

[DESCRIPTION]

[Title of Invention] ANTIVIRAL TREATMENT OF FLAVIVIRUS INFECTION

[Technical Field]

5 [0001]

The present invention generally relates to therapy of flavivirus infections and flavivirus related diseases in mammals, especially humans. More specifically, the present invention provides a novel therapeutic protocol using a target for the treatment and/or prevention of flavivirus induced infections and diseases and a screening method that can be used for the identification and development of a new substance with anti-flavivirus activity.

[Background Art]

15 [0002]

The genus Flavivirus of the family Flaviviridae includes many pathogens, such as Japanese encephalitis viruses (JEV), dengue viruses (DENV), and Zika viruses (ZIKV), which induce infections that have emerged as public health issues worldwide such as Japanese encephalitis, dengue fever, dengue hemorrhagic fever, and Zika fever.

Viruses in the genus Flavivirus are primarily arboviruses that often leads to a significant incidence rate and mortality rate in mammals and avians (Non Patent Literature 1). The geographical distribution of JEV ranges from South to Southeast Asia. JEV is zoonotic between pigs or avians and mosquitos (Non Patent Literatures 1 and 2).

[0003]

The genus Flavivirus of the family Flaviviridae includes more than 70 species of viruses, most of which are hematophagous arthropod-borne. Examples of mosquito-borne flaviviruses include Japanese encephalitis viruses (JEV), dengue viruses (DENV), Zika viruses (ZIKV), West Nile viruses (WNV), yellow fever viruses (YFV), and the like, and examples of tick-borne flaviviruses include tick-borne

encephalitis viruses (TBEV) (Non Patent Literature 3). 14
other species of flaviviruses with an unknown vector have
also been reported.

[0004]

5 A flavivirus is comprised of an enveloped virus having
a + strand RNA genome with a full length of about 11 kb,
wherein the 5' end has a cap structure and the 3' end does
not have a poly-A structure (Non Patent Literature 4). The
genome has a single reading frame. A polyprotein translated
10 from the genome is co- and post-translationally cleaved by
a host and virus protease into three structural proteins,
i.e., core, pre-membrane (prM), and envelope (E) proteins,
and 7 nonstructural proteins, i.e., NS1, NS2A, NS2B, NS3,
NS4A, NS4B, and NS5 (Non Patent Literature 5).

15 [0005]

A therapeutic drug that is specific to a flavivirus
infection has not been approved thus far. However,
development of many specific inhibitors is ongoing, with
further elucidation of the function and structure of
20 flavivirus proteins in recent years. Since NS3 has protease
activity and helicase activity that are both essential for
viral replication, protease activity inhibitors (Non Patent
Literatures 6 and 7) and helicase activity inhibitors (Non
Patent Literatures 8 and 9) have been developed. An effect
25 is also found in inhibitors against methyltransferase
activity of NS5 (Non Patent Literatures 10 and 11) and NS5
RNA dependent RNA polymerase inhibitors (Non Patent
Literatures 12 and 13). Furthermore, a structural pocket
that can be a target of an antiviral drug was recently
30 discovered in one of the structural proteins, i.e., E
protein (Non Patent Literature 14), which has led to a
search for a drug that inhibits the structural change in E
proteins upon infection (Non Patent Literatures 15 and 16).

[0006]

35 Besides the specific antiviral drugs described above,

commonly and broadly used antiviral drugs are also being studied. Since IFN is an antiviral drug that is approved by the USFDA, the effect thereof on flavivirus infections is also being studied. Although a certain effect on Saint Louis encephalitis virus infections was found in clinical trials using humans or monkeys (Non Patent Literature 17), a significant effect on a dengue virus (DENV) or Japanese encephalitis virus (JEV) infection has not been found thus far (Non Patent Literatures 18 and 19). A purine nucleoside analog ribavirin has broad antiviral activity whose effect has been studied on various viruses (Non Patent Literature 20). In *in vitro* studies on flaviviruses, an effect of suppressing viral replication due to GTP depletion by inhibition of inosine monophosphate dehydrogenase (Non Patent Literature 21) has been observed (Non Patent Literature 22). However, a significant effect was not found in a clinical trial for JEV infections in humans (Non Patent Literature 23). In view of the recent report of a nucleoside analog similar to ribavirin, i.e., ETAR, suppressing replication of flaviviruses such as DENV (Non Patent Literature 24), further study is expected.

[0007]

Antiviral drugs using new technologies such as RNAi that degrades a target RNA (Non Patent Literatures 25 and 26) and morpholino oligos (PMO) inhibiting translation (Non Patent Literatures 27 and 28) have also been studied, which were found to have certain antiviral action in animal models. Furthermore, a siRNA for JEV was fused to a rabies virus-derived peptide (Non Patent Literature 29), and siRNA for DENV was fused to a peptide specifically binding to a ligand on a dendritic cell (Non Patent Literature 30) to successfully incorporate the siRNAs specifically into the central nervous system and dendritic cells, respectively. It is understood that such technologies enable a drug to act only in a specific organ/cell with

infection/proliferation of the target virus and reduce side effects, so that application of such technologies have expectations.

In this manner, an expected therapeutic result is attained, but there is currently no therapeutic drug as a definitive measure for flavivirus infections.

[0008]

For example, pan-caspase inhibitors, cyclin-dependent kinase (CDK) inhibitors, category B anthelmintics, and proteasome inhibitors are comprehensively described as compounds with an effect of suppressing infections of a type of flavivirus, i.e., Zika virus (Patent Literature 1 and Non Patent Literature 31), which only found a phenomenon of suppressing the proliferation of Zika virus, while it is unclear which of infection, replication, packaging, or budding of viruses the inhibitors act on.

[Citation List]

[Patent Literature]

[0009]

[PTL 1] WO 2018/017426 A1

[Non Patent Literature]

[0010]

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[NPL 4] Wengler G, et al. Virology. 89: 423-37, 1978

[NPL 5] Lindenbach BD, et al., The Viruses and Their Replication. In: Fields virology (Fifth edition). Knipe DM, Howley PM (Ed), Lippincott-Raven, Philadelphia, PA, 1102-53, 2007

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- 30 [NPL 28] Stein DA, et al. J. Antimicrob. Chemother. 62: 555-65, 2008
- [NPL 29] Kumar P, et al. Nature. 448: 39-43, 2007
- [NPL 30] Subramanya S, et al. J. Virol. 84: 2490-501, 2010
- [NPL 31] Xu M, et al. Nat. Med. 1101-1107, Vol. 22, Num. 10, 35 2016

[Summary of Invention]

[Technical Problem]

[0011]

Non-human mammals and avians are carrier
5 hosts/amplifiers of viruses in the genus Flavivirus of the
family Flaviviridae, which are mediated by mosquitos or
ticks. Since it is difficult to completely insulate human
lives from contact with infectious mosquitos or ticks, it
is impossible to completely eliminate flavivirus infections.
10 In this regard, search for an antiviral therapy against
flaviviruses by the inventors and diligent study thereon
resulted in a surprising finding for inhibiting viral
replication.

[Solution to Problem]

15 [0012]

The inventors found by chance that a certain type of a
pharmacologically active compound exerts an effect on the
morphology of a nucleolus in a biological cell, and found
that this inhibits viral replication due to some type of
20 action to complete the present invention.

[0013]

Specifically, the present invention includes the
following embodiments.

<Antiviral pharmaceutical composition>

25 [1]

An anti-flavivirus pharmaceutical composition
comprising a substance exerting an effect on a morphology
of a nucleolus in a biological cell.

[2]

30 The anti-flavivirus pharmaceutical composition of [1],
comprising the substance exerting an effect on a morphology
of a nucleolus in a biological cell as an active ingredient
having anti-flavivirus activity.

[3]

35 The pharmaceutical composition of [1] or [2], wherein

the substance exerting an effect on a morphology of a nucleolus in a biological cell is a substance inducing a morphological abnormality in the nucleolus.

[4]

5 The pharmaceutical composition of any one of [1] to [3], wherein the substance exerting an effect on a morphology of a nucleolus in a biological cell is also involved in a change in localization of a core protein of a virus in the biological cell.

10 [5]

The pharmaceutical composition of any one of [1] to [4], wherein the substance exerting an effect on a morphology of a nucleolus in a biological cell induces inhibition of viral replication.

15 [0014]

[6]

The pharmaceutical composition of any one of [1] to [5], wherein anti-flavivirus activity is anti-flavivirus activity against a flavivirus selected from a Japanese encephalitis virus, a dengue virus, a Zika virus, a West Nile virus, and a yellow fever virus.

[7]

25 The pharmaceutical composition of any one of [1] to [6], wherein the substance exerting an effect on a morphology of a nucleolus in a biological cell is a substance having CDK inhibitory activity, a substance having rRNA transcription inhibitory activity, a substance having DNA topoisomerase inhibitory activity, or a substance having GSK-3 inhibitory activity.

30 [8]

The pharmaceutical composition of [7], wherein the substance having CDK inhibitory activity is selected from: alsterpaullone, 2-cyanoethyl; 5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide; (4-(2-amino-4-

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methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine;
 P276-00; A-674563; SU9516; SNS-032 (BMS-387032);
 dinaciclib; flavopiridol; AT7519; flavopiridol
 hydrochloride; AT7519 HCl; AZD5438; R547; and kenpaullone.

5 [9]

The pharmaceutical composition of [8], wherein the
 substance having CDK inhibitory activity is selected from:
 alsterpaullone, 2-cyanoethyl; 5-amino-3-((4-
 (aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-
 10 1,2,4-triazole-1-carbothioamide; and (4-(2-amino-4-
 methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine.
 [0015]

[10]

The pharmaceutical composition of [7], wherein the
 15 substance having rRNA transcription inhibitory activity is
 selected from actinomycin D, doxorubicin, and aclarubicin.
 [11]

The pharmaceutical composition of [6], wherein the
 substance having DNA topoisomerase inhibitory activity is
 20 selected from doxorubicin, aclarubicin, ellipticine,
 idarubicin, idarubicin hydrochloride, epirubicin
 hydrochloride, mitoxantrone hydrochloride, and daunorubicin
 hydrochloride.

[12]

25 The pharmaceutical composition of [11], wherein the
 substance having DNA topoisomerase inhibitory activity is
 selected from doxorubicin, aclarubicin, and ellipticine.

[13]

The pharmaceutical composition of [7], wherein the
 30 substance having GSK-3 inhibitory activity is 1-
 Azakenpaullone.

[0016]

<Screening method>

[14]

35 A method of screening for a substance having anti-

flavivirus activity, comprising the following steps:

(a) contacting a biological cell with a candidate compound of a substance having anti-flavivirus activity *in vitro*;

(b) testing whether the candidate compound exerts an effect on a morphology of a nucleolus in the biological cell; and

(c) identifying the candidate compound as a substance having anti-flavivirus activity if the candidate compound exerts an effect.

[15]

10 The method of [14], wherein an effect due to the candidate compound is a morphological abnormality of a nucleolus in the biological cell in step (c).

[16]

15 The method of [14] or [15], wherein a morphological abnormality of a nucleolus in a biological cell is identified by a change in the formation and/or localization of a protein or nucleic acid localized in the nucleolus.

[0017]

[17]

20 The method of [16], wherein the candidate compound, which has a change in the formation and/or localization of a protein localized in a nucleolus that is a change relative to an effect of a control compound in the protein localized in the nucleolus, is determined as exerting an effect on a morphology of the nucleolus.

[18]

30 The method of any one of [14] to [17], wherein a protein localized in a nucleolus in a biological cell is one or more proteins selected from nucleophosmin, nucleolin, fibrillarin, RNA polymerase I, and flavivirus core proteins.

[19]

35 The method of any one of [14] to [18], wherein the biological cell is a cultured cell line derived from a eukaryote including humans such as a liver derived Huh7 cell, a primary culture cell, a primate derived cell line

such as a monkey kidney derived Vero cell, or a cell established by a mosquito such as a C6/36 cell.

[Advantageous Effects of Invention]

[0018]

5 The present invention discloses a compound that is effective in the prevention or treatment of a flavivirus infection, and further provides a screening method that gives the compound usefulness as a drug associated with a new mechanism of action, prevention of infection, potency
10 of inhibition and target.

While there are vaccines approved for use in humans against JEV, YFV, or TBEV infections in particular, thousands to tens of thousands or more patients are still annually reported worldwide today for each of the
15 infections. Non-human animals are carriers/amplifier hosts of flaviviruses, and it is difficult to completely insulate from contact with infectious mosquitos or ticks. Thus, development of a specific therapeutic method or vaccine is an important technical problem to be addressed.

20 The present invention can create a new anti-flavivirus therapeutic drug and provide a new therapeutic substance.

[Brief Description of Drawings]

[0019]

[Figure 1] Figure 1 is a group of diagrams that are
25 substitutes for pictures, showing localization of flavivirus core proteins (also known as C proteins or capsid proteins) in biological cells. Figure 1(A) shows localization in cells transiently expressing a Japanese encephalitis virus core protein: JEV core, dengue virus
30 core protein: DENV core, Zika virus core protein: ZIKV core, and West Nile virus core protein: WNV core. Figure 1(B) shows intracellular localization upon microinjection of various flavivirus core proteins into the cytoplasm of a cell. Figure 1(C) shows localization in the cytoplasm, cell
35 nucleus (genomic DNA), and nucleolus upon microinjection of

a Japanese encephalitis virus core protein into the cytoplasm of a cell.

[Figure 2] Figure **2** is a graph showing results from a method of screening a substance having anti-flavivirus activity using a change in intracellular localization of Japanese encephalitis virus core proteins as an indicator, and a group of pictures that are substitutes for diagrams, showing intracellular localization. Figure **2 (A)** is a graphical representation of the measurement of the amounts of core protein within the cell nucleus. Figure **2 (B)** shows intranuclear localization of Japanese encephalitis virus core proteins upon treatment with DMSO (control) and three CDK inhibitors.

[0020]

[Figure 3] Figure **3** is a group of pictures that are substitutes for diagrams, showing Cdk1/2 inhibitor III (5-amino-3((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide) changing the morphology of nucleoli in cells. Figure **3 (A)** shows that Cdk1/2 inhibitor III treatment induces nucleolar morphological abnormalities compared to DMSO treatment. Figure **3 (B)** shows localization in the cytoplasm, cell nucleus (genomic DNA), and nucleolus upon microinjection of a Japanese encephalitis virus core protein into the cytoplasm of alsterpaullone, 2-cyanoethyl treated cells.

[Figure 4] Figure **4** is a group of graphs showing that identified CDK inhibitors suppress Japanese encephalitis virus replication, but do not kill biological cells at a specific concentration.

[Figure 5] Figure **5** is a group of pictures that are substitutes for diagrams, showing morphological changes in nucleoli due to a substance having rRNA transcription inhibitory activity. The pictures show images of fluorescence upon treatment with DMSO (control) and three rRNA transcription inhibitors.

[Figure 6] Figure **6(A)** is a group of pictures that are substitutes for diagrams, showing morphological changes of nucleoli due to a substance having GSK-3 inhibitory activity, 1-Azakenpaullone. Figure **6(B)** is a graph showing suppression of proliferation of Japanese encephalitis virus, dengue virus, and Zika virus due to 1-Azakenpaullone.

[Figure 7] Figure **7** is a group of pictures that are substitutes for diagrams, showing morphological changes in nucleoli due to substances having DNA topoisomerase inhibitory activity: ellipticine, idarubicin, idarubicin hydrochloride, epirubicin hydrochloride, mitoxantrone hydrochloride, and daunorubicin hydrochloride.

[Description of Embodiments]

[0021]

15 <Screening method>

The present invention provides, as one embodiment thereof, a method of screening for a substance having anti-flavivirus activity, comprising the following steps:

- (a) contacting a biological cell with a candidate compound of a substance having anti-flavivirus activity *in vitro*;
- (b) testing whether the candidate compound exerts an effect on a morphology of a nucleolus in the biological cell; and
- (c) identifying the candidate compound as a substance having anti-flavivirus activity if the candidate compound exerts an effect. This is described below in more detail.

[0022]

"Substance having anti-flavivirus activity" and "candidate compound thereof" as used herein are described.

The term "flavivirus" refers to a virus in the genus Flavivirus of the family Flaviviridae comprised of an enveloped virus with a + strand RNA genome, encompassing more than 70 species of viruses that are currently known. Examples of mosquito borne flaviviruses include Japanese encephalitis viruses (JEV), dengue viruses (DENV), Zika viruses (ZIKV), West Nile viruses (WNV), yellow fever

viruses (YFV), Saint Louis encephalitis viruses (SLEV), Kunjin viruses (KUN), Murray Valley encephalitis viruses (MVEV), and the like. Examples of tick-borne flaviviruses includes tick borne encephalitis viruses (TBEV) and the like (Gubler DJ, Kuno G, Markoff L: Flaviviruses. In: Fields virology (Fifth edition). Knipe DM, Howley PM (Ed), Lippincott-Raven, Philadelphia, PA, 1153-252, 2007). 14 other species of flaviviruses with an unknown vector have also been reported.

10 [0023]

"Flavivirus" is typically a Japanese encephalitis virus (JEV), dengue virus (DENV), Zika virus (ZIKV), or West Nile virus (WNV), and more typically a Japanese encephalitis virus (JEV), dengue virus (DENV), or Zika virus (ZIKV).

15 JEV spreads to dead-end hosts including humans by a JEV infected mosquito bite that induces infection of the central nervous system, which has a high mortality rate (Burke, D. S., and T. P. Monath. 2001. Flaviviruses, p. 1043-1125. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa). JEV has a single stranded + strand RNA genome with a length of about 11 kb, which is capped at the 5' end, but lacks modification of the 3' end due to polyadenylation (Lindenbach, B. D., and C. M. Rice. 2001. Flaviviridae: The viruses and their replication, p. 991-1041. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.). Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.). A genomic RNA encodes a single long open reading frame. A polyprotein translated from the genome is co- and post-translationally cleaved by a host and virus protease into three structural proteins, i.e., core, pre-membrane (prM), and envelope (E) proteins, and 7 nonstructural proteins, i.e., NS1, NS2A, NS2B, NS3, NS4A,

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NS4B, and NS5 (Sumiyoshi, H., et al. *Virology* 161: 497-510). A JEV core protein (also known as a C protein or capsid protein) has hardly any amino acid homology with other flaviviruses. For example, a JEV core protein has only 25%
5 homology with tick borne encephalitis viruses (TBEV), while the hydrophobicity profile, the number of basic amino acid residues, and secondary structure are very similar (Dokland, T., et al. *Structure* 12: 1157-1163.; Jones, C. T., et al. *J. Virol.* 77: 7143-7149.; Ma, L., et al. *Proc. Natl. Acad. Sci.*
10 *USA* 101: 3414-3419). Flavivirus core proteins share the property of comprising two hydrophobic sequences at the center and carboxyl terminus, and the carboxyl terminus hydrophobic region functions as a signal sequence of prM. A signal anchor sequence is cleaved by a virus protease NS2B-
15 3. The cleaving is needed for subsequent freeing of the prM amino end by a host signal peptidase (Lobigs, M., and E. Lee. 2004. *J. Virol.* 78: 178-186; Stocks, C. E., and M. Lobigs. 1998. *J. Virol.* 72: 2141-2149; Yamshchikov, V. F., and R. W. Compans. 1994. *J. Virol.* 68: 5765-5771). It is
20 understood that a mature core protein released from the endoplasmic reticulum (ER) membrane binds to genomic RNA via a basic amino acid cluster at the amino terminus and carboxyl terminus to form a nucleocapsid (Khromykh, A. A., and E. G. Westaway. 1996. *Kunjin. Arch. Virol.* 141: 685-
25 699). It is understood that the hydrophobic region at the center of a core protein is possibly associated with the ER membrane, and the interaction promotes assembly of a nucleocapsid and two membrane proteins prM and E, which buds in the ER lumen as a virion (Markoff, L., B. Falgout,
30 and A. Chang. 1997. *Virology* 233: 105-117). If the central hydrophobic region of a TBEV core protein is removed, production of sub-virus particles consisting of (pr)M and E proteins but lacking a core protein and genomic RNA increases (Kofler, R. M., F. X. Heinz, and C. W. Mandl.
35 2002. *J. Virol.* 76:3534-3543.; Kofler, R. M., et al. 2003.

J. Virol. 77: 443-451.)

[0024]

The terms "anti-flavivirus" and "anti-flavivirus activity" herein refer to any effect that reduces, inhibits, blocks, or prevents flavivirus replication, infiltration of flavivirus into a cell, and/or flavivirus infection of a cell, resulting in suppression or inhibition the life cycle of flaviviruses and elimination or reduction of viral activity.

10 [0025]

"Substance having anti-flavivirus activity" and "active ingredient having anti-flavivirus activity" as used herein refer to a substance that can treat or prevent a subject suffering from or infected with a flavivirus by eliminating or reducing flaviviruses. The terms "suffering from a flavivirus" and "infected with a flavivirus" are used in relation to a subject and refer to being infected with a flavivirus and having a flavivirus related disease. "Flavivirus related disease" refers to any disease or disorder, which is known be to or suspected of being associated with and/or directly or indirectly induced by a flavivirus. Flavivirus related diseases have fever, headache, muscle pain, and joint pain as primary symptoms. Examples thereof include, but are not limited to, particularly severe hemorrhagic fever due to a dengue virus, encephalitis due to a Zika virus, Japanese encephalitis virus, West Nile virus, or tick-borne encephalitis virus, and microcephaly due to a Zika virus.

[0026]

30 The term "candidate compound" as used herein refers to any naturally-occurring or non-naturally occurring molecule, e.g., a biological macromolecule such as a nucleic acid, polypeptide, or protein, an organic or inorganic molecule, an extract prepared from a biological material such as a cell, tissue or the like of a bacterium, fungus, plant, or

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animal (especially a mammal, including humans), or the like for testing the activity of interest. The screening method of the invention evaluates a candidate compound for its ability to have a given anti-flavivirus activity. This
5 refers to, but not limited to, a library of naturally-occurring and non-naturally occurring compounds owned by a university, company, or the like, medicinal plant extract, peptide, antibody, nucleic acid library, or the like.

[0027]

10 "Biological cells" used in the screening method of the invention refers to a cell population of substantially the same type, preferably at least about 80% and more and more preferably at least about 100% of the cells in the population are of the same cell type. Examples of cell
15 types include, but are not limited to, platelets, lymphocytes, T cells, B cells, natural killer cells, endothelial cells, tumor cells, epithelial cells, granulocytes, monocytes, mast cells, neurons, and the like. Preferred examples thereof include cultured cell strains
20 and primary culture cells derived from eukaryotes including mammals, such as human liver derived Huh7 cells and monkey kidney derived Vero cells, and cells established by a mosquito such as C6/36 cells. Any cell can be used as such cells, as long as a nucleolus thereof can be observed. As
25 described below, the screening method of the invention includes, as one embodiment, a first stage for checking for a morphological abnormality of a nucleolus and a second stage for checking for viral replication. Any cell can be the "biological cell" used in the first stage, as long as a
30 nucleolus thereof can be observed. The "biological cell" used in the second stage is preferably a virus susceptible cell, more preferably a flavivirus susceptible cell.

[0028]

"Virus susceptible cell" refers to any cell that can be
35 infected with a virus. Examples thereof include, but are

not limited to, brain cells, primary cells, dendritic cells, placental cells, endometrial cells, lymph node cells, lymphoid cells (B and T cells), peripheral blood mononuclear cells, skin cells, Langerhans cells, and
5 monocytes/macrophages.

[0029]

The cells used in the screening method of the invention may be obtained through preparation using a technology that is well known in the art such as collecting blood from a
10 patient or a healthy individual or from biopsy, or the cells may be purchased from an immunological entity or microorganism supplier such as American Type Culture Collection, Manassas, VA.

[0030]

15 The cells used herein can be cultured in accordance with a standard cell culturing technique. For example, cells are placed in an incubator containing humidified 95% air-5% CO₂ in a suitable container and grown under a 37°C sterile environment. The container can comprise an agitated
20 or static medium. Various cell cultures may be used, including media comprising undefined biological fluid (e.g., fetal bovine serum or the like) and fully defined media such as a 293 SFM serum-free medium (Invitrogen Corp., Carlsbad, CA). Cell culturing techniques are well known in
25 the art. Established protocols are available for the culture of diverse cell types (see, for example, R.I. Freshney, "Culture of Animal Cells: A Manual of Basic Technique", 2nd Edition, 1987, Alan R. Liss, Inc.).

[0031]

30 In a specific embodiment, the screening method of the invention is performed using cells contained in multiple wells of a multi-well assay plate. Such an assay plate is commercially available, for example, from Strategene Corp. (La Jolla, CA) and Corning Inc. (Acton, MA). Examples
35 thereof include 48-well, 96-well, 384-well, and 1536-well

plates.

[0032]

For example, "contacting *in vitro*" means that a candidate compound of a substance having anti-flavivirus activity adjusted to a predetermined concentration is added to cells contained in multiple wells of a multi-well assay plate. The candidate compound is subsequently tested for a given "anti-flavivirus activity".

[0033]

10 The "given anti-flavivirus activity" is tested by
(b) testing whether the candidate compound exerts an effect on a morphology of a nucleolus in the biological cell; and
(c) identifying the candidate compound as a substance having anti-flavivirus activity if the candidate compound
15 exerts an effect. Specifically, the effect due to the candidate compound is a morphological abnormality of a nucleolus in a biological cell in step (c).

[0034]

The method of determining the given anti-flavivirus activity in a candidate compound is based on the following findings that were first discovered by the inventors. The inventors were searching for an anti-virus therapy against flavivirus infections and diligently conducting studies to originally search for another target, but discovered by
20 chance that the target is not principal aspect of the event that inhibits viral replication, but instead a nucleolus that is present in the nucleus of a biological cell is associated therewith.

[0035]

30 A nucleolus that is present in the nucleus of a biological cell is a construct that is unique to a eukaryote, which can be clearly observed with an optical microscope and is primarily responsible for biosynthesis of ribosomes. The main components include ribosomal DNA (rDNA),
35 ribosomal RNA (rRNA), ribosomal protein, and the like, and

proteomic analysis of constituent proteins using a proteomic approach is ongoing (Anderson et al., Nature volume 433: 77-83). The structure of a nucleolus is divided into three regions by the difference in the electron density from observation using an electron microscope. The fibrillar center (FC) with the lowest electron density is located at the center, and is surrounded by a dense fibrillar component (DFC) with a high electron density therearound. Further, a granular component (GC) with the second highest electron density is located around the FC and DFC. A nucleolus exists as a clear intranuclear construct during the interphase, but the structure thereof changes depending on the cell cycle, e.g., the structure thereof disintegrates during the mitosis phase and is reformed during the early stages of the G1 phase where ribosome biosynthesis activity recovers. While there are 1 to 6 nucleoli within the nucleus, the number is not clearly defined because nucleoli fuse with each other after the cell enters the interphase.

[0036]

Specifically, the inventors found by chance that a certain type of pharmacologically active compound exerts an effect on the morphology of a nucleolus in a biological cell, and found that this inhibits viral replication by some type of action.

One of the constituent proteins, i.e., core protein, of flaviviruses utilizes the nucleolar function of a host cell as part of a viral replication function. This is understood to be essential for viral replication. Although not wishing to be bound by a specific theory, in the present invention, it is understood that a morphological abnormality of a nucleolus has an adverse effect on the materialization of a function of a core protein. A nucleolus is a location for the transcription of ribosomal RNA (rRNA) or the synthesis of ribosomes. When in a normal state, a nucleolus is

involved in protein synthesis of a host by localization of a core protein in the nucleolus. For example, a normal nucleolus is assumed to relatively increase translation of a viral protein by promoting synthesis of a virus derived protein or suppressing synthesis of a protein of a host cell. It is understood that the effect of a certain type of a substance on the morphology of a nucleolus discovered in the present invention is derived from the action of reducing or disrupting the normal nucleolar function.

10 [0037]

Specifically, a given anti-flavivirus activity, i.e., a morphological abnormality of a nucleolus in a biological cell is determined by a change in the presence and/or localization of a protein or nucleic acid localized in the nucleolus in the screening method of the invention.

The change in the presence and/or localization of a protein or nucleic acid localized in the nucleolus is a replacement for a change in the status of formation of the nucleolus itself, which is found in comparison to the effect of a control compound. The change in the presence and/or localization of a protein or nucleic acid localized in the nucleolus is distinguished by a nucleolus changing from a large mass to many small oil droplets, or very small particles disperse and become unobservable, or rather a mass of a nucleolus becoming an even larger mass, or the like. A candidate compound is determined as exerting an effect on the morphology of the nucleolus if there is such a change in comparison to an effect of a control compound on a protein localized in a nucleolus.

30 [0038]

As used herein, "protein localized in a nucleolus in a biological cell" refers to a protein endogenous to a nucleolus or a protein that specifically binds to a nucleolus. Examples of proteins endogenous to a nucleolus include, but are not limited to, nucleophosmin (also known

as NPM, NPM1, B23, NO38, or Numatrin), nucleolin (also known as C23 and NCL), fibrillarin (also known as FBL), and RNA polymerase I (also known as Pol1). Examples of proteins that specifically bind to a nucleolus include, but are not limited to, the core proteins of flaviviruses such as Japanese encephalitis viruses and hepatitis C viruses and corona viruses, also known as a capsid protein (Rawlinson and Moseley, 2015. Cellular Microbiology; Salvetti and Greco, 2014. Biochemica et Biophysica Acta).

Nucleophosmin is a protein which binds to a ribonucleic acid (RNA) that is present in the granular component (GC) region located on the outermost side of a nucleolus structure. Nucleolin is a protein that is localized mainly in the dense fibrillar component (DFC) of a nucleolus and is involved in the transcription of a ribosomal deoxyribonucleic acid (rDNA). Fibrillarin is a protein that is localized in the fibrillar center (FC) or dense fibrillar component (DFC) of a nucleolus, and binds to an RNA to function in the processing or modification of an rRNA precursor. RNA polymerase I is one of the enzymes (RNA polymerase) that catalyzes a reaction (transcription) for reading out the base sequence of a template strand (single strand) of DNA and synthesizing a complementary RNA. RNA polymerase I is in a nucleolus and synthesizes an rRNA precursor.

[0039]

A change in the presence and/or localization of a protein that specifically binds to a nucleolus can be observed by an immunofluorescence assay using, for example, an anti-nucleophosmin antibody (anti-NPM1 antibody (ab10530, Abcam, UK), anti-nucleolin antibody (anti-NCL antibody) (A300-710A, Bethyl Laboratories, TX), anti-fibrillarin antibody (GTX24566, GeneTex, CA), or anti-RNA polymerase I antibody (anti-POLI antibody) (13635-1-AP, Proteintech, IL). These proteins can be observed through fluorescent imaging

by ligating a nucleic acid encoding the proteins with a nucleic acid encoding a fluorescent substance such as a green fluorescent protein (GFP) and transfecting a cell with the ligated nucleic acid, or by microinjection of a recombinant protein prepared by fusing an endogenous protein with a fluorescent substance within a cell.

[0040]

"Nucleic acid localized in the nucleolus in a biological cell" refers to a nucleic acid endogenous to a nucleolus or a nucleic acid that specifically binds to a nucleolus. Examples of nucleic acids endogenous to a nucleolus include ribosomal deoxyribonucleic acids (also known as ribosomal DNA or rDNA). An rDNA is a repeat sequence with each unit being 43 kb (13.7 kb coding region and 29.3 kb non-coding region). In humans, rDNA is a DNA that is in the short arm portion of the 13th, 14th, 15th, 21st, and 22nd short armed chromosomes.

A change in the presence and/or localization of these rDNAs can be observed by FISH (Fluorescence In Situ Hybridization). FISH is, for example, an approach of using oligonucleotide probe prepared by labeling a specific genetic sequence of rDNA with a fluorescent substance or the like and hybridizing the probe with the gene or interest to detect the gene with a fluorescence microscope.

[0041]

It was known that a core protein of a flavivirus can express the morphology of a nucleolus by visualizing the localization of a nucleolus of a biological cell (Tsuda et al., 2006 Microbiol. Immunol., 50, 225-234). Use of a core protein of a flavivirus in the identification of an effect on the morphology of a nucleolus in the present invention is based on this finding.

Localization of a core protein of a flavivirus is observed by expressing the core protein within a cell with a transfection reagent such as lipofectamine (Thermo

Fisher) or injecting a recombinant protein purified from E. coli or insect cells into the cytoplasm of a biological cell by microinjection using a glass needle. A core protein of a flavivirus can be manufactured by transfecting E. coli or baculovirus with a plasmid incorporating a DNA encoding a core protein, infecting the bacterial cell for E. coli or an sf9 insect cell for baculovirus and expressing the plasmid within the insect cell, and adsorbing an antibody specific to a protein expressed by disrupting the bacterial cell or insect cell or a tag protein such as a glutathione-S-transferase tag protein with Sepharose beads crosslinked with a binding protein that is specific thereto and purifying the combination thereof.

For example, a change in localization of a core protein of a flavivirus can be observed by an immunofluorescence assay using an anti-flavivirus antibody or an anti-Flag antibody to a core protein fused to a Flag tag or the like, or by observing a cell expressing or introduced with a fusion protein prepared by fusing a fluorescent protein such as GFP using a fluorescence microscope, confocal microscope, super-resolution microscope, electron microscope, or the like.

[0042]

In this manner, the screening method of the invention encompasses the methods of the invention wherein a protein localized in a nucleolus in a biological cell is one or more proteins selected from nucleophosmin, nucleolin, fibrillarin, RNA polymerase I, and flavivirus core proteins. It is preferable to use two or more proteins localized in a nucleolus from the viewpoint of certainty in a candidate compound.

[0043]

In a specific embodiment, the method of the invention (1) contacts a biological cell with a candidate compound of a substance having anti-flavivirus activity *in vitro* and

incubates the biological cell under a condition and over a time that are sufficient for the candidate compound to exert an effect on a morphology of a nucleolus in the biological cell;

- 5 (2) tests whether the candidate compound exerts an effect on a morphology of a nucleolus in the biological cell; and
(3) identifies the candidate compound exerting an effect as a substance having anti-flavivirus activity.

[0044]

10 In a preferred embodiment, the method of the invention
(1) contacts a biological cell with a candidate compound of a substance having anti-flavivirus activity *in vitro* and incubates the biological cell under a condition and over a time that are sufficient for the candidate compound to
15 exert an effect on a morphology of a nucleolus in the biological cell;

(2) tests whether the candidate compound exerts an effect on a morphology of a nucleolus in the biological cell;

(3) sorts out the candidate compound exerting an effect;

20 (4) tests the sorted out candidate compound for a change in localization of a core protein of a virus in a biological cell; and

(5) identifies the candidate compound as a substance having anti-flavivirus activity if an effect exerted on a
25 morphology of a nucleolus and localization of a core protein are changed.

[0045]

The screening method of the invention leads to the discovery and development of an anti-flavivirus drug that
30 exerts an effect by exerting an effect on a morphology of a nucleolus in the biological cell. These drugs can be potentially useful in the treatment and/or prevention of a flavivirus infection and/or flavivirus related disease and symptom. The present invention encompasses any of the
35 compounds identified as a substance having anti-flavivirus

activity by the screening method of the invention.

[0046]

The effect exerted on a morphology of a nucleolus in a biological cell found by the inventors can be used as a target for the identification, design, and development of a new anti-flavivirus drug. Substances exerting an effect on a morphology of a nucleolus in a biological cell that is useful as an anti-flavivirus drug belong to various families of molecules. Examples thereof include, but are not limited to, organic compounds (e.g., small molecules, saccharides, steroids, and the like), monoclonal antibodies and polyclonal antibodies (e.g., antibodies binding to nuclear localization of a core protein of a flavivirus), peptides, polypeptides, and nucleic acid molecules (e.g., antisense compounds, ribozymes, triple helix molecules, SELEX RNAs, and the like).

[0047]

Screening of a library in accordance with the method of the invention provides a compound with biological activity that is desired but not optimized, which is generally referred to as a "hit" or "read" compound. The next stage of the development a useful drug candidate generally includes analysis of the relationship between the chemical structure of a hit compound and the biological or pharmacological activity thereof. The molecular structure and biological activity can be associated by observing the overall structural modification result for a given biological indicator. A small compound library can be generated by using available information on the structure-activity correlation from the first screening in the next stage, and the library is subsequently screened for a compound with higher affinity. The process for synthetic modification of a biologically active compound to satisfy stereoelectronic, physicochemical, pharmacokinetic, and toxicological factors required for clinical usefulness is

referred to as read optimization. A candidate compound identified by the screening method of the invention can be similarly applied in the early stages of read optimization. A drug candidate that is chemically modified and improved by read optimization can be encompassed by the present invention to an appropriate extent. An appropriate extent is an extent to which those skilled in the art can understand that drug candidate compounds exerting an effect on a morphology of a nucleolus in a biological cell in the present invention have a chemical structure within a certain scope and exhibit the same activity.

[0048]

The screening method described herein can be prepared as a kit. For example, a kit can be prepared by packaging biological cells disclosed herein in various containers such as a vial, tube, microtiter well plate, and bottle and including other reagents in different containers. In this regard, a positive control sample or compound, negative control sample or compound, buffer, cell culture, specific detection probe, and the like can be prepared, as a whole, as a kit.

[0049]

<Anti-flavivirus pharmaceutical composition>

As another embodiment, the present invention provides an anti-flavivirus pharmaceutical composition comprising a substance exerting an effect on a morphology of a nucleolus in a biological cell, preferably an anti-flavivirus pharmaceutical composition comprising a substance exerting an effect on a morphology of a nucleolus in a biological cell as an active ingredient, specifically an anti-flavivirus pharmaceutical composition comprising a substance inducing a morphological abnormality in a nucleolus, and preferably an anti-flavivirus pharmaceutical composition comprising a substance inducing a morphological abnormality in a nucleolus as an active ingredient having

anti-flavivirus activity.

The term "flavivirus" has the meaning described above. The terms "anti-flavivirus" or "anti-flavivirus activity" are interchangeably used, referring to any effect that
5 reduces, inhibits, blocks, or prevents flavivirus replication, flavivirus infiltration into a cell, and/or flavivirus infection of a cell, resulting in the suppression or inhibition of the life cycle of flaviviruses and elimination or reduction of viral activity.

10 [0050]

Thus, "anti-flavivirus pharmaceutical composition" refers to a pharmaceutical composition that can treat or prevent a subject suffering from or infected with a flavivirus by eliminating or reducing flaviviruses. The
15 terms "suffering from a flavivirus" and "infected with a flavivirus" are used in relation to a subject and refer to being infected with a flavivirus and having a flavivirus related disease. The term "flavivirus infection" can refer to introduction of flavivirus genetic information into a
20 target cell (e.g., by fusion of a target cell membrane and flavivirus or flavivirus envelope glycoprotein positive cell). "Flavivirus related disease" refers to any disease or disorder, which is known to be or suspected of being associated with and/or directly or indirectly induced by a
25 flavivirus. Flavivirus related diseases have fever, headache, muscle pain, and joint pain as primary symptoms. Examples thereof include, but are not limited to, particularly severe hemorrhagic fever due to a dengue virus, encephalitis due to a Zika virus, Japanese encephalitis
30 virus, West Nile virus, or tick-borne encephalitis virus, and microcephaly due to a Zika virus. Anti-flavivirus activity is specifically anti-flavivirus activity against a flavivirus selected from a Japanese encephalitis virus, a dengue virus, a Zika virus, a West Nile virus, and a yellow
35 fever virus.

[0051]

"Treatment" as used herein refers to a method or process intended to: (1) delay or prevent onset of a disease or condition (e.g., flavivirus infection or
5 flavivirus related disease); (2) slow or stop the progression, exacerbation, or aggravation of a symptom of a disease or condition; (3) induce remission of a symptom of a disease or condition; or (4) heal a disease or condition. Treatment may be administered before the onset of a disease
10 or condition as a preventive measure, or administered after the onset of a disease.

[0052]

The term "prevent, inhibit, or block a flavivirus infection", in the context of using a substance having
15 anti-flavivirus activity, refers to reducing the amount of flavivirus genetic information introduced into a susceptible cell or susceptible cell position compared to the amount that would be introduced in the absence of a drug.

20 [0053]

"Pharmaceutical composition" as used herein comprises an effective amount of at least one substance having anti-flavivirus activity and at least one pharmaceutically acceptable carrier or excipient. The term "effective
25 amount" as used herein refers to any amount of compound or composition that is sufficient to satisfy the intended objective thereof, such as a desired biological or pharmacological response in a cell, tissue, system, or subject. In a specific embodiment of the invention, the
30 objective refers to, for example, having anti-flavivirus activity, preventing a flavivirus infection, preventing the onset of a flavivirus related disease, slowing, alleviating, or stopping the progression, exacerbation, or aggravation of a symptom of a flavivirus related disease, inducing
35 remission of a symptom of a disease, and/or healing a

flavivirus related disease.

[0054]

"Pharmaceutically acceptable carrier or excipient" refers to a carrier medium that does not interfere with the efficacy of the biological activity of an active ingredient and is not excessively toxic to the host at the administered concentration. The term encompasses solvents, dispersion media, coating agents, antimicrobial agents, antifungal agents, isotonizing agents, adsorption delaying agent, and the like. Use of such a medium and drug for a pharmaceutically active substance is well known in the art.

[0055]

"Substance exerting an effect on a morphology of a nucleolus in a biological cell" as used herein refers to a substance that exerts an effect on a nucleolus, which is a location for the transcription of ribosomal RNA (rRNA) or the synthesis of ribosomes, and changes the morphology thereof in a biological cell herein, and specifically to a substance inducing a morphological abnormality in a nucleolus. A morphological abnormality of a nucleolus in a biological cell is identified by a change in the formation and/or localization of a protein localized in the nucleolus of the invention as described above. Preferably, these substances are sorted out by the screening method of the invention.

[0056]

In one embodiment, a substance exerting an effect on a morphology of a nucleolus in a biological cell in the present invention exerts anti-flavivirus activity by inhibition of viral replication. The present invention is preferably a pharmaceutical composition wherein inhibition of viral replication involves a change in localization of a core protein of a virus in a biological cell. This is because inhibition of viral replication, which is indicative of the final anti-flavivirus activity, involves

localization of a core protein of a flavivirus.

[0057]

A specific "substance exerting an effect on a morphology of a nucleolus in a biological cell" is selected from substances having cyclin-dependent kinase (CDK) inhibitory activity, substances having rRNA transcription inhibitory activity, substances having DNA topoisomerase inhibitory activity, and substances having glycogen synthase kinase-3 (GSK-3) inhibitory activity.

10 [0058]

"CDK" in a substance having CDK inhibitory activity refers to a kinase, which becomes active only after binding to cyclin among a group of kinases that regulate the cell cycle, including CDK1 (or cdc2), CDK2, CDK4, CDK6, and the like, which are each activated at different points in the cell cycle. Cyclin is a protein that induces progression of a cell cycle in a eukaryotic cell. Cyclin A, B, D, and E are involved in the rotation of a cell cycle. Cyclin A primarily binds to CDK1 or CDK2 and forms a complex to regulate the end of the DNA synthesis phase. Cyclin B primarily binds to CDK2 and forms a complex to regulate the progression to the mitosis phase. Cyclin D primarily binds to CDK4 and CDK6 and forms a complex to regulate transition of the DNA synthesis preparation phase/DNA synthesis phase. Cyclin E primarily binds to CDK2 and forms a complex to regulate transition to the DNA synthesis phase. Many substances having CDK inhibitory activity generally exhibit an action of inhibiting CDK activity to suppress cell proliferation and are developed as an anticancer agent by utilizing such an action, including the CDK4/6 inhibitor Ibrance (generic name palbociclib, Pfizer Japan Inc.). In the present invention, a substance inhibiting CDK1, CDK2, or CDK9 is preferred.

[0059]

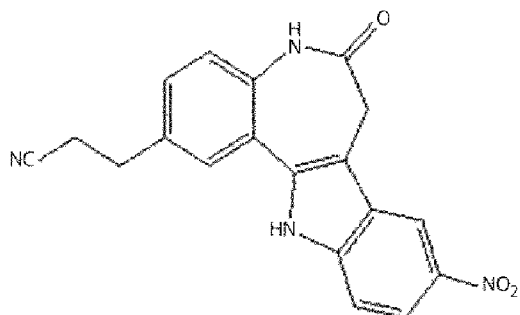
35 More specifically, a substance having CDK inhibitory

activity is selected from: alsterpauellone, 2-cyanoethyl; 5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide; (4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine; P276-00; A-674563; SU9516; SNS-032 (BMS-387032); dinaciclib (SCH727965); flavopiridol (alvocidib); AT7519; flavopiridol hydrochloride (flavopiridol HCl); P276-00; AT7519 HCl; AZD5438; R547; and kenpauellone. A preferred substance having CDK inhibitory activity is selected from: alsterpauellone, 2-cyanoethyl; 5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide; (4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine; P276-00; A-674563; and SU9516. A more preferred substance having CDK inhibitory activity is selected from: alsterpauellone, 2-cyanoethyl; 5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide; and (4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine.

[0060]

Said substances are specifically, alsterpauellone, 2-cyanoethyl: 3-(6-oxo-9-nitro-5,6,7,12-tetrahydroindolo[3,2-d][1]benzazepin-2-yl)propionitrile (Kunick, C., et al. 2005. ChemBioChem. 6, 541-549.)

[Chemical Formula 1]

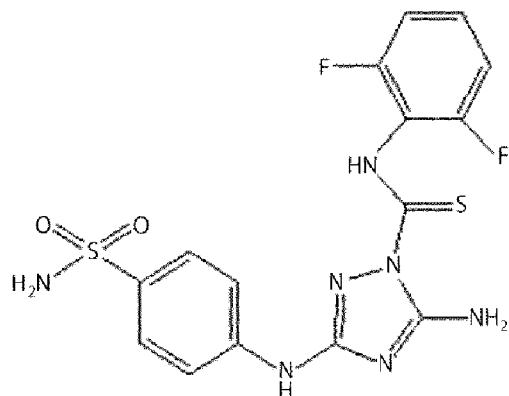


[0061]

5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-

difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide (Lin, R., et al. 2005. J. Med. Chem. 48, 4208-4211.)

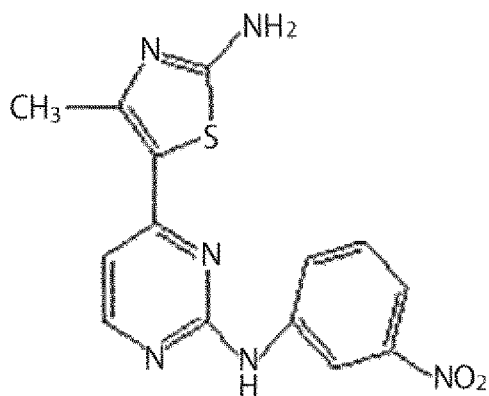
[Chemical Formula 2]



5 [0062]

(4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine (Kontopidis, G., et al. 2006. Chem. Biol. 13, 201.)

[Chemical Formula 3]



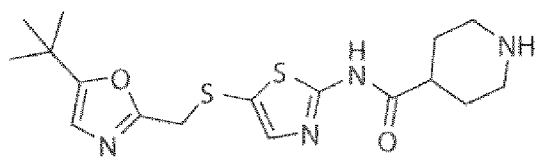
10

[0063]

SNS-032 (BMS-387032): Chen R, et al. 2009. Blood, 113(19), 4637-45; Ali MA, et al., 2007. Neoplasia, 9(5), 370-81; Conroy A, et al. 2009, Cancer Chemother Pharmacol, 64(4), 723-32; Walsby E, et al. 2011. Leukemia, 25(3), 411-9.

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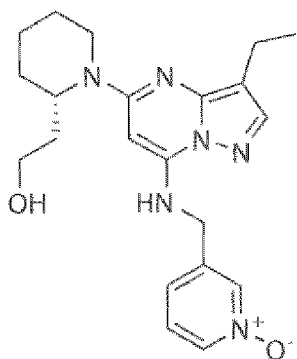
[Chemical Formula 4]



[0064]

Dinaciclib (Parry D, et al. 2010. Mol Cancer Ther. 9(8), 2344-2453; Guzi TJ, et al., 2011. Mol Cancer Ther., 10(4), 591-602; Abdullah C, et al. 2011. Cell Cycle., 10(6), 977-988; Fu W, et al., 2011. Mol Cancer Ther., 10(6), 1018-1027)

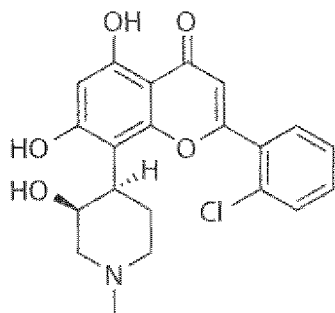
[Chemical Formula 5]



10 [0065]

Flavopiridol (alvociclib) (Kim KS, et al. 2000. J Med Chem, 43(22), 4126-4134; Lu H, et al. 2005. J Med Chem, 48(3), 737-743; Montagnoli A, et al. 2008. Nat Chem Biol, 4(6), 357-365; Carlson BA, et al. 1996. Cancer Res, 56(13), 2973-2978)

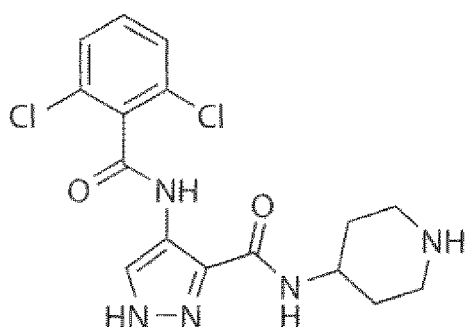
[Chemical Formula 6]



[0066]

AT7519: (Squires MS, et al. 2009. Mol Cancer Ther, 8(2), 324-332; Santo L, et al. 2010. Oncogene, 29(16), 2325-2336)

[Chemical Formula 7]



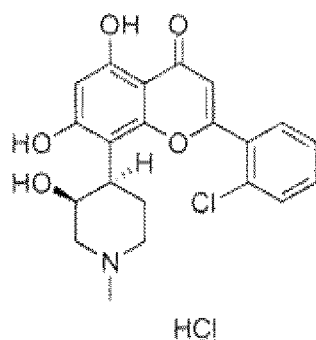
5

[0067]

Flavopiridol hydrochloride (Senderowicz AM, 2002. Oncologist, 7 Suppl 3:12-9; Carlson BA, et al, 1996. Cancer Res, 56(13), 2973-2978; Parker BW, et al, 1998. Blood, 91(2), 458-465; Drees M, et al, 1997. Clin Cancer Res, 3(2), 273-279)

10

[Chemical Formula 8]

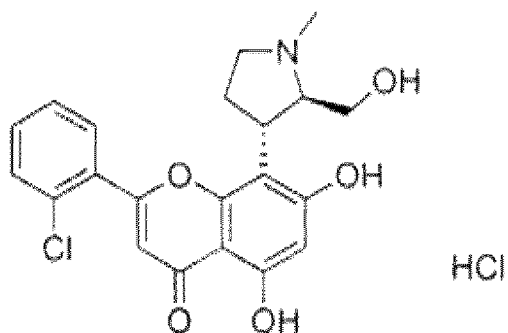


[0068]

P276-00: (Joshi KS, et al. 2007. Mol Cancer Ther, 6(3), 918-925; Joshi KS, et al. 2007. Mol Cancer Ther, 6(3), 926-934)

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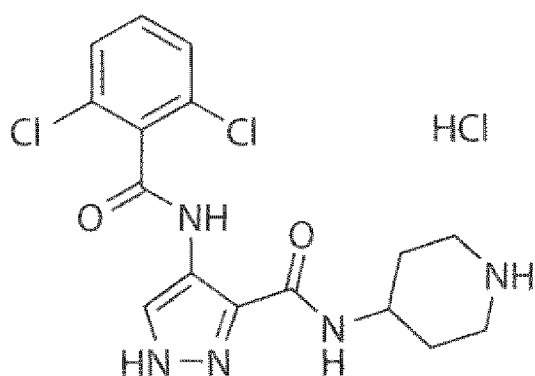
[Chemical Formula 9]



[0069]

AT7519 HCl: (Squires MS, et al. 2009. Mol Cancer Ther, 8(2), 324-332; Santo L, et al. 2010. Oncogene, 29(16), 2325-2336)

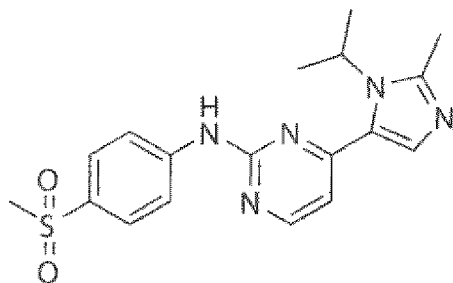
5 [Chemical Formula 10]



[0070]

AZD5438: (Byth KF, et al. 2009. Mol Cancer Ther. 8(7), 1856-1866)

10 [Chemical Formula 11]

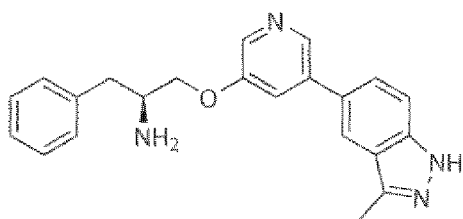


[0071]

A-674563: (Luo Y, et al, 2005. Mol Cancer Ther, 4(6), 977-986; Zhu QS, et al, 2008. Cancer Res, 68(8), 2895-2903;

Tatsuya Okuzumi, et al, 2010. Mol Biosyst, 6(8), 1389)

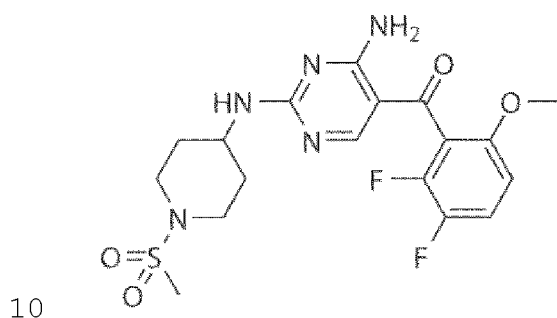
[Chemical Formula 12]



[0072]

- 5 R547: (Rodriguez A, et al. 2006. Mol Cancer Ther, 5(11), 2644-2658; Chu XJ, et al. 2006. J Med Chem, 49(22), 6549-6560; Berkofsky-Fessler W, et al. 2009. Mol Cancer Ther, 8(9), 2517-25)

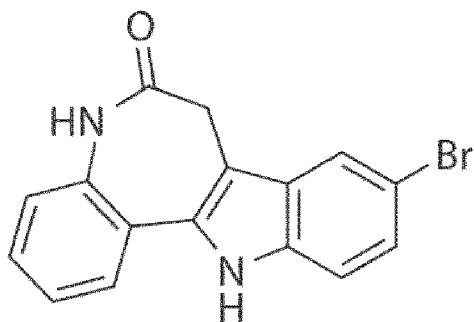
[Chemical Formula 13]



[0073]

Kenpaullone (Zaharevitz et al. 1999. Cancer Res. 59(11): 2566-2569; Adam COLE, et al. 2004. Biochem. J. 377(1): 249-255; Leclerc S, et al. 2001. J Biol Chem. 276(1): 251-260)

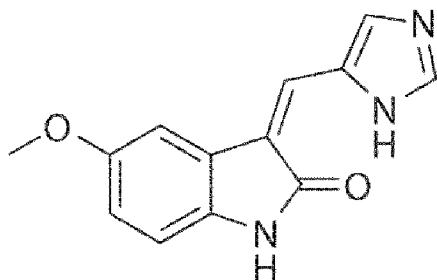
- 15 [Chemical Formula 14]



[0074]

SU9516: (Lane ME, et al. 2001. Cancer Res. 61(16), 6170-6177; Gao N, et al. 2006. Mol Pharmacol. 70(2), 645-655; Uchiyama H, et al. 2010. Cancer Sci. 101(3), 728-734; Leontovich AA, et al. 2013. Oncol Rep. 29(5), 1785-1788)

5 [Chemical Formula 15]



[0075]

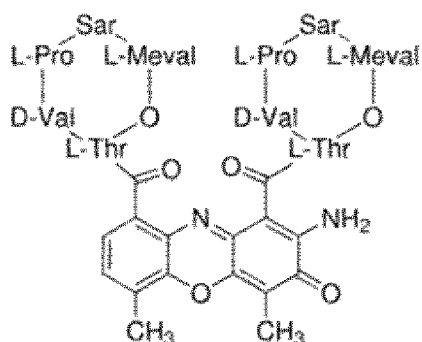
rRNA transcription refers to a reaction that forms a transcription initiation complex comprising RNA polymerase I on a promoter of rDNA encoding a ribosomal RNA (rRNA) to synthesize an rRNA. A substance having rRNA transcription inhibitory activity generally exhibits an action of suppressing or inhibiting transcription of an rRNA such as RNA polymerase I, resulting in inhibition of rRNA synthesis.

15 A substance having rRNA transcription inhibitory activity is typically selected from actinomycin D, doxorubicin, and aclarubicin described in detail below. Actinomycin D treatment is known to induce nucleolar disintegration (Scheer et al., 1975. 65: 163-179).

20 [0076]

Actinomycin D: Inhibition of rRNA transcription activity by actinomycin D results in nucleolar segregation wherein DFC or GC forms a cap-like structure around the nucleolus (Scheer et al., 1975. 65:163-179). (Waksman S.A. et al., 1940. Proc. Soc. Exp. Biol. Med. 45, 609-614; Goldberg I. H. et al., 1962. Science. 136, 315-316; Sobell H. M. et al., 1985. Proc. Natl. Acad. Sci. USA. 82, 5328-5331)

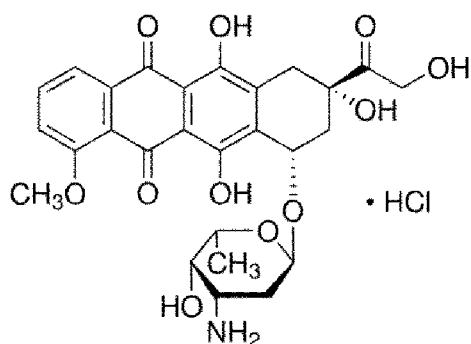
[Chemical Formula 16]



[0077]

Doxorubicin (Arcamone F., et al, 1969. Tetrahedron Lett. 13, 1007-1010; Taatjes D. J., et al, 1996. J. Med. Chem. 39, 4135-4138; Zeman S. M., et al, 1998. Proc. Natl. Acad. Sci. USA. 95, 11561-11565)

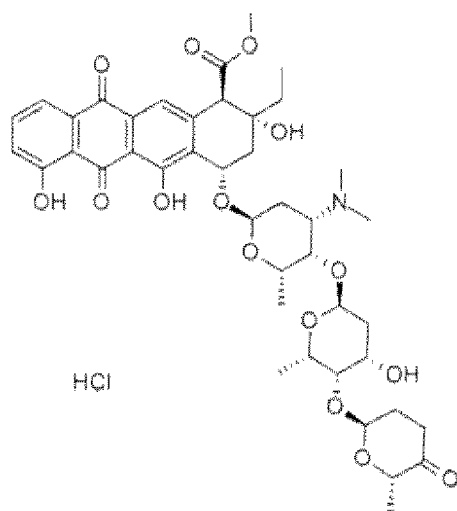
[Chemical Formula 17]



[0078]

10 Aclarubicin (Isoe T., et al., 1992. Biochim. Biophys. Acta. 1117, 131-135; Figueiredo-Pereira M. E., et al., 1996. J. Biol. Chem. 271, 16455-16459; Natale R. B., et al., 1993. Cancer Chemother. Pharmacol. 31, 485-488,)

[Chemical Formula 18]



[0079]

DNA topoisomerase is an enzyme that temporarily cleaves one or both of the double strands of DNA and ligates the cleavage point again. DNA topoisomerase is roughly classified into two types (type I and type II). Type I cleaves only a single strand of double stranded DNA and then ligates the cleaved DNA again. Type II cleaves and ligates the double strands. A DNA topoisomerase eliminates the twist or distortion in a helical structure of a DNA upon replication by such a property. A substance inhibiting the activity of a DNA topoisomerase generally inhibits the action of cleaving and reconnecting a DNA and inhibits DNA replication by forming a crosslink between DNAs. A substance having DNA topoisomerase inhibitory activity is typically selected from doxorubicin, aclarubicin, ellipticine, idarubicin, idarubicin hydrochloride (idarubicin HCl), epirubicin hydrochloride (epirubicin HCl), mitoxantrone hydrochloride (mitoxantrone HCl), and daunorubicin hydrochloride (daunorubicin HCl) described in detail below. A preferred substance having DNA topoisomerase inhibitory activity is ellipticine.

[0080]

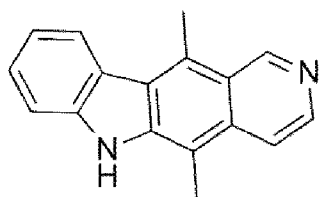
Doxorubicin (same as above);

Aclarubicin (same as above);

[0081]

Ellipticine (Stiborova M, et al. Biomed Pap Med Fac Univ
Palacky Olomouc Czech Repub. 2006 Jul; 150(1): 13-23.)

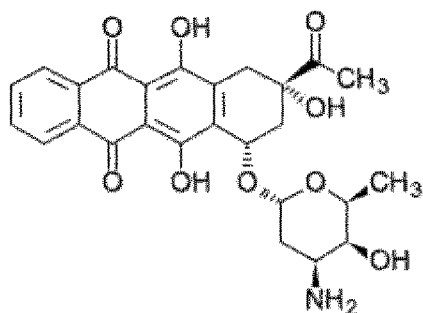
5 [Chemical Formula 19]



[0082]

Idarubicin

[Chemical Formula 20]



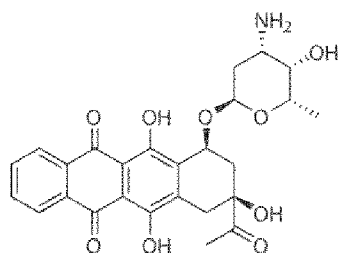
10

D08062

[0083]

Idarubicin hydrochloride (Orlandi P, et al. J Chemother.
2005, 17(6), 663-667.)

[Chemical Formula 21]

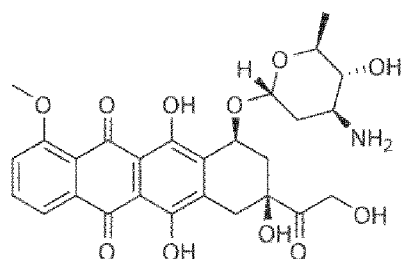


HCl

[0084]

Epirubicin hydrochloride (Ozkan A, et al. Pol J Pharmacol, 2004, 56(4), 435-444.)

5 [Chemical Formula 22]

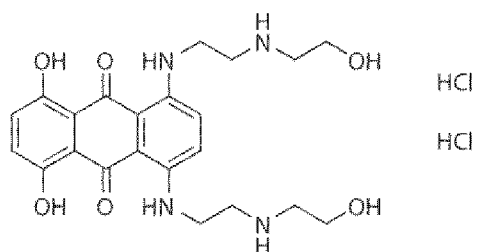


HCl

[0085]

Mitoxantrone hydrochloride (Nieciecka D., et al. Electrochimica Acta, 2015, 165, 430-442])

10 [Chemical Formula 23]



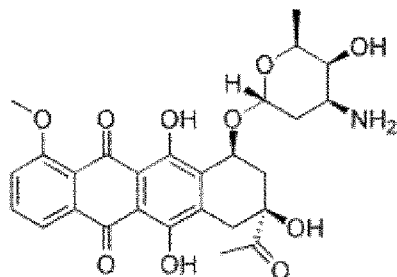
HCl

HCl

[0086]

Daunorubicin hydrochloride (Fu Y, et al. Thromb Haemost, 2010, 104(6), 1235-1241.)

[Chemical Formula 24]



HCl

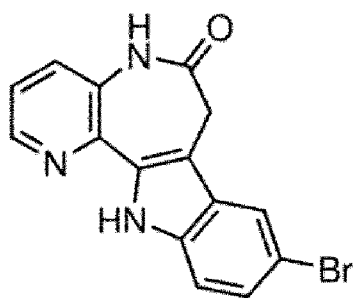
[0087]

GSK-3 was identified as a serine/threonine protein
 5 kinase that phosphorylates and inactivates glycogen
 synthase. GSK-3 is classified into two isoforms, i.e., α
 and β , in humans, and is known to be involved in not only
 glycogen metabolism but also in the regulation of many
 cellular functions. A substance inhibiting the activity of
 10 GSK-3 is typically 1-Azakenpaullone.

[0088]

1-Azakenpaullone (Kunick C, et al. Bioorg Med Chem Lett.
 2004, 14(2), 413-416.)

[Chemical Formula 25]



15

[0089]

As described above, "substance exerting an effect on a
 morphology of a nucleolus in a biological cell" herein has
 various known biological activities such as CDK inhibition,
 20 rRNA transcription inhibition, DNA topoisomerase inhibition,
 and GSK-3 inhibition, and has, as described above, various
 chemical structures. Compounds with various known

activities and various chemical structures are all proven to share in common the action of "exerting an effect on a morphology of a nucleolus in a biological cell" and thereby inhibiting replication of flaviviruses. This is, in other words, evidence showing that any compound having the common action of "exerting an effect on a morphology of a nucleolus in a biological cell" inhibits replication of flaviviruses and has anti-flavivirus activity. Although not wishing to be bound by a specific theory, it is understood that a core protein of a flavivirus utilizes the nucleolar function of a host cell as a part of the viral replication function, and a morphological abnormality in a nucleolus that was first found in the present invention has an adverse effect on the materialization of a function of the core protein, resulting in the action of reducing or destructing normal nucleolar function that is responsible for the promotion of synthesis of a virus derived protein or the like.

[0090]

In general, the substance or composition having anti-flavivirus activity of the invention is administered at an effective amount, i.e., amount sufficient to satisfy the intended objective. The precise amount of the substance or pharmaceutical composition having anti-flavivirus activity to be administered varies among subjects, depending on the age, sex, body weight, and overall health status of the subject to be treated, desired biological or medical response (e.g., prevention of a flavivirus infection or treatment of a flavivirus related brain disease), or the like. In many embodiments, the effective amount is an amount that exerts an effect on a morphology of a nucleolus in a biological cell, and an amount that inhibits or prevents replication of a flavivirus in a susceptible cell of a subject, whereby a flavivirus infection is treated or prevented.

[0091]

The substance and composition having anti-flavivirus activity of the invention may be used in various therapeutic or preventive methods. In particular, the present invention provides a method for treating or preventing a flavivirus related disease or pathological condition in a subject, the method comprising administering an effective amount of the substance having anti-flavivirus activity of the invention or a composition thereof that exerts an effect on a morphology of a nucleolus of the biological cell of the invention, wherein the administration inhibits a flavivirus from infiltrating into or infecting a cell of the subject, which can result in remission, prevention, or treatment of a brain disease or pathological condition in the subject.

[0092]

In a specific embodiment, the present invention administers the substance or composition having anti-flavivirus activity alone. In another embodiment, the substance or composition having anti-flavivirus activity of the invention is administered in combination with at least one additional therapeutic drug. The substance or composition having anti-flavivirus activity of the invention can be administered before, concomitantly with, or after administration of a therapeutic drug.

[0093]

A therapeutic drug that can be administered in combination with the substance or composition having anti-flavivirus activity of the invention can be selected from various biologically active compounds that are known in the art as having a beneficial effect in the treatment, management, or prevention of a flavivirus infection or a flavivirus related disease or condition. Such a drug comprises, in particular, an antiviral drug, including interferon (e.g., interferon alpha and PEGylated interferon

alpha), anti-flavivirus (monoclonal or polyclonal) antibodies, RNA polymerase inhibitors, protease inhibitors, IRES inhibitors, helicase inhibitors, antisense compounds, ribozymes, and any combination thereof.

5 [0094]

The substance having anti-flavivirus activity of the invention can be optionally formulated with one or more suitable pharmaceutically acceptable carriers or excipients and administered through any suitable route to a subject in
10 need thereof at a desired dosage. Various delivery systems are known and can be used for the administration of the substance having anti-flavivirus activity of the invention, including, for example, a tablet, capsule, injection, agent encapsulated in a liposome, powder, microcapsule, and the
15 like. The administration method includes transdermal, intradermal, intramuscular, intraperitoneal, intralesional, intravenous, subcutaneous, intranasal, pulmonary, epidural, intraocular, and oral routes. Administration can be systemic or topical.

20 [0095]

The substance or composition having anti-flavivirus activity of the invention can be administered at a dosage at which the delivered amount is effective for the intended objective. The route of administration, formulation, and
25 dosage can be dependent on the desired therapeutic effect, severity of the flavivirus infection or flavivirus related condition to be treated (if it already exists), presence of any other infection, age, sex, body weight, and overall health status of the patient, potency, bioavailability, *in*
30 *vivo* half-life, use of (or lack of) concomitant therapy, and other clinical factors of the substance or composition having anti-flavivirus activity to be used. These factors can be readily determined by the primary physician in the course of therapy. As a test is conducted using the
35 substance having anti-flavivirus activity of the invention,

more information related to a suitable dosage level and treatment time would be available.

[0096]

The treatment of the invention can consist of a single
5 dose or multiple doses. In this manner, the substance
having anti-flavivirus activity of the invention or a
composition thereof can be administered constantly or
periodically over a specific period and at a specific
interval, such as once in several hours, once daily, once
10 weekly, or once monthly (e.g., timed release form).
Alternatively, delivery can be sustained delivery over a
certain period such as intravenous delivery.

[0097]

In general, the amount of substance having anti-
15 flavivirus activity to be administered can be preferably
about 1 ng/kg to about 100 mg/kg of body weight of a
subject, e.g., in the range of about 100 ng/kg to about 50
mg/kg of body weight of a subject or about 1 µg/kg to about
10 mg/kg of body weight of a subject.

20 [0098]

It should be noted that the present invention is
described hereinafter in more detail with Reference
Examples and Examples, but the Reference Examples and
Examples are merely exemplification, which do not limit the
25 scope of the invention.

[Examples]

[0099]

Reference Example 1: Nucleolar localization of flavivirus
core protein

30 Flavivirus core proteins were expressed in cells to
study the localization thereof.

Reference Example 1-1: Construction of transient culture
cell expression plasmid

Core (core) protein cDNAs of Japanese encephalitis
35 viruses (JEV), dengue viruses (DENV), and Zika viruses

(ZIKV) were provided by the National Institute of Infectious Diseases. Synthesis of core protein cDNA of West Nile viruses (WNV) was commissioned to Integrated DNA Technologies (IDT, Inc., Illinois). Each core protein cDNA was amplified by a PCR reaction using the primers described below. Each amplified cDNA was incorporated into an EcoRI part of a pCAGGS vector (Miyazaki et al., (1989) Gene. 79: 269-77) comprising a Flag-tag using an In-Fusion HD Cloning Kit (Takara Bio Inc. Japan) to construct pCAGGS/flag-JEVcore, pCAGGS/flag-DENVcore, pCAGGS/flag-ZIKAc core, and pCAGGS/flag-WNVcore.

[0100]

JEV core:

Fw GCTGTACAAGCTCGAGATGACTAAAAACCAGGAGG (SEQ ID NO: 1)

15 Rv ACAGGTTTTCCTCGAGTCTTTTGTTTGCTTCTGC (SEQ ID NO: 2)

DENV core:

Fw GCTGTACAAGCTCGAGATGAATAACCAACGAAAAA (SEQ ID NO: 3)

Rv ACAGGTTTTCCTCGAGTCTGCGTCTCCTATTCAAGA (SEQ ID NO: 4)

ZIKV core:

20 Fw GCTGTACAAGCTCGAGATGAAAAACCCAAAGAAGAA (SEQ ID NO: 5)

Rv ACAGGTTTTCCTCGAGACGTCTCTTCTCTCTTTCC (SEQ ID NO: 6)

WNV core:

Fw GCTGTACAAGCTCGAGATGTCTAAGAAACCAGGAGG (SEQ ID NO: 7)

Rv ACAGGTTTTCCTCGAGTCTTTTCTTTTGTTTGAGC (SEQ ID NO: 8)

25 [0101]

Reference Example 1-2: Transient culture cell expression

Liver cancer cells Huh7 (provided by the National Institute of Infectious Diseases) was cultured in a Dulbecco's Modified Eagle's medium (containing 10% fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin) at 37°C under 5% CO₂. 1 x 10⁵ Huh7 cells were seeded on a 35 mm dish (IWAKI, Japan) containing a 15 mm round micro cover glass (Matsunami, Japan). After 24 hours of culture, pCAGGS/flag-JEVcore, pCAGGS/flag-DENVcore, pCAGGS/flag-ZIKAc core, and pCAGGS/flag-WNVcore were each

introduced using a TransIT-LT1 Transfection Reagent (Mirus Bio LLC, WI). After 24 hours, the cells were immobilized with 4% paraformaldehyde and then treated for 5 minutes with 0.5% Triton-X100. After incubating for 60 minutes with
5 anti-Flag-tag antibodies diluted 1000-fold (SIGMA), the cells were washed with PBS, then an anti-mouse secondary antibody (Invitrogen) diluted 2000-fold was added, and the cells were incubated for 60 minutes. After washing with PBS, the cells were observed with a fluorescence microscope
10 (Olympus). Figure 1 (A) shows the obtained results. The cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride, DOJINDO, Japan, final concentration of 0.2 µg/mL). Flag (top row) shows intracellular localization of the studied core proteins
15 (JEV core, DENV core, ZIKV core, WNV core). It can be understood that each of the proteins is localized in the cytoplasm and nucleus (nucleolus). DAPI (bottom row) shows the genomic DNA (cell nucleus).

[0102]

20 Reference Example 1-3: Intracellular localization of core proteins derived from baculovirus

In the same manner as Reference Example 1-1, core protein cDNAs of each of JEV, DENV, ZIKV, and WNV were amplified by PCR and incorporated into an EcoRI part of a
25 baculovirus expression vector pFastBac1 (Thermo Fisher Scientific Inc., MA), in a form where AcGFP (Clontech) is fused to the N-terminus and FLAG-One-STrEP (FOS) tag (Kouwaki T, et al. (2016) Journal of virology 90: 3530-3542) is fused to the C-terminus, using an In-Fusion HD
30 Cloning Kit (Takara Bio Inc., Japan) to construct pFastBac1/AcGFP-JEVcore-FOS, pFastBac1/AcGFP-DENVcore-FOS, pFastBac1/AcGFP-ZIKAc core-FOS, and pFastBac1/AcGFP-WNVcore-FOS.

[0103]

35 Competent E. coli (DH10 Bac (Thermo Fisher Scientific

Inc.)) was transformed using baculovirus expression plasmids pFastBac1/AcGFP-JEVcore-FOS, pFastBac1/AcGFP-DENVcore-FOS, pFastBac1/AcGFP-ZIKAc core-FOS, and pFastBac1/AcGFP-WNVcore-FOS. 100 μ l of each of 0.1 M isopropyl- β -D-thiogalactopyranoside (IPTG) and 25 mg/mL 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) was applied to a 2 $\times$ YT agar medium supplemented with kanamycin, gentamicin, and tetracycline, and DH10 Bac transformed with the baculovirus expression plasmids described above was seeded and cultured for 3 days at 37°C. After culturing the colony, plasmid DNA was extracted and transfected into Sf9 insect cells (Thermo Fisher Scientific Inc.) using an X-treme GENE 9 DNA transfection reagent (Roche Applied Science, Germany). After 4 days of culture at 27°C, Sf9 insect cells were retrieved from 24 10 cm petri dishes and suspended in 12 mL cell lysis buffer (20 mM Tris-HCl (pH7.4), 135 mM NaCl, 1% Triton X-100, 10% glycerin). After centrifugation of the cell suspension, 300 μ L Strep-Tactin Sepharose was added to the supernatant, and the mixture was incubated for 12 hours at 4°C. After washing the Strep-Tactin Sepharose with buffer W (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA), a protein was eluted with 2.4 mL buffer E (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin).

[0104]

Each of the AcGFP fused core proteins purified from a baculovirus was microinjected into the cytoplasm of Huh7 cells with an injection marker mouse IgG (Santa Cruz Biotechnology, Texas, USA) and incubated for 30 minutes. Mouse IgG was detected with an Alexa Fluor 594 anti-mouse secondary antibody (Thermo Fisher Scientific Inc.). Figure 1(B) shows the obtained results. Injection marker (bottom row) shows intracellular localization of mouse IgG. Since mouse IgG injected into the cytoplasm cannot migrate into the nucleus, IgG remained in the cytoplasm. It can be

understood therefrom that a sample is correctly injected into the cytoplasm and the nuclear membrane is not physically disrupted by microinjection. GFP (top row) shows that mouse IgG and core proteins (GFP-JEV core, GFP-DENV core, GFP-ZIKV core, GFP-WNV core) microinjected into the cytoplasm have activity to localize within the nucleus.

[0105]

Reference Example 1-4: Evidence of intranuclear migration activity and nucleolar localization activity of JEV core protein purified from a baculovirus

AcGFP-JEV core and injection marker anti-mouse secondary antibody (Alexa Fluor Plus 555, Thermo Fisher Scientific Inc.) were admixed and microinjected into the cytoplasm of Huh7 cells. After 30 minutes of incubation, cells were immobilized, and an immunofluorescence assay was performed using an anti-nucleophosmin antibody (anti-NPM1 antibody) (ab10530, Abcam, UK). An anti-mouse secondary antibody (Alexa Fluor 645, Thermo Fisher Scientific Inc.) was used as the secondary antibody, and the genomic DNA (cell nucleus) was stained with DAPI.

Figure **1(C)** shows the obtained results, wherein Injection marker shows that fluorescence of injection marker Alexa Fluor Plus 555 mouse IgG is present in the cytoplasm. This shows that the injection marker remains in the cytoplasm where the marker was injected and does not migrate into the nucleus. JEV core shows that the JEV core protein microinjected into the cytoplasm with Alexa Fluor Plus 555 mouse IgG is colocalized with a nucleolus marker NPM1. This indicates that a JEV core protein has activity to migrate to the nucleus and localize in a nucleolus. NPM1 and DAPI show the presence of a nucleolus and genomic DNA (cell nucleus), respectively.

[0106]

Example 1

Screening of substance having anti-flavivirus activity

using a change in intracellular localization of JEV core as an indicator

A substance having anti-flavivirus activity was screened using GFP fluorescence (green) area within the cell nucleus that indicates a change in intracellular localization of a JEV core as an indicator.

Example 1-1: Preparation of JEV core protein stably expressing cell strain

In the same manner as Reference Example 1-1, a JEV core gene cDNA amplified by a PCR reaction was incorporated into an EcoRI part of a lentivirus vector pLV-CAG (Okada Y, et al. (2007) Nat. Biotechnol 25: 233-237) comprising a super folder GFP-11 (sfGFP11) using an In-Fusion HD Cloning Kit (Takara Bio Inc.) to construct a plasmid for preparing stably expressing cell strain pLV-CAG/sfGFP-JEVcore. Synthesis of sfGFP1-10-SV40NLS gene cDNA was commissioned to Integrated DNA Technologies (IDT, Inc., Illinois), and the cDNA was incorporated into the EcoRI part of a lentivirus vector pFUGW (Addgene, plasmid #14883) with a neomycin resistant gene under an IRES (ribosomal entry site inside an mRNA) sequence using an In-Fusion HD Cloning Kit (Takara Bio Inc.) to construct the plasmid pFUGW/sfGFP1-10-SV40NLS.

[0107]

pLV-CAG/sfGFP-JEVcore cDNA and pFUGW/sfGFP1-10-SV40NLS cDNA were then introduced into Huh7 cells using a TransIT-LT1 transfection reagent (Mirus Bio LLC), and 1 mg/mL G418 solution (nacalai tesque) was added to selectively retrieve transfected cells. G418 resistant Huh7 (nacalai tesque) was seeded at a low cell density because they form a colony. A single colony with GFP fluorescence was retrieved under a fluorescence microscope to prepare a JEV core protein stably expressing cell strain.

[0108]

Example 1-2: Screening of substance having anti-flavivirus

activity

1 x 10⁴ sfGFP-JEVcore stably expressing Huh7 cells prepared in Example 1-1 were seeded on a 96-well microplate (Greiner bio-one) and cultured for 24 hours in a 37°C 5% CO₂ incubator, and then cultured for an additional 24 hours after adding small molecule compounds of SCADS Inhibitor Kit III ver 1.5 (Table 1: Kinase Inhibitor Kit, Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Innovative Areas, "Scientific Support Programs for Cancer Research", from The Ministry of Education, Culture, Sports, Science and Technology, Japan (<https://scads.jfcr.or.jp/kit/kit.html>)) to the medium so that the final concentration of each compound would be 1 μM.

After culturing and then washing the cells with PBS, the cells were incubated for 15 minutes in a 3.7% formalin solution to immobilize the cells, and the genomic DNA (cell nucleus) was stained with (DOJINDO, Japan, final concentration of 0.2 μg/mL). A plate was run through a high throughput cell function searching system CV7000S (Yokogawa Electric Corporation). Three fluorescence pictures were taken per well to analyze the GFP fluorescence intensity and intracellular localization.

[0109]

[Table 1-1]. List of candidate substances having anti-flavivirus activity

Category	Candidate substance	IC50(μM)[target]	Distributor
blank	none(DMSO)		
AK	ABT-702	0.05 [AK]	Calbio Chem
AKT	Akt Inhibitor IV		Calbio Chem
AKT	Akt Inhibitor VIII, Isozyme-Selective, Akti-1/2	0.058 [Akt1], 0.21 [Akt2], 2.1 [Akt3]	Calbio Chem
AKT	Akt Inhibitor XI	0.1 [Akt]	Calbio Chem
AMPK	compound C	0.1 [AMPK]	Calbio Chem
ATM	ATM/ATR kinase inhibitor	0.2 [ATM]	Calbio Chem
ATM	ATM kinase inhibitor	0.013 [ATM]	Calbio Chem
Aurora	Aurora kinase/ck1 inhibitor	0.011 [aurora-A], 0.015 [aurora-B]	Calbio Chem
Aurora	Aurora kinase inhibitor II	0.31 [aurora-A], 0.24 [aurora-B]	Calbio Chem
Aurora	Aurora kinase inhibitor III	0.042 [aurora-A]	Calbio Chem

Bcr-abl	AG957	0.75 (K) [Bcr-abl]	Calbio Chem
BTk	LFM-A13	2.5 [BTk]	Calbio Chem
BTk	Terreic acid	3 [BTk]	TOCRIS
CAMKII	KN-93	0.37 (K) [CAMKII]	WAKO
CAMKII	KN-62	0.9 [CAMKII]	WAKO
CAMKII	Lavendustin C	0.2 [CAMKII], 0.2 [c-src]	Calbio Chem
CDK	Kenpaulone	CDK1/cycB	Calbio Chem
CDK	purvalanol A	0.004 [cdc2], 0.07 [cdk2/A], 0.035 [cdk2/E], 0.075 [cdk5]	Sigma
CDK	Olomoucine		WAKO
CDK	Alsterpaulone, 2-cyanoethyl	230pM [Cdk1]	Calbio Chem
CDK	Cdk1/2 inhibitor III	600pM [Cdk1], 500 pM [Cdk2]	Calbio Chem
CDK	Cdk2/9 inhibitor	0.002 [Cdk2], 0.004 [Cdk9]	Calbio Chem
CDK	NU6102	0.01 [Cdk1], 0.005 [Cdk2]	Calbio Chem
CDK	Cdk4 inhibitor	0.076 [Cdk4]	Calbio Chem
CDK	NSC625987	0.2 [Cdk4]	TOCRIS
Chk	SB218078		Calbio Chem
Chk	isogranulatimide	0.1 [Chk1]	Calbio Chem
Chk	Chk2 inhibitor	0.008 [Chk2]	Calbio Chem
Chk	Chk2 inhibitor II	0.015 [Chk2]	Calbio Chem
CK	Ellagic acid	0.04 [CK2]	Calbio Chem
CK	TBB	0.9 [CKII]	Calbio Chem
CK	DMAT	0.14 [CK2]	Calbio Chem
CK	D4476	0.2 [CK1]	Calbio Chem
Clk	TG003	0.01 [Clk1]	Calbio Chem

[0110]

[Table 1-2]. List of candidate substances having anti-flavivirus activity

Category	Candidate substance	IC50(μM)[target]	Distributor
DGK	Diacylglycerol kinase inhibitor II	0.12 [DGK]	Calbio Chem
DNA-PK	IC60211	0.43 [DNA-PK]	SantaCruz
eEF2	TX-1918	0.44 [eEF2-K]	Calbio Chem
EGFR	BPIQ-II	8 pM [EGFR]	SCB
EGFR	AG1478	0.003 [EGFR]	Calbio Chem
EGFR	AG490	JAK-2	ALEXIS
FGFR	SJ4984		ALEXIS
FGFR	SJ5402		Calbio Chem
Flt-3	Flt-3 Inhibitor	0.042 [Flt-3]	Calbio Chem
Fms	cFMS Receptor Tyrosine Kinase Inhibitor	0.03 [cFMS]	Calbio Chem
Fyn	SJ6656	0.17 [Fyn], 0.28 [Src]	Calbio Chem
GSK	GSK-3 inhibitor IX	0.005 [GSK-3a]	Calbio Chem
GSK	1-Azakenpaulone	0.018 [GSK-3b]	Calbio Chem
GSK	indirubin-3'-monoxime	0.022 [GSK-3b], 0.18 [Cdk1], 0.1 [Cdk5]	Calbio Chem
HER2	AG825	0.35 [HER2], 19 [EGFR]	Calbio Chem

IGF-IR	AG1024	IGF-IR, IR	Calbio Chem
IGF-IR	AGL 2263	0.4 [IR], 0.43 [IGF-1R]	Calbio Chem
IKK	BMS-345541	0.3 [IKK-2]	Calbio Chem
IKK	IKK-2 inhibitor VI	0.013 [IKK-2]	Calbio Chem
IRAK	IRAK-1/4 inhibitor	0.3 [IRAK]	Calbio Chem
Jak	JAK Inhibitor I	0.015 [JAK1], 0.001 [JAK2], 0.005 (K) [JAK3], 0.001 [Tyk2]	Calbio Chem
Jak	JAK3 Inhibitor VI	0.027 [JAK3]	Calbio Chem
JNK	SP600125	0.04 [JNK]	TOCRIS
JNK	JNK inhibitor VIII	0.002 [JNK]	Calbio Chem
Lck	Damcanthal	p56lck	Calbio Chem
Lck	PP2	0.004 [Lck]	Calbio Chem
MAPK	ERK inhibitor II	0.51 [ERK1], 0.33 [ERK2]	Calbio Chem
MEK	PD98059	MEK	WAKO
MEK	U-0126	0.072 [MEK1], 0.058 [MEK2]	WAKO
MEK	MEK inhibitor I	0.012 [MEK]	Calbio Chem
Met	SJ11274	0.02 [Met]	Calbio Chem
MLCK	ML-7	0.3 [MLCK]	Calbio Chem

[0111]

[Table 1-3]. List of candidate substances having anti-flavivirus activity

Category	Candidate substance	IC50(μM)[target]	Distributor
p38	SB202190	0.016 [p38b]	Calbio Chem
p38	SB239063	0.044 [p38a, b]	Calbio Chem
PDGFR	AG1296	1 [PDGFRa], 0.8 [PDGFRb]	WAKO
PDGFR	SJ11652	0.003 [PDGFR], 0.027 [VEGFR2], 0.17 [VEGFR1]	Calbio Chem
PDGFR	PDGF receptor tyrosine kinase inhibitor V	0.004 [PDGFR]	Calbio Chem
PDGFR	PDGF receptor tyrosine kinase inhibitor IV	0.004 [PDGFR]	Calbio Chem
PI3K	LY-294002	1.4 [PI3K]	CAYMAN
PI3K	Wortmannin		Calbio Chem
PKA	H-89		LKT
PKA	4-cyano-3-methylisoquinoline	0.03 [PKA]	Calbio Chem
PKC	Bisindolymaleimide I, HCl	0.01 [PKC]	Calbio Chem
PKC	Go7874	0.004 [PKC]	Calbio Chem
PKG	Rp-8-CPT-cGMPs		Enzo
PKG	KT5823	0.23 [PKG]	ALEXIS
PKR	PKR inhibitor	0.21 [PKR]	Calbio Chem
Raf	RAF1 kinase inhibitor I	0.009 [Raf]	Calbio Chem
Raf	ZM 336372	0.07 [Raf]	Calbio Chem
ROCK	H-1152	0.002 [ROCK]	Calbio Chem
ROCK	Y-27632	0.14 [ROCK]	Calbio Chem
Hsp90	radicol	0.27nM [v-src]	Enzo
Src	PP1 analog	1.5nM [v-src]	Calbio Chem

Syk	Syk inhibitor	0.014 [Syk]	Calbio Chem
TGF- β RI	SB431542		Sigma
TGF- β RI	TGF- β RI kinase inhibitor II	0.023 [TGF- β RI]	Calbio Chem
Tpl2	Tpl2 kinase inhibitor	0.05 [Tpl2]	SCB
TrkA	TrkA inhibitor	0.006 [TrkA]	Calbio Chem
VEGFR	VEGFR receptor tyrosine kinase inhibitor II	0.02 [KDR], 0.18 [Flt-1], 0.24 [c-Kit]	Calbio Chem
VEGFR	VEGF receptor 2 kinase inhibitor I	0.07 [VEGFR2]	Calbio Chem
VEGFR	SU1498	0.7 [Flk-1]	Calbio Chem

[0112]

Figure 2 (A) shows the obtained results. Figure 2 (A) is a graphical representation of the mean area of dots (green fluorescence) per cell (per nucleus) from measuring the GFP fluorescence (green) area within the cell nucleus by using DAPI staining as an indicator. The horizontal axis indicates the well, and the vertical axis indicates the mean area of GFP fluorescence. The arrows indicate wells where three types of CDK inhibitors ((Alsterpaullone, 2-cyanoethyl), Cdk1/2 inhibitor III (5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide), and Cdk2/9 inhibitor ((4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine)) were administered, showing that the fluorescence area actually becomes smaller.

[0113]

Figure 2 (B) shows images of fluorescence when treated with DMSO (control) and the three CDK inhibitors described above. It can be seen that sfGFP-JEVcore is localized in a nucleolus that is clearly present within the nucleus when treated with DMSO. Meanwhile, fluorescence of sfGFP-JEV core appears as small dots in CDK inhibitor treated cells. It can also be seen that the number of dots and brightness of fluorescence vary for each CDK inhibitor.

[0114]

The three types of CDK inhibitors are described in detail below.

*Alsterpaullone, 2-cyanoethyl: 3-(6-oxo-9-nitro-5,6,7,12-

tetrahydroindolo[3,2-d][1]benzazepin-2-yl)propionitrile

(Kunick, C., et al. 2005. ChemBioChem. 6, 541-549.)

*Cdk1/2 inhibitor III: 5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-
 5 1,2,4-triazole-1-carbothioamide (Lin, R., et al. 2005. J. Med. Chem. 48, 4208-4211.)

*Cdk2/9 inhibitor: (4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine (Kontopidis, G., et al. 2006. Chem. Biol. 13, 201.)

10 [0115]

Example 1-3: Screening of CDK inhibitors that change the nucleolar localization of JEV core protein

1 x 10⁵ sfGFP-JEVcore stably expressing Huh7 cells prepared in Example 1-1 on a 35 mm dish (IWAKI, Japan)
 15 containing a 15 mm round micro cover glass (Matsunami, Japan) were cultured for 24 hours. CDK inhibitors (Selleck Customized Library of 30 compounds: Cherry Pick Library (96-well)-L2000-Z237098-100uL (Selleck.co.jp, Japan, Table 2)) were then added so the final concentration would be 1
 20 µM, and the cells were cultured for an additional 24 hours.
 [0116]

After culture, the cells were immobilized with 4% paraformaldehyde and observed with a fluorescence microscope (Olympus). Table 2 shows CDK inhibitors
 25 resulting in a change in nucleolar localization of sfGFP-JEVcore.

1 x 10⁵ Huh7 cells on a 35 mm dish (IWAKI, Japan) containing a 15 mm round micro cover glass (Matsunami, Japan) were cultured for 24 hours. pCAGGS/flag-JEVcore
 30 prepared in Reference Example 1-1 was then introduced using a TransIT-LT1 Transfection Reagent (Mirus Bio LLC, WI). After 24 hours, CDK inhibitors (Selleck Customized Library of 30 compounds (Selleck.co.jp, Japan, see Table 2)) were added so the final concentration would be 1 µM, and the
 35 cells were cultured for an additional 24 hours. After

culture, the cells were immobilized and stained by the immunofluorescence assay performed in Reference Example 1-2. Table 2 shows CDK inhibitors resulting in a change in nucleolar localization of JEVcore (Flag-core).

5 [0117]
[Table 2]

CDK inhibitor	CDK1	CDK2	CDK3	CDK4	CDK5	CDK6	CDK7	CDK9
SNS-032 (BMS-387032)		+++			+		++	++++
Dinaciclib	++++	++++			++++			++++
Flavopiridol (alvocidib)	+++	+++		+++		+++	+	
AT7519	++	++	+	++	+++	++		+++
Flavopiridol hydrochloride	+++	+++		+++		+++	+	
P276-00	++	+		++		+	+	+++
AT7519 HCl	++	++	+	++	+++	++		+++
AZD5438	+++	++++						+++
A-674563		++						
R547	++++	++++		++++				
Kenpaulone	+	+			+			
SU9516	+++	+++		++				

In Table 2, "+" represents specificity to each CDK in an inhibitor. Greater number of "+" indicates higher specificity and effect. As can be seen from Table 2, studied CDK inhibitors have an effect on multiple CDKs.

[0118]

Example 2

Change in morphology of nucleolus due to Cdk1/2 inhibitor
15 III

Example 1 demonstrated that three types of CDK inhibitors, alsterpaulone, 2-cyanoethyl, Cdk1/2 inhibitor III, and Cdk2/9 inhibitor actually reduce the fluorescence area.

20 The cells used were of an sfGFP-JEVcore stably expressing cell strain with a core protein already present

within the nucleus (nucleolus). In this Example, a CDK inhibitor was added to the cell strain at a later time. The following experiment was conducted to study what action changes the localization of a core protein.

5 Example 2-1: Nucleolar morphological abnormality due to Cdk1/2 inhibitor III treatment

After culturing Huh7 cells for 24 hours in the presence of Cdk1/2 inhibitor III at a final concentration of 1 μ M, an immunofluorescence assay was performed with an anti-NPM1
10 antibody. An anti-mouse secondary antibody (Alexa Fluor 645) was used as the secondary antibody, and the genomic DNA (cell nucleus) was stained with DAPI. Figure 3(A) shows the obtained results.

It can be understood by comparing DMSO treatment (first
15 diagram from the left on the top row, NPM1 staining (using $\times$ 40 objective lens) and second diagram from the left on the top row, NPM1 staining (high power diagram), control) with Cdk1/2 inhibitor III treatment (third diagram from the left on the top row, NPM1 staining (using $\times$ 40 objective
20 lens) and fourth diagram from the left on the top row, NPM1 staining (high power diagram), inhibitor treatment) that NPM1 is localized in a large and clear nucleolus within the nucleus for the control, whereas NPM1 is present throughout the nucleoplasm and in small constructs for the inhibitor
25 treatment. The nucleoplasm is identified from genomic DNA staining using DAPI (four diagrams on the bottom row, showing the same cells as the NPM1 staining on the top row). Since NPM1 is a nucleolus specific marker protein, the small constructs within the nucleus can be considered
30 nucleoli. It was found in view of the above that Cdk1/2 inhibitor III treatment resulted in a morphological abnormality of a nucleolus.

[0119]

Example 2-2: Nucleolar morphological abnormality due to
35 alsterpaullone, 2-cyanoethyl treatment

2 x 10⁴ Huh7 cells were seeded on a 12-well plate containing a 15 mm round micro cover glass (Matsunami) and were cultured for 24 hours in a 37°C 5% CO₂ incubator. After culture, alsterpaullone, 2-cyanoethyl (SCADS Inhibitor Kit III ver 1.5 (kinase inhibitor kit)) was added to the medium so that the final concentration would be 1 µM, and the cells were cultured for 24 hours. An AcGFP-JEV core recombinant protein (final concentration of 100 ug/ml) purified from a baculovirus and an injection marker goat anti-mouse IgG (H+L) high cross-adsorbed secondary antibody Alexa Fluor Plus 555 (Thermo Fisher Scientific Inc., final concentration of 100 ug/mL) were admixed and microinjected into the cytoplasm of Huh7 using a glass needle. After culturing for 30 minutes in a 37°C 5% CO₂ incubator, the cells were incubated for 15 minutes in a 3.7% formalin solution to immobilize the cells. After washing the cells with PBS, a DAPI solution (final concentration of 0.2 ug/mL) was added, the cells were incubated for 20 minutes at room temperature, and the genomic DNA (cell nucleus) was stained. Cells were observed using a confocal microscope TCS SP8 (Leica).

[0120]

Figure **3(B)** shows the obtained results, wherein Injection marker shows that fluorescence of an injection marker Alexa Fluor Plus 555 mouse IgG is in the cytoplasm. This shows that the injection marker remains in the cytoplasm where the marker was injected. JEV core shows that AcGFP-JEVcore is within the nucleus and is localized in a small construct that is different from a normal nucleolus. This shows that the JEV core protein is microinjected into the cytoplasm with Alexa Fluor Plus 555 mouse IgG, so that nuclear migration of AcGFP-JEVcore is not inhibited by alsterpaullone, 2-cyanoethyl treatment. Meanwhile, it can be understood that a JEV core protein within the nucleus is localized in a small nucleolus that

is different from normal. This indicates that alsterpaullone, 2-cyanoethyl treatment induces a morphological abnormality of a nucleolus, and a JEV core protein migrates into the nucleus and localizes in a nucleolus exhibiting an abnormal morphology. The genomic DNA (cell nucleus) is stained with DAPI. merge is a combination of images of Injection marker, JEV core, and DAPI.

[0121]

10 Example 3

Suppression of JEV replication due to identified CDK inhibitors

3 × 10⁴ Huh7 cells were seeded on a 24-well plate and cultured for 24 hours in a 37°C 5% CO₂ incubator. Subsequently, the cells were infected with JEV (AT31 strain) for 2 hours at multiplicity of infection (Moi) = 1. The JEV infected Huh7 cells were washed with PBS and cultured for 24 hours in media comprising three types of CDK inhibitors, i.e., Cdk1/2 inhibitor III, Cdk2/9 inhibitor, and alsterpaullone, 2-cyanoethyl. The infectious titer and cell viability were analyzed through a focus forming assay and CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega Corporation, WI), respectively.

[0122]

25 A focus forming assay was performed by the following method. First, the culture supernatant of JEV infected Huh7 cells was collected and serially diluted 10-fold with PBS. 5 × 10⁴ Vero cells (Mori Y., et al. (2005) Journal of virology 79: 3448-3458) were seeded on a 24-well plate on the day before the assay and cultured for 2 hours after adding the culture supernatant sample diluted 10-fold at an amount of 100 µl/well. The cells were washed with PBS, and Dulbecco's Modified Eagle's medium (containing 10% fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin) comprising 1% methylcellulose was added. The

cells were further cultured for 2 days and then immobilized with 4% paraformaldehyde. The immobilized cells were treated for 5 minutes with 0.5% Triton-X100, and incubated for 30 minutes with an anti-NS3 mouse antibody (Mori Y., et al. (2005) Journal of virology 79: 3448-3458) diluted 2000-fold. After washing with PBS, a biotin crosslinked anti-mouse secondary antibody (Vector Laboratories, USA) diluted 500-fold was added, and the cells were incubated for 30 minutes. After washing with PBS, Strept ABC Peroxidase Kit (nacalai tesque, Japan) was added. Vector VIP Peroxidase Substrate (Vector Laboratories, CA) was continuously added for 30 minutes, and the cells were incubated for 10 minutes. Lastly, the cells were washed, dried, and the number of foci was counted.

[0123]

Figure 4 shows the obtained results. A CDK inhibitor was added to JEV infected Huh7 cells at the timing of (a) and (b) indicated in Figure 4 (A), and the cells were incubated for 24 hours. The cell viability 48 hours after infection is shown in (B), and the infectious titer is shown in (C). It can be understood that treatment with a CDK inhibitor does not affect cell viability, but viral replication is suppressed.

[0124]

Example 4

Morphological change in nucleolus due to substances having rRNA transcription inhibitory activity

The following experiment was conducted to study whether rRNA transcription inhibitor treatment changes the nucleolar localization of a Japanese encephalitis virus core protein in a biological cell.

The cells used were of the sfGFP-JEVcore stably expressing Huh7 cell strain prepared in Example 1-1. 1×10^4 cells were seeded on a 96-well microplate (Greiner bio-one) and cultured for 24 hours in a 37°C 5% CO₂ incubator.

rRNA transcription inhibitors actinomycin D, doxorubicin, and aclarubicin were then added so that the final concentration would be 1 μ M, and the cells were cultured for an additional 24 hours.

5 After the culture, the cells were washed with PBS. The cells were then incubated for 15 minutes in a 3.7% formalin solution to immobilize the cells, and the genomic DNA (cell nucleus) was stained with (DOJINDO, Japan, final concentration of 0.2 μ g/mL). A plate was run through a high
10 throughput cell function searching system CV7000S (Yokogawa Electric Corporation). Three fluorescence pictures were taken per well to analyze the GFP fluorescence intensity and intracellular localization.
[0125]

15 Figure 5 shows the obtained results, showing images of fluorescence when treated with DMSO (control) and the three rRNA transcription inhibitors described above. It can be seen that sfGFP-JEVcore is localized in a nucleolus that is clearly present within the nucleus when treated with DMSO.
20 Meanwhile, fluorescence of sfGFP-JEV core appears as small dots for each of the rRNA transcription inhibitors actinomycin D, doxorubicin, and aclarubicin.
[0126]

Example 5

25 Suppression of flavivirus proliferation due to substances having GSK-3 inhibitory activity

Screening was performed substantially in accordance with the methodology of Example 1, other than using small molecule compounds obtained from a GSK-3 inhibitor library
30 (<https://www.selleck.co.jp/GSK-3.html>) as candidate substances having anti-flavivirus activity.

Figure 6 (A) shows the obtained results, which revealed that 1-Azakenpaullone suppresses GFP fluorescence intensity.
[0127]

35 Suppression of proliferation of Japanese encephalitis

viruses, dengue viruses, and Zika viruses due to the identified 1-Azakenpaullone was then studied.

3 x 10⁴ Huh7 cells were seeded on a 24-well plate and cultured for 24 hours in a 37°C 5% CO₂ incubator. The cells were then infected with Japanese encephalitis viruses (JEV, AT31 strain), dengue viruses (DENV, H241 strain), and Zika viruses (ZIKV, MR766 strain) for 2 hours at multiplicity of infection (Moi) = 1. The infected Huh7 cells were washed with PBS and cultured for 24 hours in a medium comprising 1-Azakenpaullone (final concentration of 1 μM). The infectious titer was analyzed through a focus forming assay (see Example 3).

[0128]

Figure 6(B) shows the obtained results. 1-Azakenpaullone was added to the virus infected Huh7 cells, and the cells were incubated for 24 hours. The infectious titer 24 hours after infection is shown. It can be understood that 1-Azakenpaullone treatment suppresses viral proliferation.

Example 6

Further screening of substances having anti-flavivirus activity

Screening was performed substantially in accordance with the methodology of Example 1, other than using small molecule compounds obtained from LOPAC1280 (provided by the Graduate School of Pharmaceutical Sciences, Kyoto University) and FDA-approved Drug Library (provided by the Graduate School of Pharmaceutical Sciences, Osaka University) as candidate substances having anti-flavivirus activity.

This revealed that substances having DNA topoisomerase activity: ellipticine, idarubicin, idarubicin hydrochloride, epirubicin hydrochloride, mitoxantrone hydrochloride, and daunorubicin hydrochloride suppress GFP fluorescence intensity.

[0129]

Next, a study was conducted to find a morphological change of a nucleolus, more specifically whether the nucleolar localization of a Japanese encephalitis virus core protein in a biological cell is changed, due to the identified substances having DNA topoisomerase inhibitory activity.

The cells used were of a sfGFP-JEVcore stably expressing Huh7 cell strain prepared in Example 1-1. 1×10^4 cells were seeded on a 96-well microplate (Greiner bio-one) and cultured for 24 hours in a 37°C 5% CO₂ incubator. Substances having DNA topoisomerase inhibitory activity, i.e., ellipticine, idarubicin, idarubicin hydrochloride, epirubicin hydrochloride, mitoxantrone hydrochloride, and daunorubicin hydrochloride, were then added so that the final concentration would be 1 μ M, and the cells were cultured for an additional 24 hours.

After culture, the cells were washed with PBS. The cells were then incubated for 15 minutes in a 3.7% formalin solution to immobilize the cells, and the genomic DNA (cell nucleus) was stained with (DOJINDO, Japan, final concentration of 0.2 μ g/mL). A plate was run through a high throughput cell function searching system CV7000S (Yokogawa Electric Corporation). Three fluorescence pictures were taken per well to analyze the GFP fluorescence intensity and intracellular localization.

[0130]

Figure 7 shows the obtained results, showing images of fluorescence when treated with DMSO (control) and the DNA topoisomerase inhibitors described above. It can be seen that sfGFP-JEVcore is localized in a nucleolus that is clearly present within the nucleus when treated with DMSO. Meanwhile, fluorescence of sfGFP-JEV core is a small dot, or fluorescence is lost, dispersed and localized in the nucleoplasm for each of ellipticine, idarubicin, idarubicin

hydrochloride, epirubicin hydrochloride, mitoxantrone hydrochloride, and daunorubicin hydrochloride.

[Industrial Applicability]

[0131]

5 Due to global warming and advancement in transportation means, the habitat of disease bearing mosquitos and the geographical distribution of flaviviruses can change in the future. West Nile viruses led to many infected patients when the US was first invaded thereby in 1999. In this
10 manner, invasion into a new region by viruses is highly likely to result in many victims. For this reason, further development in the therapeutic means against flaviviruses is desirable not only in Japan, but also in every country in the world. While there are vaccines approved for use in
15 humans against Japanese encephalitis virus, yellow fever virus, and tick-borne encephalitis virus infections, thousands to tens of thousands or more patients are still annually reported worldwide today for each of the infections. The screening method of the invention provides
20 new findings for the replication mechanism or immune response/evasion mechanism and leads to the development of new antiviral drugs.

[Claims]

[Claim 1]

An anti-flavivirus pharmaceutical composition comprising a substance exerting an effect on a morphology
5 of a nucleolus in a biological cell.

[Claim 2]

The anti-flavivirus pharmaceutical composition of claim 1, comprising the substance exerting an effect on a morphology of a nucleolus in a biological cell as an active
10 ingredient having anti-flavivirus activity.

[Claim 3]

The pharmaceutical composition of claim 1 or 2, wherein the substance exerting an effect on a morphology of a nucleolus in a biological cell is a substance inducing a
15 morphological abnormality in the nucleolus.

[Claim 4]

The pharmaceutical composition of any one of claims 1 to 3, wherein the substance exerting an effect on a morphology of a nucleolus in a biological cell is also
20 involved in a change in localization of a core protein of a virus in the biological cell.

[Claim 5]

The pharmaceutical composition of any one of claims 1 to 4, wherein the substance exerting an effect on a morphology of a nucleolus in a biological cell induces
25 inhibition of viral replication.

[Claim 6]

The pharmaceutical composition of any one of claims 1 to 5, wherein anti-flavivirus activity is anti-flavivirus activity against a flavivirus selected from a Japanese
30 encephalitis virus, a dengue virus, a Zika virus, a West Nile virus, and a yellow fever virus.

[Claim 7]

The pharmaceutical composition of any one of claims 1 to 6, wherein the substance exerting an effect on a
35

morphology of a nucleolus in a biological cell is a substance having CDK inhibitory activity, a substance having rRNA transcription inhibitory activity, a substance having DNA topoisomerase inhibitory activity, or a substance having GSK-3 inhibitory activity.

[Claim 8]

The pharmaceutical composition of claim 7, wherein the substance having CDK inhibitory activity is selected from: alsterpaullone, 2-cyanoethyl; 5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide; (4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine; P276-00; A-674563; SU9516; SNS-032 (BMS-387032); dinaciclib; flavopiridol; AT7519; flavopiridol hydrochloride; AT7519 HCl; AZD5438; R547; and kenpaullone.

[Claim 9]

The pharmaceutical composition of claim 8, wherein the substance having CDK inhibitory activity is selected from: alsterpaullone, 2-cyanoethyl; 5-amino-3-((4-(aminosulfonyl)phenyl)amino)-N-(2,6-difluorophenyl)-1H-1,2,4-triazole-1-carbothioamide; and (4-(2-amino-4-methylthiazol-5-yl)pyrimidin-2-yl)-(3-nitrophenyl)amine.

[Claim 10]

The pharmaceutical composition of claim 7, wherein the substance having rRNA transcription inhibitory activity is selected from actinomycin D, doxorubicin, and aclarubicin.

[Claim 11]

The pharmaceutical composition of claim 7, wherein the substance having DNA topoisomerase inhibitory activity is selected from doxorubicin, aclarubicin, ellipticine, idarubicin, idarubicin hydrochloride, epirubicin hydrochloride, mitoxantrone hydrochloride, and daunorubicin hydrochloride.

[Claim 12]

The pharmaceutical composition of claim 11, wherein the

substance having DNA topoisomerase inhibitory activity is selected from doxorubicin, aclarubicin, and ellipticine.

[Claim 13]

5 The pharmaceutical composition of claim 7, wherein the substance having GSK-3 inhibitory activity is 1-Azakenpaullone.

[Claim 14]

A method of screening for a substance having anti-flavivirus activity, comprising the following steps:

- 10 (a) contacting a biological cell with a candidate compound of a substance having anti-flavivirus activity *in vitro*;
(b) testing whether the candidate compound exerts an effect on a morphology of a nucleolus in the biological cell; and
(c) identifying the candidate compound as a substance
15 having anti-flavivirus activity if the candidate compound exerts an effect.

[Claim 15]

The method of claim 14, wherein an effect due to the candidate compound is a morphological abnormality of a
20 nucleolus in the biological cell in step (c).

[Claim 16]

The method of claim 14 or 15, wherein a morphological abnormality of a nucleolus in a biological cell is identified by a change in the formation and/or localization
25 of a protein or nucleic acid localized in the nucleolus.

[Claim 17]

The method of claim 16, wherein the candidate compound, which has a change in the formation and/or localization of a protein localized in a nucleolus that is a change
30 relative to an effect of a control compound in the protein localized in the nucleolus, is determined as exerting an effect on a morphology of the nucleolus.

[Claim 18]

The method of any one of claims 14 to 17, wherein a
35 protein localized in a nucleolus in a biological cell is

one or more proteins selected from nucleophosmin, nucleolin, fibrillarin, RNA polymerase I, and flavivirus core proteins.
[Claim 19]

5 The method of any one of claims 14 to 18, wherein the biological cell is a cultured cell line derived from a eukaryote including humans such as a liver derived Huh7 cell, a primary culture cell, a primate derived cell line such as a monkey kidney derived Vero cell, or a cell established by a mosquito such as a C6/36 cell.

10