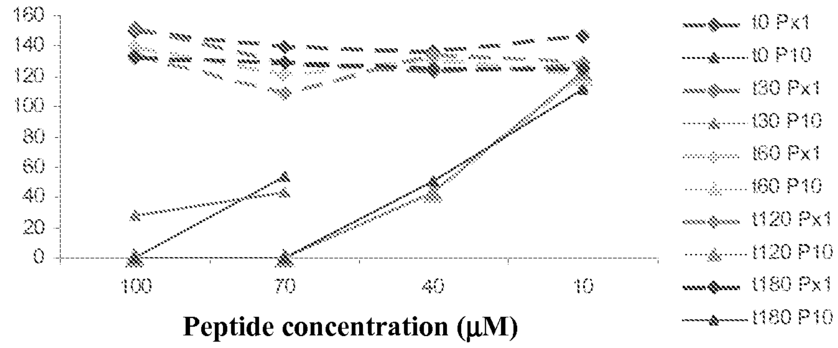


Figure 3.

A



B

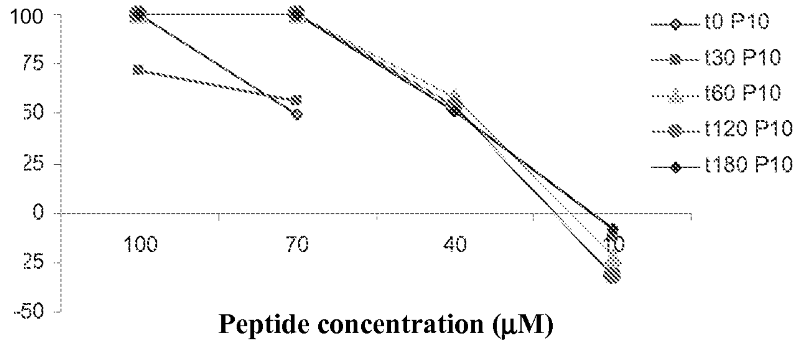
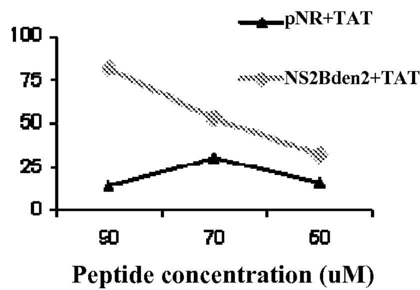


Figure 4.

A



B

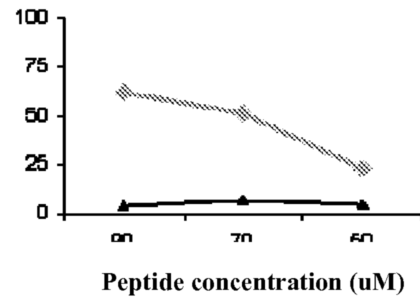


Figure 5.

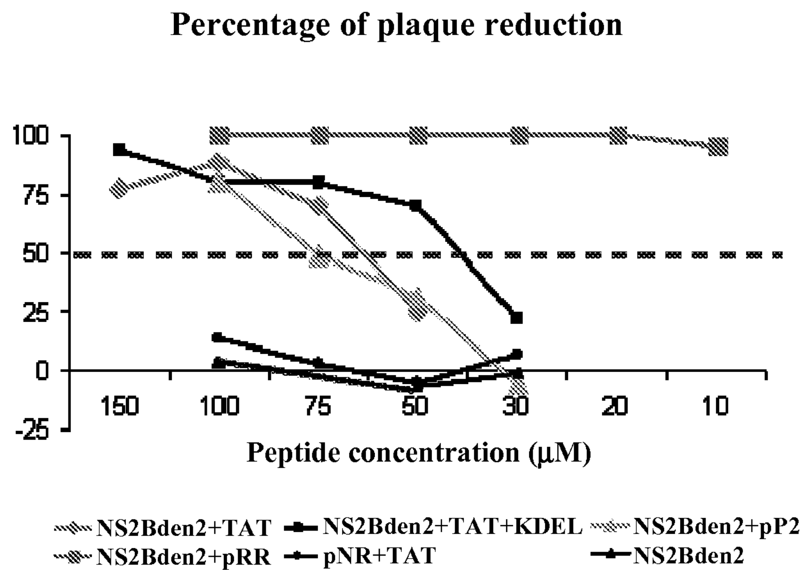
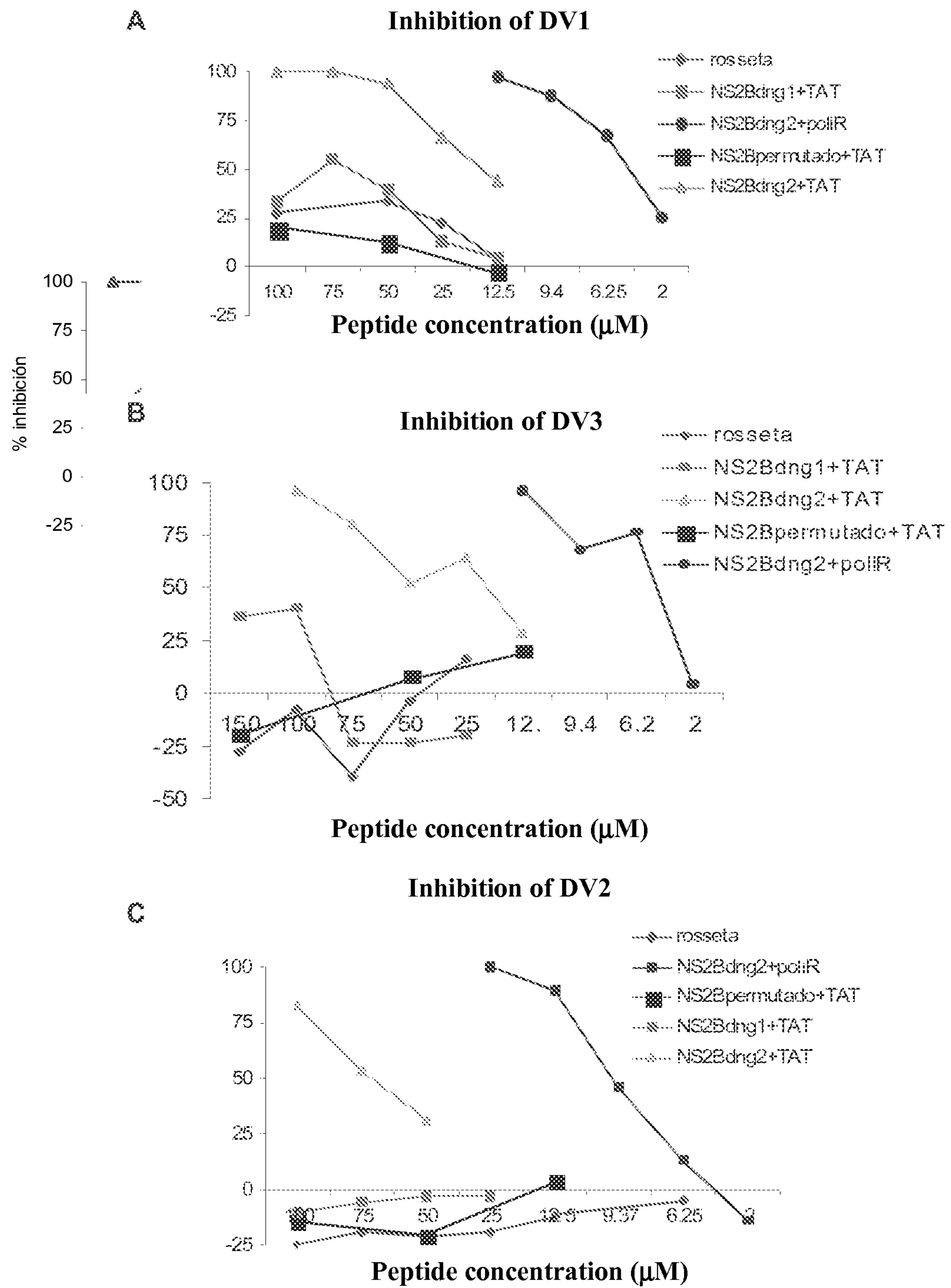


Figure 6.



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