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(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101 Rue de Tolbiac, 75013 Paris (FR). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (CNRS)** [FR/FR]; 3, Rue Michel Ange, 75016 Paris (FR). **ECOLE NORMALE SUPÉRIEURE DE LYON** [FR/FR]; 15 Parvis René Descartes, 69007 Lyon (FR). **SORBONNE UNIVERSITÉ** [FR/FR]; 21 rue de l'Ecole de Médecine, 75006 Paris (FR). **UNIVERSITÉ CLAUDE BERNARD - LYON 1** [FR/FR]; 43 Boulevard du 11 novembre 1918, 69100 Villeurbanne (FR).

(72) Inventors: **COSSET, Francois-Loic**; U1111 - TOUR INSERM, 69365 Lyon - CEDEX 07 (FR). **CARBONE, Alessandra**; UMR7238, Equipe « Génomique Analytique », 4 Place Jussieu, 75005 Paris (FR). **PEREZ-VARGAS OBREGON, Jimena**; 877 West 7th Avenue-Unit 14, Vancouver, BC V5Z1C2 (CA). **TEPPA, Roxana Elin**; Toulouse Biotechnology Institute Groupe Molecular Modelling & Design, Biocatalysis Department -135, avenue de Rangueil, 31077 Toulouse cedex 04 (FR).

(74) Agent: **INSERM TRANSFERT**; 7 rue Watt, 75013 Paris (FR).

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(54) Title: METHODS FOR TREATING HEPATITIS B AND D VIRUS INFECTION

(57) Abstract: In the present invention, a combination of computational and experimental approaches was used to unravel the main determinants of hepatitis B virus (HBV) membrane fusion process. They discovered that ERp57 is a host factor critically involved in triggering HBV fusion and infection. Then, through modelling approaches, they uncovered a putative allosteric cross-strand disulfide (CSD) bond in the HBV S glycoprotein and demonstrate that its stabilization could prevent membrane fusion. These results underscore a membrane fusion mechanism that could be triggered by ERp57, allowing a thiol/disulfide exchange reaction to occur and regulate isomerization of a critical CSD, which ultimately leads to the exposition of the fusion peptide. Finally, targeting ERp57 with a pharmacological agent or with oligo antisense approach in a infection assay with human cell line (with HDV and HBV particle) induced a strongly decrease the level of HBV and HDV infection. Furthermore, these results were confirmed in vivo in a liver humanized mice (HuHep mice) and inhibition of ERp57 with a pharmacological agent prevent HBV propagation and infection. Accordingly, the present invention relates to an inhibitor of ERp57 for use in the treatment of Hepatitis B virus (HBV) or Hepatitis B virus (HBV) infection said inhibitor said ERp57 inhibitor inhibit the virus entry by membrane fusion.

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# METHODS FOR TREATING HEPATITIS B AND D VIRUS INFECTION

## FIELD OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for treating Hepatitis B and/or D infections, using an inhibitor of ERp57 (cellular protein disulfide isomerase) in particular said ERp57 inhibitor inhibit the virus entry by membrane fusion.

## BACKGROUND OF THE INVENTION:

Hepatitis B is a major public health problem; it affects over 250 million people worldwide and 850,000 deaths occur each year as a result of hepatitis B complications (WHO, March 2015). The structure of its etiological agent, the hepatitis B virus (HBV), features a nucleocapsid that is surrounded by a lipid bilayer containing the envelope glycoproteins (GPs) designated as the small (S), medium (M) and large (L), which are the product of a single open reading frame. They share the C-terminal S-domain that contains four putative transmembrane domains. The L and M proteins have N-terminal extensions (preS1/prS2 and preS2, respectively) that mediate diverse functions in nucleocapsid binding and receptor recognition (Baumert et al., 2014). The first 2-75 amino acids sequence of the L protein preS1 domain of the L protein (Blanchet and Sureau, 2007) and the antigenic loop of the S domain (Le Duff et al., 2009) have been identified as essential for HBV infectivity.

Entry process of enveloped viruses into cells can be defined as the sequence of all events occurring from the attachment of the virus to the host cell until the release of the genome in the cytoplasm, via fusion between viral and cellular membrane. Like for most enveloped viruses, HBV entry into cells is a finely regulated and complex process consisting in different steps, in which several viral and cellular factors are involved. Its first step involves low-affinity binding to heparan sulfates proteoglycans (HSPGs) residing on the hepatocytes' surface. This attachment is mediated by the preS1 region of the L protein and/or the antigenic loop of the S protein (Ni et al., 2014; Schulze et al., 2007). Afterwards, the virus interacts with its high-affinity receptor, the sodium taurocholate-cotransporting polypeptide (NTCP) (Ni et al., 2014; Yan et al., 2012) through the amino-terminal end of the L protein preS1 domain (Glebe et al., 2005a; Gripon et al., 2005; Yan et al., 2012). NTCP is an integral membrane protein, expressed at the basolateral membrane of hepatocytes, which explains the tropism of HBV for the liver.

The post-binding entry steps of HBV occur through endocytosis; however, the exact mechanism is still unclear and somehow controversial. One early study showed that HBV in

HepaRG cells is internalized via caveolin-mediated endocytosis (Macovei et al., 2010a). Nevertheless, inhibition of caveolin-mediated endocytosis or silencing of caveolin-1 did not impair HBV infection in Tupaia hepatocytes (Bremer et al., 2009) or HepaG2-NTCP cells (Herrscher et al., 2020). Contrastingly, several other studies presented evidence that HBV endocytosis is clathrin-dependent (Herrscher et al., 2020; Huang et al., 2012). Recent studies reported that HBV infection of HepaRG cells depends on Rab5 and Rab7 (Macovei et al., 2013a), which are GTPases involved in the biogenesis of endosome and that the Epidermal growth factor receptor (EGFr) is a host-entry cofactor that interacts with NTCP and mediates HBV internalization (Iwamoto et al., 2019). These findings support the hypothesis that HBV is transported from early to mature endosomes. After the early endosome stage, translocation is associated with a gradually decreasing pH, from about 6.2 in early endosomes to close to 5.5 in late endosomes, which allows fusion of many enveloped viruses with the endosomal membrane. However, in the case of HBV, pharmacological agents that raise or neutralizes the pH in the endocytic pathway do not affect infection (Macovei et al., 2010a, 2013a; Rigg and Schaller, 1992a). Furthermore, treatment with protease inhibitors have no effect on infection (Macovei et al., 2013a), suggesting that HBV transport into the degradative branch of the endocytic pathway is not required *per se* to initiate this process.

Virus entry by membrane fusion involves protein–protein interactions between viral fusion proteins and host receptors, which result in conformational changes of the virus envelope proteins. However, the molecular determinants and mechanism of membrane fusion of HBV remains to be defined. Previous results indicated the essential role of the cysteine residues of the antigenic loop (AGL), as shown by the reduction of virus entry levels by inhibitors of thiol/disulfide exchange reaction (Abou-Jaoudé and Sureau, 2007), hence suggesting a redox state responsible for conformational changes that can have a role during fusion step.

## SUMMARY OF THE INVENTION:

In the present invention, using a combination of computational and experimental approaches, inventors tried to decipher how HBV induces the fusion of its lipid membrane with that of the infected cell. Specifically, using a coevolution analysis of HBV GPs and molecular modelling combined with experimental investigations ex vivo in molecular virology and in vivo in liver humanized mice, inventors provide evidence that the mechanism triggering HBV membrane fusion involves ERp57, a cellular protein disulfide isomerase. Furthermore, their results highlight the role of specific cysteines in the AGL determinant and well as a sequence

(aa 48 to 66) in the preS1 determinant that could ultimately act as a fusion peptide mediating HBV membrane fusion.

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Accordingly the present invention relates to an inhibitor of ERp57 for use in the treatment of Hepatitis B virus (HBV) and/or Hepatitis D virus (HBV) infection.

In particular embodiment, said inhibitor prevents or reduces the virus entry into hepatocytes at membrane fusion step.

The present invention also relates to a method for screening a plurality of candidate compounds useful for treating Hepatitis B virus (HBV) and/or Hepatitis D virus (HBV) infection comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit ERp57 and (b) and positively selecting the candidate compounds capable of inhibiting said ERp57.

## DETAILED DESCRIPTION OF THE INVENTION:

### ***Therapeutic Method and use***

In the present study the inventors used a combination of computational and experimental approaches to unravel the main determinants of hepatitis B virus (HBV) membrane fusion process. They discovered that ERp57 is a host factor critically involved in triggering HBV fusion and infection. Then, through modelling approaches, they uncovered a putative allosteric cross-strand disulfide (CSD) bond in the HBV S glycoprotein and demonstrate that its stabilization could prevent membrane fusion. Finally, inventors identified and characterized a potential fusion peptide in the preS1 domain of the HBV L glycoprotein. These results underscore a membrane fusion mechanism that could be triggered by ERp57, allowing a thiol/disulfide exchange reaction to occur and regulate isomerization of a critical CSD, which ultimately leads to the exposition of the fusion peptide.

Finally, targeting ERp57 with a pharmacological agent (NTZ and EGCG) or with oligo antisense approach in an infection assay with human cell line (with HDV and HBV particle induced a strongly decrease the level of HBV and HDV infection (figure 5). Furthermore, these results were confirmed in vivo in a liver humanized mice (HuHep mice) and inhibition of Erp57 with a pharmacological agent (NTZ) prevent HBV propagation and infection (figure 6).

Altogether, these results indicate that the inhibition of ERp57 induces a strong inhibition of HBV infection in hepatic cells and thus highlighting a roadmap to new avenues for therapies targeting ERp57 of the HBV and HDV infection disease

Altogether, these results indicate that the inhibition of ERp57 induces a strong inhibition of HBV infection in hepatic cells and represents an attractive new therapy for HBV infections.

Accordingly, the present invention relates to an inhibitor of ERp57 for use in the treatment of Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV) infection.

In particular embodiment, said inhibitor prevents or reduces the viral membrane fusion activity of HBV.

As used herein, the term " HBV infection " refers to an infectious disease commonly known in the art that is caused by the hepatitis B virus (HBV) and affects the liver. HBV infection can be an acute or a chronic infection. Some infected persons have no symptoms during the initial infection and some develop a rapid onset of sickness with vomiting, yellowish skin, tiredness, dark urine and abdominal pain ("Hepatitis B Fact sheet N°204". who.int. July 2014. Retrieved 4 November 2014). Often these symptoms last a few weeks and can result in death. It may take 30 to 180 days for symptoms to begin. In those who get infected around the time of birth 90% develop a chronic hepatitis B infection while less than 10% of those infected after the age of five do ("Hepatitis B FAQs for the Public - Transmission", U.S. Centers for Disease Control and Prevention (CDC), retrieved 2011-11-29). Most of those with chronic disease have no symptoms; however, cirrhosis and liver cancer may eventually develop (Chang, 2007, Semin Fetal Neonatal Med, 12: 160-167). These complications result in the death of 15 to 25% of those with chronic disease ("Hepatitis B Fact sheet N°204". who.int. July 2014, retrieved 4 November 2014). Herein, the term "HBV infection" includes the acute and chronic hepatitis B infection. The term "HBV infection" also includes the asymptotic stage of the initial infection, the symptomatic stages, as well as the asymptotic chronic stage of the HBV infection.

In particular embodiment HBV infection is a chronic infection. In particular embodiment HBV and/or HDV infection is a chronic infection. In particular embodiment HBV and HDV infections are chronic infections.

The hepatitis B virus (HBV) is an enveloped, partially double-stranded DNA virus. The compact 3.2 kb HBV genome consists of four overlapping open reading frames (ORF), which encode for the core, polymerase (Pol), envelope and X-proteins. The Pol ORF is the longest and the envelope ORF is located within it, while the X and core ORFs overlap with the Pol ORF. The lifecycle of HBV has two main events: 1) generation of closed circular DNA (cccDNA) from relaxed circular (RC DNA), and 2) reverse transcription of pregenomic RNA (pgRNA) to produce RC DNA.

As used herein, the term " HDV infection " or "Hepatitis D" refers to an infectious disease commonly known in the art that is caused by the by the hepatitis delta virus (HDV), a

small spherical enveloped particle that shares similarities with both a viroid and virusoid (Magnius L, et al (2018). "ICTV Virus Taxonomy Profile: Deltavirus". The Journal of General Virology. 99 (12): 1565–1566). HDV is one of five known hepatitis viruses: A, B, C, D, and E. HDV is considered to be a satellite (a type of subviral agent) because it can propagate only in the presence of the hepatitis B virus (HBV) (Makino S, et al (1987). "Molecular cloning and sequencing of a human hepatitis delta (delta) virus RNA". Nature. 329 (6137): 343–6). Transmission of HDV can occur either via simultaneous infection with HBV (coinfection) or superimposed on chronic hepatitis B or hepatitis B carrier state (superinfection).

HDV and HBV infecting a person simultaneously is considered the most serious type of viral hepatitis due to its severity of complications. These complications include a greater likelihood of experiencing liver failure in acute infections and a rapid progression to liver cirrhosis, with an increased risk of developing liver cancer in chronic infections (Fattovich G, et al (2000). Gut. 46 (3): 420–6) In combination with hepatitis B virus, hepatitis D has the highest fatality rate of all the hepatitis infections, at 20%.

As used herein, the terms "treatment", "treating", and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment", as used herein, covers any treatment of a disease in a subject, and includes: (a) increasing survival time; (b) decreasing the risk of death due to the disease; (c) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (d) inhibiting the disease, i.e., arresting its development (e.g., reducing the rate of disease progression); and (e) relieving the disease, i.e., causing regression of the disease.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of subjects at risk of contracting the disease or suspected to have contracted the disease as well as subjects who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By "therapeutic regimen" is meant the pattern of treatment of an illness, e.g., the pattern of dosing used during therapy. A therapeutic regimen may include an

induction regimen and a maintenance regimen. The phrase "induction regimen" or "induction period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the initial treatment of a disease. The general goal of an induction regimen is to provide a high level of drug to a subject during the initial period of a treatment regimen. An induction regimen may employ (in part or in whole) a "loading regimen", which may include administering a greater dose of the drug than a physician would employ during a maintenance regimen, administering a drug more frequently than a physician would administer the drug during a maintenance regimen, or both. The phrase "maintenance regimen" or "maintenance period" refers to a therapeutic regimen (or the portion of a therapeutic regimen) that is used for the maintenance of a subject during treatment of an illness, e.g., to keep the subject in remission for long periods of time (months or years). A maintenance regimen may employ continuous therapy (e.g., administering a drug at a regular intervals, e.g., weekly, monthly, yearly, etc.) or intermittent therapy (e.g., interrupted treatment, intermittent treatment, treatment at relapse, or treatment upon achievement of a particular predetermined criteria [e.g., disease manifestation, etc.]).

Accordingly, herein “treating a HBV infection” includes treating and preventing a HBV infection from occurring in a subject, and treating and preventing the occurrence of symptoms of a HBV infection. In the present invention in particular the prevention of HBV infection in children from HBV infected mothers are contemplated. Also contemplated is the prevention of an acute HBV infection turning into a chronic HBV infection.

The terms "subject," and "patient," used interchangeably herein, refer to a mammal, particularly a human who has been previously diagnosed with HBV/HDV or who is at risk for having or developing HBV/HDV.

As used herein the term “ERp57” also known as “Protein disulfide-isomerase A3” (PDIA3) or glucose-regulated protein, 58-kD (GRP58), has its general meaning in the art and is an disulphide isomerase enzyme (EC 5.3.4.1) that in human is encoded by PDIA3 gene (ID: 2923). ERp57 protein localizes to the endoplasmic reticulum (ER), amongst other cellular locations, and interacts with lectin chaperones calreticulin and calnexin (CNX) to modulate folding of newly synthesized glycoproteins. It is thought that complexes of lectins and this protein mediate protein folding by promoting formation of disulfide bonds in their glycoprotein substrates. The ERp57 protein is a thiol oxidoreductase that has protein disulfide isomerase activity (Dong G, et al (2009). Immunity. 30 (1): 21–32) ERp57/PDIA3 is also part of the major histocompatibility complex (MHC) class I peptide loading complex, which is essential for formation of the final antigen conformation and export from the endoplasmic reticulum to the

cell surface. This protein of the endoplasmic reticulum interacts with lectin chaperones such as calreticulin and CNX in order to modulate the folding of proteins that are newly synthesized. It is believed that ERp57 plays a role in protein folding by promoting the formation of disulfide bonds, and that CNX facilitates the positioning substrates next to the catalytic cysteines ((Dong G, et al (2009). . Immunity. 30 (1): 21–32) This function allows it to serve as a redox sensor by activating mTORC1, which then mediates mTOR complex assembly to adapt cells to oxidative damage. Thus, ERp57 regulates cell growth and death according to oxygen concentrations, such as in the hypoxic microenvironment of bones.

One example of ERp57 human amino acid sequence (UniProtKB - P30101) is provided in SEQ ID NO:1 (NCBI Reference Sequence: NP_005304.). One example of nucleotide sequence encoding wild-type ERp57 is provided in SEQ ID NO:2 (NCBI Reference Sequence: NM_005313).

An " inhibitor of ERp57" or "ERp57 antagonist" refers to a molecule (natural or synthetic) capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with the biological activities of ERp57 including, for example, reduction or blocking the HBV entry at membrane fusion step and accordingly to reduce or block the HBV infection. ERp57 inhibitors or antagonists include antibodies and antigen-binding fragments thereof, proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, bioorganic molecules, peptidomimetics, pharmacological agents and their metabolites, transcriptional and translation control sequences, and the like. Inhibitors or antagonists also include, antagonist variants of the protein, siRNA molecules directed to a protein, antisense molecules directed to a protein, aptamers, and ribozymes against a protein. For instance, the ERp57 inhibitor or antagonist may be a molecule that binds to ERp57 and neutralizes, blocks, inhibits, abrogates, reduces or interferes with the biological activity of ERp57 (such as inducing HBV entry at membrane fusion step).

In the context of the present invention, the direct ERp57 inhibitor (i) directly binds to ERp57 (protein or nucleic sequence (DNA or mRNA)) and (ii) inhibits HBV entry into hepatocyte at membrane fusion step.

More particularly, the direct ERp57 inhibitor according to the invention is:

1) an inhibitor of ERp57 activity (such as small organic molecule, antibody, aptamer, polypeptide) and/ or

2) an inhibitor of ERp57 gene expression (such as antisense oligonucleotide, nuclease, siRNA, …)

In some embodiments, the present invention relates to an inhibitor of ERp57 for use in the treatment of Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV) infection wherein the said inhibitor of ERp57 prevents or reduces the virus entry into hepatocytes at membrane fusion step and wherein said antagonist is an inhibitor of ERp57 activity and/or an inhibitor of ERp57 gene expression. In some embodiments, the present invention relates to an inhibitor of ERp57 for use in the treatment of Hepatitis B virus (HBV) and Hepatitis D virus (HDV) infection wherein the said inhibitor of ERp57 prevents or reduces the virus entry into hepatocytes at membrane fusion step and wherein said antagonist is an inhibitor of ERp57 activity and/or an inhibitor of ERp57 gene expression.

According to the present invention, by "biological activity of ERp57" is meant inducing / promoting HBV entry at membrane fusion step.

Tests for determining the capacity of a compound to be inhibitor of ERp57 are well known to the person skilled in the art. Inhibition of the biological activity of ERp57 may be determined by any assays well known in the art in order to assay the virus entry at membrane fusion step. For example the assay may consist in determining the ability of the agent to alter HBV entry at membrane fusion step. In the present study, the inventors designed a cell-cell fusion assay to directly measuring HBV membrane fusion using HBV GP-transfected cells (donor cells) expressing a luciferase marker gene and transfected with an expression plasmid encoding the wild-type HBV glycoproteins S, M and L (see Example section). Such cell-cell fusion assay may be used as a test for determining the capacity of a compound to be on inhibitor of ERp57 by inhibiting HBV entry into hepatocyte at membrane fusion step.

Then a competitive assay may be settled to determine the ability of the agent to inhibit biological activity of ERp57. The functional assays may be envisaged such evaluating the ability to induce or inhibit the HBV entry at membrane fusion step (see example with NTZ compound and Figures 5-6).

The skilled in the art can easily determine whether an ERp57 inhibitor neutralizes, blocks, inhibits, abrogates, reduces or interferes with a biological activity of the ERp57. To check whether the ERp57 inhibitor alters the HBV entry into hepatocyte at membrane fusion step and inhibits the HBV infection in the same way than the initially characterized NTZ compound may be performed with each inhibitor. For instance, HBV infection in hepatic cells can be measured by analysis of viral parameters such as cccDNA quantification and/or pgRNA quantification. For cccDNA quantification total DNA is digested by T5 exonuclease (New England Biolabs) then submitted to qPCR using Taqman Fast Advanced Master Mix (Life Technologies. For pgRNA quantification, RT-qPCR may be performed using Taqman Fast

Advanced Master Mix (Life Technologies). PgRNA levels is normalized to GUSB using a commercial probe primer mix (Life Technologies #Hs99999908_m1).

HBV infection can also be measured by analysis of viral parameters such as quantification of secreted HBe and HBs antigens by Elisa (chemiluminescence immunoassay kit Autobio,): level of secreted HBe and HBs antigens are standard secreted markers of HBV infection of hepatic cells) and/or assessment of Intracellular total HBV DNA or RNA extracted from infected cells by qPCR or RT-qPCR with specific HBV primers.

A) Inhibitor of ERp57 activity

- small organic molecule

In one embodiment, the ERp57 I inhibitors is a small organic molecule. As used herein, the term "small organic molecule" refers to a molecule of size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e.g., proteins, nucleic acids, etc.); preferred small organic molecules range in size up to 2000 Da, and most preferably up to about 1000 Da.

In a particular embodiment, the ERp57 inhibitor or antagonist according to the invention is a small organic molecule such as:

- Punicalagin, a polyphenolic compound isolated from pomegranate fruit recently described as a new inhibitor of PDIA3 reductase activity. Punicalagin binds with high affinity to ERp57 and inhibits its redox activity and also strongly affects PDIA3 reductase activity in vitro as a non-competitive inhibitor (Giamogante F et al Biochimie . 2018 Apr;147:122-129);

- The small molecule known as 16F16 compound known to inhibit of PDIA3 function (Hoffstrom BG, et al Nat Chem Biol. 2010 Dec; 6(12):900-6). The compound 16F16 contains a chloroacetyl group that covalently modifies free cysteine thiols

- The small molecule LOC14 compound a reversible inhibitor of PDI's reductase activity. This compound, although targeting similar residues of PDI as the irreversible inhibitor 16F16, forces the protein to adopt a different conformation that resembles the native oxidized form. LOC14 has improved solubility, potency, and *in vitro* metabolism properties compared with other reported PDI inhibitors. Kaplan A et al. "Small molecule-induced oxidation of protein disulfide isomerase is neuroprotective" PNAS 2015 Apr 28; 112(17): E2245–E2252.

- Antibody

In another embodiment, the ERp57 inhibitor or antagonist may consist in an antibody directed against the ERp57, in such a way that said antibody binding to ERp57 and able of

neutralizing, blocking, inhibiting, abrogating, reducing or interfering with the biological activities of ERp57 I ("neutralizing antibody").

Then, for this invention, neutralizing antibody of ERp57 are selected as above described for their capacity to (i) bind to ERp57 (protein) and/or (ii) and inhibits HBV entry into hepatocyte at membrane fusion step.

In one embodiment of the antibodies or portions thereof described herein, the antibody is a monoclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a polyclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a humanized antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a chimeric antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a light chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a heavy chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fab portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a F(ab')2 portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fc portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fv portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a variable domain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises one or more CDR domains of the antibody.

As used herein, "antibody" includes both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and monovalent and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. The antibody may be a human or nonhuman antibody. A nonhuman antibody may be humanized by recombinant methods to reduce its immunogenicity in man.

Antibodies are prepared according to conventional methodology. Monoclonal antibodies may be generated using the method of Kohler and Milstein (Nature, 256:495, 1975). To prepare monoclonal antibodies useful in the invention, a mouse or other appropriate host animal is immunized at suitable intervals (e.g., twice-weekly, weekly, twice-monthly or monthly) with antigenic forms of ERp57. The animal may be administered a final "boost" of

antigen within one week of sacrifice. It is often desirable to use an immunologic adjuvant during immunization. Suitable immunologic adjuvants include Freund's complete adjuvant, Freund's incomplete adjuvant, alum, Ribi adjuvant, Hunter's Titermax, saponin adjuvants such as QS21 or Quil A, or CpG-containing immunostimulatory oligonucleotides. Other suitable adjuvants are well-known in the field. The animals may be immunized by subcutaneous, intraperitoneal, intramuscular, intravenous, intranasal or other routes. A given animal may be immunized with multiple forms of the antigen by multiple routes.

Briefly, the recombinant ERp57 I may be provided by expression with recombinant cell lines or bacteria. Recombinant form of ERp57 I may be provided using any previously described method. Following the immunization regimen, lymphocytes are isolated from the spleen, lymph node or other organ of the animal and fused with a suitable myeloma cell line using an agent such as polyethylene glycol to form a hydridoma. Following fusion, cells are placed in media permissive for growth of hybridomas but not the fusion partners using standard methods, as described (Coding, Monoclonal Antibodies: Principles and Practice: Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology, 3rd edition, Academic Press, New York, 1996). Following culture of the hybridomas, cell supernatants are analyzed for the presence of antibodies of the desired specificity, i.e., that selectively bind the antigen. Suitable analytical techniques include ELISA, flow cytometry, immunoprecipitation, and western blotting. Other screening techniques are well-known in the field. Preferred techniques are those that confirm binding of antibodies to conformationally intact, natively folded antigen, such as non-denaturing ELISA, flow cytometry, and immunoprecipitation.

Significantly, as it is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) The Experimental Foundations of Modern Immunology Wiley & Sons, Inc., New York; Roitt, I. (1991) Essential Immunology, 7th Ed., Blackwell Scientific Publications, Oxford). The Fc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')2 fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments

are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDRS). The CDRs, and in particular the CDRS regions, and more particularly the heavy chain CDRS, are largely responsible for antibody specificity.

It is now well-established in the art that the non CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody.

This invention provides in certain embodiments compositions and methods that include humanized forms of antibodies. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567,5,225,539,5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby incorporated by reference. The above U.S. Pat. Nos. 5,585,089 and 5,693,761, and WO 90/07861 also propose four possible criteria which may be used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid reside at the framework positions at which the amino acid is predicted to have a side chain atom within 3A of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs.

The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies. One of ordinary skill in the art will be familiar with other methods for antibody humanization.

In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgG1, IgG2, IgG3, IgG4, IgA and IgM molecules. A "humanized" antibody retains a similar antigenic specificity as the original antibody. However, using certain methods of humanization, the affinity and/or specificity of binding of the antibody may be increased using methods of "directed evolution", as described by Wu et al., /. Mol. Biol. 294:151, 1999, the contents of which are incorporated herein by reference.

Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of which are incorporated herein by reference. These animals have been genetically modified such that there is a functional deletion in the production of endogenous (e.g., murine) antibodies. The animals are further modified to contain all or a portion of the human germ-line immunoglobulin gene locus such that immunization of these animals will result in the production of fully human antibodies to the antigen of interest. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (KAMA) responses when administered to humans.

In vitro methods also exist for producing human antibodies. These include phage display technology (U.S. Pat. Nos. 5,565,332 and 5,573,905) and in vitro stimulation of human B cells (U.S. Pat. Nos. 5,229,275 and 5,567,610). The contents of these patents are incorporated herein by reference.

As the ERp57 in the context of the present invention is located into hepatocyte cells (intracellular target), the antibody of the invention acting as an activity inhibitor could be an antibody fragment without Fc fragment.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab') 2 Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')2 fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

The various antibody molecules and fragments may derive from any of the commonly known immunoglobulin classes, including but not limited to IgA, secretory IgA, IgE, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgGl, IgG2, IgG3 and IgG4.

In another embodiment, the antibody according to the invention is a single domain antibody. The term “single domain antibody” (sdAb) or "VHH" refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains. Such VHH are also called “nanobody®”. According to the invention, sdAb can particularly be llama sdAb.

The skilled artisan can use routine technologies to use the antigen-binding sequences of these antibodies (e.g., the CDRs) and generate humanized antibodies for treatment of pathological conditions associated with intense stress (such as Post-Traumatic Stress Disorder (PTSD) as disclosed herein.

The skilled artisan can use routine technologies to use the antigen-binding sequences of these antibodies (e.g., the CDRs) and generate humanized antibodies for treatment of pathological conditions associated with intense stress (such as Post-Traumatic Stress Disorder (PTSD) as disclosed herein.

Several monoclonal antibodies to ERp57 I have been characterized and shown to inhibit ERp57 I activity are disclosed in Holbrook LM et al. (2011) “The platelet-surface thiol isomerase enzyme ERp57 modulates platelet function” Journal of Thrombosis and Haemostasis 10(2):278-88; Wu Y; et al “The disulfide isomerase ERp57 mediates platelet aggregation, hemostasis, and thrombosis “Blood . 2012 Feb 16;119(7):1737-46.

Examples of commercial neutralizing monoclonal antibodies that can be used according to the invention are also available.

- Aptamer

In another embodiment, the ERp57 inhibitor antagonist is an aptamer directed against ERp57. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

Then, for this invention, neutralizing aptamers of ERp57are selected as above described for their capacity to (i) bind to ERp57and/or (ii) and inhibits HBV entry at membrane fusion step.

Example of methods of screening for neutralizing RNA aptamers such as ERp57 that can be used according to the invention are disclosed in WO2014100434.

B) Inhibitor of ERp57 gene expression

In still another embodiment, the ERp57 inhibitor or antagonist is an inhibitor of ERp57 gene expression. An "inhibitor of expression" refers to a natural or synthetic compound that has a biological effect to inhibit the expression of a gene. Therefore, an "inhibitor of ERp57gene expression" denotes a natural or synthetic compound that has a biological effect to inhibit the expression of ERp57gene.

In a preferred embodiment of the invention, said inhibitor of ERp57gene expression is antisense oligonucleotide, nuclease, siRNA, shRNA or ribozyme nucleic acid sequence.

Inhibitors of ERp57gene expression for use in the present invention may be based on antisense oligonucleotide constructs. Antisense oligonucleotides, including antisense RNA molecules and antisense DNA molecules, would act to directly block the translation of ERp57mRNA by binding thereto and thus preventing protein translation or increasing mRNA degradation, thus decreasing the level of ERp57, and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding ERp57can be synthesized, e.g., by conventional

phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of ERp57gene expression for use in the present invention. ERp57gene expression can be reduced by using small double stranded RNA (dsRNA), or a vector or construct causing the production of a small double stranded RNA, such that ERp57 gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschi, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836).

Examples of siRNAs against ERp57 that can be used according to the invention are disclosed in Kim Y. et al. "Protein Disulfide Isomerases as potential therapeutic targets for Influenza A and B Viruses" Virus Res. 2018 Mar 2; 247: 26–33 (see Table 1) and Li S,: "ERp57‐small interfering RNA silencing can enhance the sensitivity of drug‐resistant human ovarian cancer cells to paclitaxel". Int J Oncol 54: 249-260, 2019.

Examples of shRNAs against ERp57 used in the present study are the following:

CCGGGCTGCACTGTTTATGGAAATACTCGAGTATTTCCATAAACAGTGCAG CTTTTTG (SEQ ID N°3)

CCGGCGATTTGCACATACGAATGTTCTCGAGAACATTCGTATGTGCAAATC GTTTTTTG (SEQ ID N°4)

Inhibitors of ERp57gene expression for use in the present invention may be based nuclease therapy (like Talen or Crispr).

The term "nuclease" or "endonuclease" means synthetic nucleases consisting of a DNA binding site, a linker, and a cleavage module derived from a restriction endonuclease which are used for gene targeting efforts. The synthetic nucleases according to the invention exhibit increased preference and specificity to bipartite or tripartite DNA target sites comprising DNA binding (i.e. TALEN or CRISPR recognition site(s)) and restriction endonuclease target site while cleaving at off-target sites comprising only the restriction endonuclease target site is prevented.

The guide RNA (gRNA) sequences direct the nuclease (i.e. Cas9 protein) to induce a site-specific double strand break (DSB) in the genomic DNA in the target sequence.

Restriction endonucleases (also called restriction enzymes) as referred to herein in accordance with the present invention are capable of recognizing and cleaving a DNA molecule at a specific DNA cleavage site between predefined nucleotides. In contrast, some endonucleases such as for example FokI comprise a cleavage domain that cleaves the DNA unspecifically at a certain position regardless of the nucleotides present at this position. Therefore, preferably the specific DNA cleavage site and the DNA recognition site of the restriction endonuclease are identical. Moreover, also preferably the cleavage domain of the chimeric nuclease is derived from a restriction endonuclease with reduced DNA binding and/or reduced catalytic activity when compared to the wildtype restriction endonuclease.

According to the knowledge that restriction endonucleases, particularly type II restriction endonucleases, bind as a homodimer to DNA regularly, the chimeric nucleases as referred to herein may be related to homodimerization of two restriction endonuclease subunits. Preferably, in accordance with the present invention the cleavage modules referred to herein have a reduced capability of forming homodimers in the absence of the DNA recognition site, thereby preventing unspecific DNA binding. Therefore, a functional homodimer is only formed upon recruitment of chimeric nucleases monomers to the specific DNA recognition sites. Preferably, the restriction endonuclease from which the cleavage module of the chimeric nuclease is derived is a type IIP restriction endonuclease. The preferably palindromic DNA recognition sites of these restriction endonucleases consist of at least four or up to eight contiguous nucleotides. Preferably, the type IIP restriction endonucleases cleave the DNA within the recognition site which occurs rather frequently in the genome, or immediately adjacent thereto, and have no or a reduced star activity. The type IIP restriction endonucleases as referred to herein are preferably selected from the group consisting of: PvuII, EcoRV, BamHI, BcnI, BfaSORF1835P, BfiI, BglI, BglII, BpuJI, Bse6341, BsoBI, BspD6I, BstYI, Cfr101, Ecl18kI, EcoO109I, EcoRI, EcoRII, EcoRV, EcoR124I, EcoR124II, HinP11, HincII, HindIII, Hpy99I, Hpy188I, MspI, MunI, MvaI, NaeI, NgoMIV, NotI, OkrAI, PabI, PacI, PspGI, Sau3AI, SdaI, SfiI, SgrAI, ThaI, VvuYORF266P, DdeI, Eco57I, HaeIII, HhaII, HindII, and NdeI.

Example of commercial gRNAs against ERp57 include, but are not limited to: Human ERp57 (PDIA3) Human Gene Knockout Kit (CRISPR) (KN205940BN) from CliniSciences, ERp57CRISPR Plasmids (human) gene knockout, with ERp57-specific 20 nt guide RNA sequences from Santa Cruz Biotechnology (ref: sc-401497).

Examples of gRNAs gRNAs against ERp57 are also described in Liu CC et al “Integrins and ERp57 Coordinate to Regulate Cell Surface Calreticulin in Immunogenic Cell Death” Front Oncol. 2019; 9: 411.

Other nuclease for use in the present invention are disclosed in WO 2010/079430, WO2011072246, WO2013045480, Mussolino C, et al (Curr Opin Biotechnol. 2012 Oct;23(5):644-50) and Papaioannou I. et al (Expert Opinion on Biological Therapy, March 2012, Vol. 12, No. 3: 329-342) all of which are herein incorporated by reference.

Ribozymes can also function as inhibitors of ERp57gene expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of ERp57mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using, e.g., ribonuclease protection assays.

Antisense oligonucleotides, siRNAs and ribozymes useful as inhibitors of ERp57gene expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramadite chemical synthesis. Alternatively, antisense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides, siRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide, siRNA or ribozyme nucleic acid to the cells and preferably cells expressing ERp57. Preferably, the vector transports the nucleic acid within cells with reduced degradation relative to the extent of degradation that

would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide, siRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vectors and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rouse sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in KRIEGLER (A Laboratory Manual," W.H. Freeman C.O., New York, 1990) and in MURRY ("Methods in Molecular Biology," vol.7, Humana Press, Inc., Cliffton, N.J., 1991).

Preferred viruses for certain applications are the adenoviruses and adeno-associated viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hematopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than

100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g., SANBROOK et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUCl9, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In a preferred embodiment, the antisense oligonucleotide, nuclease (i.e. CrispR), siRNA, shRNA or ribozyme nucleic acid sequences are under the control of a heterologous regulatory region, e.g., a heterologous promoter. The promoter may be specific for the hepatocyte cells.

- Pharmaceutical compositions

The inhibitor of ERp57 may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

Thus, in some embodiments, the present invention also relates to a pharmaceutical composition for use in a method for treating Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV) infection in a subject in need thereof, comprising an inhibitor of ERp57. In some embodiments, the present invention relates to a pharmaceutical composition for use in a method

for treating Hepatitis B virus (HBV) and Hepatitis D virus (HDV) infection in a subject in need thereof, comprising an inhibitor of ERp57.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local, inhaled or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

Preferably, the pharmaceutical compositions contain vehicles, which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The inhibitor of ERp57 of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric

hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media, which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the

subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The inhibitor of ERp57 or expression of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; liposomal formulations; timerelease capsules; and any other form currently used.

**Method of screening**

The present invention also relates to a method for screening a plurality of candidate compounds useful for treating Hepatitis B virus (HBV) infection comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit ERp57 and (b) and positively selecting the candidate compounds capable of inhibiting biological activity of said ERp57. In some embodiments, the present invention relates to a method for screening a plurality of candidate compounds useful for treating Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV) infection comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit ERp57 and (b) and positively selecting the candidate compounds capable of inhibiting biological activity of said ERp57. In some embodiments, the present invention relates to a method for screening a plurality of candidate compounds useful for treating Hepatitis B virus (HBV) and Hepatitis D virus (HDV) infection comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit ERp57 and (b) and positively selecting the candidate compounds capable of inhibiting biological activity of said ERp57

Typically, the candidate compound is selected from the group consisting of small organic molecules, peptides, polypeptides or oligonucleotides.

Testing whether a candidate compound can inhibit ERp57 can be determined using or routinely modifying reporter assays known in the art.

For example, the method may involve contacting cells expressing ERp57 with the candidate compound, and assessing the ability of ERp57 to induce HBV entry into hepatocyte at membrane fusion step (e.g., using the cell-cell fusion assay describe above), and comparing the level of fusion to a standard level of fusion. Typically, the standard level of fusion is

measured in absence of the candidate compound. A decrease level of fusion over the standard indicates that the candidate compound is an inhibitor of ERp57.

The candidate compounds that have been positively selected may be subjected to further selection steps in view of further assaying its properties on hepatocytes cells isolated from subjects suffering from HBV infections (or dHeparRG cells infected by HBV see Grippon et al 2002). For example, the candidate compounds that have been positively selected with the screening method as above described may be further selected for their ability to inhibit HBV entry at membrane fusion step in HBV target cells (hepatocyte). Typically, the screening method may further comprise the steps of i) bringing into contact hepatocytes from patients with HBV infection with a positively selected candidate compound ii) determining the amount of cccDNA and/or pgRNA in said HBV infected cell and iii) comparing the amount of cccDNA and/or pgRNA determined at step ii) with the amount of cccDNA and/or pgRNA determined when step i) is performed in the absence of the positively selected candidate compound. Step i) as above described may be performed by adding an amount of the candidate compound to be tested to the culture medium of the hepatocytes. Usually, a plurality of culture samples are prepared, so as to add increasing amounts of the candidate compound to be tested in distinct culture samples. Generally, at least one culture sample without candidate compound is also prepared as a negative control for further comparison.

Finally, the candidate compounds that have been positively selected may be subjected to further selection steps in view of further assaying its properties on animal models for HBV infections. Typically, the positively selected candidate compound may be administered to the animal model and the progression of HBV infections is determined and compared with the progression of HBV infections is in an animal model that was not administered with the candidate compound.

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

**FIGURES:**

**Figure 1. HBV GP fusion trigger is independent of acid pH and NTCP.** (**A**) Huh7 "donor" cells transfected with the pT7HB2.7 plasmid allowing expression of HBV GPs (HBV) and a luciferase marker gene driven by the HIV-1 promoter were co-cultured with either Huh7-tat (H-tat) or Huh7-NTCP-tat (N-tat) "indicator" cells that express the HIV Tat protein. After

24h of co-culture, the cells were treated at pH4 (or pH 5 for VSV-G) *vs.* pH7 for 3 minutes. The luciferase activity induced by fusion between donor and indicator cells was then measured 24h later. A control plasmid that does not allow GP expression (Empty) was used to determine the background of luciferase expression. The CCHFV Gn/Gc (CCHFV) or VSV-G GPs (VSV) were used as positive controls for fusion at low pH. Fusion mediated by HBV GPs with Huh7-tat was taken as 100%. The bars represent the means (N=3). Error bars correspond to standard deviation. (**B**) Huh7 "donor" cells transfected plasmids allowing expression of L, M or S HBV GPs alone, both L and S GPs (noM) or all HBV GPs (Wt) and a luciferase marker gene driven by the HIV-1 promoter were co-cultured with Huh7-tat or Huh7-NTCP-tat "indicator" cells that express HIV Tat protein. Cell co-cultures were then processed as above described to determine cell-cell fusion activity. Fusion mediated by HBV GP at pH7 with Huh7-tat cells was taken as 100%. The bars represent the means (N=3). Error bars correspond to standard deviation.

**Figure 2. DTNB, a thiol-specific oxidizing reagent, inhibits HBV membrane fusion.** (**A**) DTNB (2 mM) was added to the cell supernatant containing HDV particles at the onset of infection (0h) or at the indicated times post-infection and was removed 8hr later. VSV-Δp, *i.e.*, HDV particles generated with VSV-G GP rather than HBV GPs (Perez-Vargas et al., 2019), were used as control for a virus entry process that is not affected by DNTB. As negative control, pSVLD3 was co-transfected with an empty plasmid (referred to as "NoGP"). At 7 days post-infection, HDV RNAs were extracted from infected cells and quantified by RTqPCR. The results are expressed after normalization with GAPDH RNAs as means ± SD (N=3) per mL of cell lysates containing $10^6$ cells. The results of infection in the absence of DTNB are shown [DTNB(-)]. (**B**) Huh7 "donor" cells co-expressing HBV GPs and a luciferase marker gene driven by the HIV-1 promoter were co-cultured with Huh7-NTCP-tat "indicator" cells that express HIV Tat protein. Different concentrations of DTNB were added at 0h *vs.* at 16h after initiating the cell co-culture, as indicated. No cytotoxicity could be detected in these conditions (data not shpown). The luciferase activity induced by fusion between donor and indicator cells was then measured 24h later. Fusion mediated by HBV GPs without DTNB was taken as 100%. The graphs represent the average of four independent experiments.

**Figure 3. Disulfides conformation models.** (**A**) Cysteine-rich regions on the "a" determinant (residues 261-324) of the HBV S GP. Four sub-regions that are rich in cysteine are coloured: I (blue), II (green), III (yellow) and IV (red). Jpred secondary structure prediction different from random-coil is indicated: β-strand (arrow) and α-helix (zigzag line). (**B**) Probability of contacts predicted by RaptorX between the four cysteine-rich regions. The

probabilities higher than 0.7 are highlighted in red (data not shown). (**C**) Predominant disulfide conformations obtained by molecular dynamics simulation of the modelled 294-317 region of the HBV surface protein. Note that the ß-strand on the wt sequence (left) adopts a loop conformation with an allosteric disulfide conformer between the C301-C310 bond, which is specifically classified as a -/+RHHook conformation. The T303C/G308C double mutant (right) may generate an additional disulfide bond, resulting in two structural disulfides of +/-RHStaple and -/+LHSpiral conformations that form the C301-310 and C303-C308 bonds, respectively.

**Figure 4. PDI inhibitors in HBV entry.** (**A**) HDV particles harboring wt or TG/CC mutant (T303C/G308C) HBV GPs were incubated with Huh7 or Huh7-NTCP cells that were pre-treated for 2h with the indicated inhibitors that block different PDI proteins or with DMSO, used as vehicle. Binding of either virus particles to the cells was quantified by RTqPCR and expressed after normalization with GAPDH RNAs as mean ± SD (N=3) per mL of cell lysates containing $10^6$ cells. (**B**) HDV particles or (**C**) HBV virus were used to infect Huh7-NTCP cells that were pre-incubated for 2h with the indicated inhibitors that block different PDI proteins or with DMSO, used as vehicle. Infected cells were grown for 7 days before total intracellular RNA or DNA was purified. The results of HDV RNA and HBV DNA quantification by RTqPCR and qPCR, respectively, are expressed after normalization with GAPDH RNAs as means ± SD (N=3) per mL of cell lysates containing $10^6$ cells. (**D**) Huh7 "donor" cells co-expressing HBV GPs and a luciferase marker gene driven by the HIV-1 promoter were co-cultured with Huh7-NTCP-tat "indicator" cells that express HIV Tat protein. The indicated PDI inhibitors were added when "donor" and "indicator" cells were mixed for co-cultures and the luciferase activity induced by cell-cell fusion was measured 24h later. DMSO was used as vehicle. Fusion mediated by HBV GPs without inhibitor was taken as 100%. The graphs represent the average of four independent experiments. The PDI inhibitors were used at the following concentrations: NTZ, 30 µg/mL; EGCG, 5 µM; Rutin, 5 µM; Bacitracin, 5 mM; PX-12, 30 µg/mL. No cytotoxicity could be detected in these conditions (data not shown)

**Figure 5. ERp57 down-regulation inhibits in HBV entry.** (**A**) HDV or (**B**) HBV particles were used to infect Huh7-NTCP cells in which the indicated PDI were down-regulated by lentiviral vectors carrying shRNA. Naïve Huh7-NTCP cells were used as controls. Infected cells were grown for 7 days before total intracellular RNA or DNA was purified. The results of HDV RNA and HBV DNA quantification by RTqPCR and qPCR, respectively, are expressed after normalization with GAPDH RNAs as means ± SD (N=3) per mL of cell lysates containing $10^6$ cells. (**C**) Huh7 "donor" cells co-expressing HBV GPs and a luciferase marker gene driven by the HIV-1 promoter were co-cultured with Huh7-NTCP-tat "indicator" cells that express

HIV Tat protein in which the indicated PDI were down-regulated by lentiviral vectors carrying shRNA. After 24h, the cells were treated at pH4 or pH7 for 3 minutes. The luciferase activity induced by the fusion between donor and indicator cells was measured 24h later. Fusion mediated by HBV GPs at pH7 with naïve Huh7-NTCP-tat cells (Ctrl) was taken as 100%. A control plasmid that does not allow GP expression (Empty) was used to determine the background of luciferase expression. The bars represent the means (N=3). Error bars correspond to standard deviations.

**Figure 6. *In vivo* assessment of ERp57 inhibition.** (**A**) 4-8 weeks old NOD-FRG mice were engrafted with primary human hepatocytes (PHH). After *ca.* 2-3 months, the animals displaying HSA levels >15 mg/mL were split in 4 different groups (N=3 to N=5 animals, see Table in the inset) that were infected with HBV ($10^8$ GE/mouse), using the displayed NTZ treatment schedule. (**B**) At different time points post-infection, blood samples (50 µl) were collected and the viremia in sera was monitored by qPCR (GE/mL of serum). The graphs show the results of viremia (means ± SD) of HBV.

**Figure 7. Down-regulation of PDI family members.** Naïve Huh7-NTCP cells (Ctrl+) or shRNA-expressing Huh7-NTCP cells were subjected to flow cytometry (left) and western blot (right) analyses, in order to evaluate the expression levels of the indicated PDIs (**A**) ERp57, (**B**) ERp72 and (**C**) ERp46 before or after down-regulation. Huh7-NTCP cells stained with secondary antibody only (Neg) were used to provide the background of flow cytometry analyses

**EXAMPLE 1:**

**Material & methods:**

**Plasmids.** Plasmid pSVLD3 harboring a trimer of the HDV gt1 genome (accession number M21012.1), pCiS encoding the S protein, pCiL encoding the L protein (Komla-Soukha and Sureau, 2006) and pT7HB2.7 encoding the three HBV envelope proteins were a gift from Camille Sureau (Sureau, 2010; Sureau et al., 1994). To induce the expression of S and L only, the pT7HB2.7 plasmid was modified at the M start codon and the Kozak consensus sequence in order to silence the expression of M protein, resulting in pT7HB2.7Mless construct. The pCiM plasmid encoding the M protein was constructed by deleting the preS1 region from pCiL until the N-terminal methionine of preS2. All mutations in pT7HB2.7 plasmid were introduced by point directed mutagenesis. The phCMV-VSV-G encoding the G protein from vesicular stomatitis virus (VSV) and pCAGGS-GP encoding the Gn and Gc glycoproteins from Crimean-Congo hemorrhagic fever virus (CCHFV) were described previously (Freitas et al., 2020). The plasmid encoding the luciferase reporter under control of an HIV-1 long terminal repeat internal

promoter (pLTR-luc) was described before (Lavillette et al., 2007). shRNAs sequences against ERp57 are described in Table 1.

**Cells.** Huh7 hepatocarcinoma cells and Huh7-NTCP cells which were generated by transduction of Huh7 cells with a retroviral vector transducing the NTCP plasmid (pLX304NTCP, Dharmacon) and selected for blasticidin resistance, cells were grown in William's E medium (WME) (Gibco, France) supplemented with non-essential amino acids, 2 mM L-Glutamine, 10 mM HEPES buffer, 100 U/mL of penicillin, 100 µg/mL of streptomycin and 10% fetal bovine serum. 293T kidney cells (ATCC CRL-1573) CHO-K1 (CHO) Chinese hamster ovary cells (ATCC CCL-61) and CHO-pgsB-618 cells (ATCC CRL-2241), which do not produce glycosaminoglycans, were grown in Dulbecco's modified minimal essential medium (DMEM, Gibco) supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 10% fetal calf serum. Huh7-Tat and Huh7-NTCP-Tat indicator cells expressing HIV Tat were generated by transduction of Huh7 and Huh7-NTCP cells, respectively, with the LXSN-tat retroviral vector and selected for G418 resistance. HepG2.2.15 cells were used to produce HBV virus, there were maintained in WME complemented with 10% fetal bovine serum.

**PDI inhibitors**. 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB), nitazoxanide (NTZ), (−)-Epigallocatechin 3-gallate (EGCG), rutin and PX-12 were purchased from Sigma-Aldrich and dissolved in DMSO, ethanol or water according to the manufacturer's instructions.

**Antibodies.** For western-blot analysis, HBs antigen and calnexin were detected with mouse anti-HBV antibody (murex, DiaSorin) coupled to horseradish peroxidase (HRP) and rabbit calnexin polyclonal antibody (Enzo. The mouse anti-ERp57 (Abcam), mouse anti-ERp72 (Santa Cruz Biotechnology), and the rabbit anti-ERp46 (Abcam) antibodies were used for detecting protein disulfide isomerase proteins by flow cytometry and western blot. NTCP was detected with polyclonal NTCP/SLC10A1 antibody (Bioss Antibodies) coupled to PE for flow cytometry.

**shRNA-expressing stable cell lines.** 293T cells were seeded 24h prior to transfection with VSV-G plasmid, pTG-5349 packaging plasmid, and pLKO.1 expression vector carrying shRNA against ERp72, ERp57 or ERp46 using calcium phosphate precipitation. Medium was replaced 16h post-transfection. Vector supernatants were harvested 24h later, filtered through a 0.45 µm filter. Stable knockdown of ERp72, ERp57 or ERp46 in Huh7-NTCP, Huh7-tat, and Huh7-NTCP-tat cells was performed by selection with puromycin after lentiviral transduction. The knockdown was validated by flow cytometry and western blot using antibodies against ERp72, ERp57 or ERp46.

**Cell-cell fusion assays.** Huh7 "donor" cells ($2.5x10^5$ cells/well seeded in six-well tissue culture dishes 24 h prior to transfection) were co-transfected using FuGENE 6 transfection reagent (Promega) with 3 µg of pT7HB2.7- wt or mutated glycoproteins and 50 ng of pLTR-luc reporter plasmid. For a positive control, cells were co-transfected with 3 µg of pCAGGS-GP or 1 µg of phCMV-VSV-G and with 50 ng of the pLTR-luc plasmid. For negative controls, cells were co-transfected with 2 µg of an empty phCMV plasmid and 50 ng of the pLTR-luc plasmid. Twelve hours later, transfected cells were detached with Versene (0.53 mM EDTA; Gibco), counted, and reseeded at the same concentration ($10^5$ cells/well) in twelve-well plates. Huh7-Tat or Huh7-NTCP-Tat indicator cells, detached with EDTA and washed, were then added to the transfected cells ($3x10^5$ cells per well). After 24h of cocultivation, the cells were washed with PBS, incubated for 3 min in fusion buffer (130 mM NaCl, 15 mM sodium citrate, 10 mM MES [2-(N-morpholino)ethanesulfonic acid], 5 mM HEPES) at pH4, pH5 or pH7, and then washed three times with normal medium. The luciferase activity was measured 24h later using a luciferase assay kit according to the manufacturer's instructions (Promega).

**HDV particles production and infection**. Huh7 cells were seeded in 10 cm plates at a density of $10^6$ cells per plate and were transfected with a mixture of 2.5 µg of pSVLD3 plasmid and 10 µg of plasmid allowing the expression of surface envelope glycoproteins of VSV or HBV using FuGENE 6 transfection reagent (Promega), as described previously (Perez-Vargas et al., 2019). Transfected cells were grown for up to 9 days in primary hepatocyte maintenance medium containing 2% DMSO to slow cell growth.

The supernatants of virus producer cells were filtrated through 0.45 nm-pore filters and were analyzed by RTqPCR for detection of HDV RNA, using the primers described below. These supernatants were also used for infection experiments in Huh7-NTCP cells or PDI-down-regulated Huh7-NTCP cells, which were seeded in 48-well plates at a density of $1.5x10^4$ cells per well. Infected cells were cultured in primary hepatocyte maintenance medium containing 2% DMSO following infection. RTqPCR assays were used to assess infectivity of viral particles at 7 days post-infection.

For inhibition assays, drugs were incubated with cells for 2h at 37°C before virus addition or at different times post-infection and the infectivity was assessed 7 days post-infection by RT-qPCR.

**Binding assays.** HDV wt particles ($10^7$ GE) were added to Huh7-NTCP cells and incubated for 1h at 4°C. Unbound virions were removed by three washes with cold PBS, and RTqPCR was used to assess the amount of bound viral particles.

**RTqPCR detection of HDV RNAs in virus-producer and in infected cells**. Cells were washed with phosphate-buffer saline (PBS) and total RNA was extracted with TRI Reagent according to the manufacturer's instructions (Molecular Research Center). RNAs were reverse transcribed using random oligonucleotides primers with iScript (Bio-Rad). Specific primers were used: for HDV RNA quantification (not shown). qPCR was performed using FastStart Universal SYBR Green Master (Roche) on a StepOne Real-Time PCR System (Applied Biosystems). As an internal control of extraction, in vitro-transcribed exogenous RNAs from the linearized Triplescript plasmid pTRI-Xef (Invitrogen) were added to the samples prior to RNA extraction and quantified with specific primers (data not shown). All values of intracellular HDV RNAs were normalized to GAPDH gene transcription. For GAPDH mRNAs quantification, we used also specific primer (not shown).

**Western-blot analyses.** The proteins from pelleted cell supernatants or extracted from total cell lysates were denatured in Lammeli buffer at 95°C for 5 min and were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5% nonfat dried milk in PBS and incubated at 4°C with a rabbit or mouse antibody diluted in PBS-0.01% milk, followed by incubation with a IRdye secondary antibody (Li-Cor Biosciences). Membranes visualization was performed using an Odyssey infrared imaging system CLx (LI-COR Biosciences).

For cell surface biotinylation**.** Huh7 cells were transfected into 10 cm plates with plasmid encoding wt or mutant HBV GPs. After 48h, the cell monolayers were rinsed three times with ice-cold PBS and overlaid with 0.5 ml biotin solution (0.5 mg sulpho-N-hydroxysuccinimide–biotin (Pierce) per ml of PBS, pH 7.2). The cells were then labeled for 30 min at 4°C. The biotin solution was removed and the cells were rinsed once with ice-cold 100 mM glycine solution and then incubated for 15 min with 100 mM glycine at 4 °C to stop the reaction. The last washing step was performed with ice-cold PBS. Proteins were solubilized by the addition of 1 ml RIPA buffer and equivalent quantities of protein lysates from each sample (Nanodrop quantification, Thermofisher) were immunoprecipitated with Biotin-agarose beads. Proteins were electrophoresed under reducing conditions in SDS–PAGE followed by electrophoretic transfer to nitrocellulose. Surface-biotinylated proteins were detected with anti-HBV antibody (murex) coupled to horseradish peroxidase (HRP) and enhanced chemiluminescence (ECL; Roche). The membranes of biotinylated samples were routinely re-probed with anti-calnexin antibody to confirm the absence of the intracellular protein calnexin.

In addition, 10% of each lysate was denatured and loaded onto separate gels. Immunoblotting for calnexin on the membranes of lysate was done to confirm uniform protein loading.

Densitometry analysis (Image Lab BioRad software) was used to estimate the relative total amount of L, M and S mutant proteins, which were expressed relative of the wild type L, M and S total proteins.

**Flow cytometry**. The surface expression of NTCP, ERp46, ERp57 and ERp72 was quantified by FACS analysis from $10^6$ live cells using antibodies added to cells for 1h at 4°C. After washing, the binding of antibody to the cell surface was detected using PE (Phycoerythrin)-conjugated anti-mouse antibodies.

**Fusion peptide prediction.** The HBV surface sequence used was taken from the UniProt database, with accession number P03138. Hydropathy plots were obtained with Membrane Protein eXplorer software (Snider et al., 2009) using as input the reference sequence. Hydropathy plots were also used to evaluate the effect of residue mutations. Sequences with a propensity to partition into the lipid bilayer were identified using interfacial settings and pH=5.0.

**Contact prediction on the Cys rich region.** Contact prediction was performed using RaptorX (Wang et al., 2017)(Teppa et al., 2020). RaptorX integrates evolutionary coupling and sequence conservation information through an ultra-deep neural network formed by two deep residual neural networks. RaptorX predicts pairs of residues, whose mutations have arisen simultaneously during evolution.

**Structural models and molecular dynamic simulation studies.** The HBV surface protein sequence was taken from the UniProt database, with accession number P03138. Secondary structure prediction was performed with Jpred (Cole et al., 2008). The S protein region 294-317 was modelled using MODELLER (Sali and Blundell, 1993). The template crystal structure of the Newcastle disease virus fusion protein (PDB code: 1G5G) was retrieved from the PDB database (Berman et al., 2000). Sequence alignment was generated with Clustal X (Larkin et al., 2007). The model evaluation was conducted using the Ramachandran plot (Ramachandran et al., 1963). The model of the wild-type sequence was further used to create two structural models with mutations using UCSF Chimera package (Pettersen et al., 2004). One model contains the double mutations T303C/G308C, which may create an extra disulfide bond. The overall effect of those mutations would be to "shift" the disulfide bridge of two amino acids towards the turn of the β-hairpin motif. After mutations, the models were energy minimized by applying Molecular Modelling Toolkit (MMTK) with Amber parameters for standard residues, and 100 steepest descent minimization steps with a step size of 0.02 Å. To investigate the stability of the disulfide bonds, molecular dynamic (MD) simulations of the

three models were carried out by GROMACS version 2020 (Abraham et al., 2015) in conjunction with OPLS-AA/L all-atom force field. The models were immersed in the cubic boxes filled with water molecules with a minimal distance of 1.0 nm between the peptide surface and box. Each system was equilibrated to the desired temperature through a step-wise heating protocol in NVT ensemble followed by 100.0 ps equilibration in NPT ensemble with position restraints on the protein molecule. The final productive MD was performed for each system for 10 ns under periodic boundary conditions without any restraints on the protein with a time step of 2 fs at constant pressure (1 atm) and temperature (300 K). Coordinates were saved every 10 ps, yielding 1000 frames per MD trajectory. All the frames were further investigated to differentiate between allosteric and structurally-stabilizing disulfides. Disulfide bonds were classified based on the five relevant torsion angles ($\chi_1$, $\chi_2$, $\chi_3$, $\chi_{2'}$ and $\chi_{1'}$) (data not shown), disulfides were treated as symmetrical. In this system, twenty conformational categories are possible (Marques et al., 2010; Schmidt and Hogg, 2007; Schmidt et al., 2006). The three central angles ($\chi_2$, $\chi_3$ and $\chi_2$) define the basic shape: Spiral, Hook and Staple (Eklund et al., 1984). The $\chi_3$ defines the orientationally motif: left-handed (LH) or right-handed (RH) if the sign is negative or positive, respectively (Eklund et al., 1984). The $\chi_1$ and $\chi_{1'}$ determines the sign of the nomenclature (Qi and Grishin, 2005).

***In vivo* experiments.** All experiments were performed in accordance with the European Union guidelines for approval of the protocols by the local ethics committee (Authorization Agreement C2EA-15, "Comité Rhône-Alpes d'Ethique pour l'Expérimentation Animale", Lyon, France - APAFIS#27316-2020060810332115 v4). Primary human hepatocytes (PHH, Corning, BD Gentest) were intrasplenically injected in NFRG mice (Azuma et al., 2007), a triple mutant mouse knocked-out for fumarylacetoacetate hydrolase ($fah^{-/-}$), recombinase activating gene 2 ($rag2^{-/-}$), interleukin 2 receptor gamma chain ($IL2rg^{-/-}$). 48h after adeno-uPA conditioning (Bissig et al., 2010; Calattini et al., 2015). Mice were subjected to NTBC cycling during the liver repopulation process, as described previously (Calattini et al., 2015). Mice with human serum albumin (HSA) levels >15 mg/mL, as determined using a Cobas C501 analyzer (Roche Applied Science), were inoculated with virus preparations by intra-peritoneal injection. Sera were collected at different time points before and after infection. Mice were sacrificed 6 weeks post-infection.

**Statistical analysis.** Statistical analyses were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software (San Diego California, USA). The Mann-Whitney or Wilcoxon tests were used for statistical comparisons. A p-value of 0.05 or less was considered as significant. When applicable, data are presented as mean ± standard deviation and results of

the statistical analysis are shown as follows: ns, not significant ($P > 0.05$); *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

**Results:**

**HBV membrane fusion is independent of acid pH and receptor expression**. To investigate the fusion activation mechanism and to identify the fusion determinants of HBV, we designed a cell-cell fusion assay whereby Huh7 "donor" cells, expressing a luciferase reporter gene under control of the HIV-1 promoter, were co-cultured with either Huh7-tat or Huh7-NTCP-tat "indicator" cells, expressing the HIV-1 transactivator of transcription (Tat) protein, which induces luciferase expression only in fused donor and indicator cells (Lavillette et al., 2007). We transfected donor cells with pT7HB2.7 (Sureau et al., 1994), an expression plasmid encoding the wild-type HBV glycoproteins S, M and L. The transfected donor cells where then co-cultivated with Huh7-tat or Huh7-NTCP-tat indicator cells for 1 day. The medium of the co-cultures was then acidified at pH4 for 3 min to trigger fusion and the next day, the luciferase activity in the lysates of co-cultured cells was measured as a read-out of membrane fusion (Figure 1A). The GPs of vesicular stomatitis virus (VSV) or of Crimean-Congo hemorrhagic fever virus (CCHFV) were used as controls for viruses that need acidic pH to promote membrane fusion (Figure 1A). We found that HBV GPs induced similar levels of fusion in co-cultures that were exposed to either acidic or neutral pH, as well as in co-cultures lacking or expressing NTCP receptor (Figure 1A). Since HBV entry requires heparan sulfate proteoglycans (HSPG) to mediate the capture of its viral particles through HBsAg (Leistner CM, et al., Cell Microbiol 2008;10:122–133 and Schulze A, et al. Hepatology 2007;46:1759–1768), we addressed whether blocking of HBsAg/HSPG interaction could inhibit cell-cell fusion using heparin as competitor. Yet, while the applied doses of heparin could prevent cell-free entry, as shown in Schulze A, et al. Hepatology 2007, heparin present during the cell-cell fusion assay did not prevent the levels of fusion, whether the indicator cells expressed or not NTCP (data not shown). We confirmed these results by using CHO and CHO-pgsB618 (Richard C. et al. Cell Biology and Metabolism Vol. 270, Issue 41, p24188-24196, Oct. 1995) cells as donor and/or indicator cells. While both cell types do not express NTCP, only the former express HSPGs. We found that cell-cell fusion could be detected for either indicator cell type to the same extent as for Huh7 cells (data not shown).

These results indicated that cell-cell fusion mediated by HBV GPs is independent of both acid pH and requires neither HSPG nor NTCP receptor, which underscored an alternative fusion trigger.

**The preS1 domain of HBsAg harbors a critical determinant of membrane fusion**. The GPs of HBV (L, M and S proteins) are produced by a single open reading frame and share a common C-terminal S-domain. M and L proteins harbor additional N-terminal extensions (preS2 and preS1/preS2, respectively), with preS1 harboring the NTCP-binding determinant (Glebe et al., 2005b; Gripon et al., 2005). Noteworthy, the fusion determinants of HBV GPs and, particularly, the fusion peptide that could induce merging of viral and endosomal membranes has not yet been functionally identified.

First, to address which GP form is responsible for HBV membrane fusion, we evaluated the role of either protein in cell-cell fusion assays (Figure 1B). Huh7 cells were transfected with plasmids encoding wt HBV GPs, *i.e.*, S, M and L (pT7HB2.7), *vs.* only S, M or L (using pCiS, pCiM and pCiL plasmids, respectively) (Komla-Soukha and Sureau, 2006). To analyze the expression of either protein at the cell surface, transfected cells were labeled with sulfo-NHS-SS-biotin, a chemical compound that is unable to penetrate biological membranes. After lysis and immuno-precipitation of biotinylated proteins, we found that the individually expressed L, M or S proteins were detected at similar levels as compared to HBV GPs (L, M and S) expressed simultaneously, as in cells transfected with the wt pT7HB2.7 plasmid (data not shown). Then, to determine the fusion activity of either protein, we performed cell-cell fusion assays as described above. We found that none of the S, M or L proteins expressed alone were able to induce membrane fusion (Figure 1B). Furthermore, when we tested the pT7HB2.7Mless plasmid, which induces co-expression of S and L only (data not shown), we detected a cell-cell fusion activity at the same level that for wt HBV GPs (Figure 1B). This indicated that M is not necessary for membrane fusion, in agreement with previous results (Sureau et al., 1994) showing that M is dispensable for infectivity of viral particles (data not shown). Altogether, these results suggested that the determinants of membrane fusion are harbored within S and L GPs.

Next, aiming to identify a fusion peptide in either protein, we used a computational approach to pinpoint regions of the HBV GPs that may potentially interact with membrane bilayers. Using Membrane Protein Explorer (MPEx), a tool based on the Wimley-White Interfacial Hydrophobicity Scale (Snider et al., 2009), five regions of high interfacial hydrophobicity were identified (data not shown). Two out of the five hydrophobic regions did not correspond to HBV GP transmembrane regions (TM1, TM2, and TM3/TM4), and therefore were considered as candidate fusion peptides (data not shown). The first predicted segment comprised amino acids 48 to 66 that partially overlap with to the preS1 domain. The second segment, which includes amino acids 127 to 145, is included in the preS2 region. Our prediction

analyzes indicated that the first segment (ΔG=-3.38) was more likely to be a fusion peptide than the second one (ΔG=-0.85) (data not shown). Considering the Wimley-White scale, a set of mutants was designed to alter the hydrophobicity of the two predicted segments as candidate peptide fusion (48-66 (preS1): GAGAFGLGFTPPHGGLLGW (SEQ ID N°5) and 127-145 (preS2): GLYFPAGGSSSGTVNPVLT (SEQ ID N°6)). In the first segment, three mutants were studied by changing the aromatic residues to an alanine or glutamate: F52A, F56A, W66A, F52A/W66A (FW/AA) and F52E/W66E (FW/EE), or a glycine to an alanine (G53A). In the second segment, four mutants were considered: Y129A, F130A, S136E, L144A; while the first two mutants targeted aromatic residues, S136 and L144 were also considered important because they are at the center of the predicted region and have a relatively high hydrophobicity. To evaluate the role of these two sequences in HBV fusion, we introduced these single or double mutations in both regions and inserted them in the pT7HB2.7 HBV GP expression plasmid. Each mutant was compared to wt HBV GPs in both infection assays, using HDV particles (Sureau, 2010; Perez-Vargas et al., 2019), and cell-cell fusion assays, as above-described. We found that HDV particles carrying these mutant GPs were produced by Huh7 cells at levels similar to those produced with wt GPs (data not shown), hence ruling out gross misfolding induced by the mutations that would otherwise prevent HBV GP incorporation on viral particles (Abou-Jaoudé and Sureau, 2007). Interestingly, no infectivity could be detected for most of the mutations introduced in the preS1 peptide (data not shown), whereas the HDV particles with mutations in the preS2 peptide showed levels of infectivity that were similar to those obtained with the wt GPs (data not shown). Correlating with the results of these infection assays, we found that the mutants in the preS1 peptide that prevented HDV infectivity also abrogated cell-cell fusion activity (data not shown) in a manner unrelated to the levels of cell surface expression (data not shown). In contrast, mutations in the preS2 peptide displayed the same levels of cell-cell fusion activity as compared to wt (data not shown). Altogether, these results indicated that the preS1 region harbors a potential fusion peptide.

**Modification of the disulfide bond network of HBV S protein prevents membrane fusion steps.** Next, we sought to investigate the mechanisms that could induce fusion-activating conformational changes in the HBV GPs, leading to exposure of the fusion peptide t. As neither the HBV receptor interaction nor the acidic pH could trigger membrane fusion (Figure 1), we sought that conformational rearrangement of HBV GPs may involve reshuffling of their disulfide bonds. Indeed, previous studies showed that cysteine residues of the HBV S antigenic loop (AGL) are essential for HDV infectivity and that viral entry is blocked by inhibitors of thiol/disulfide exchange reactions, such as TCEP, DTT, DTNB or AMS (Abou-Jaoudé and

Sureau, 2007). Thus, to extend the notion that thiol/disulfide exchange reactions are implicated during membrane fusion and entry, we performed infection assays with HDV particles in the presence of DTNB, an alkylator agent. First, using different DTNB concentrations that were added either at the onset of infection or at 16h post-infection, we confirmed that DTNB could block HDV infection in a dose-dependent manner but only when it was added at the onset of infection (Abou-Jaoudé and Sureau, 2007) (data not shown). Second, using time-of-addition experiments, we found that DTNB could inhibit infection only if added within the first 2 hours post-inoculation HDV particles (Figure 2A). These results suggested that DTNB blocks a thiol/disulfide exchange reaction that could be necessary at an early step of infection, such as a trigger of the fusion mechanism, though not at a later stage of the entry process. Third, to evaluate the effect of DTNB in membrane fusion, we performed cell-cell fusion assays in presence of DTNB, which was added at the onset of cell co-cultures *vs.* at 16h after seeding the cell co-cultures. We showed that DTNB added during the co-culture neither induced cytotoxicity (data not shown) nor affected expression of HBV glycoproteins on the cell surface (data not shown) We found a dose-dependent reduction of the level of cell-cell fusion when DTNB was added immediately after cell-cell contact, whereas we detected a much lower effect in fusion activity when DTNB was added at 16h after cell contact (Figure 2B).

Altogether, these results suggested a role of the disulfide bonds network during HBV membrane fusion steps, perhaps at the level of the fusion trigger.

To address this possibility and to identify potential mechanisms involved in fusion triggering, we focused on the "a" determinant of protein S that exhibits eight conserved Cys, which, for some of them, are in strong proximity in the sequence (Figure 3A). To avoid trivial contact predictions between consecutive Cys, we defined four Cys-containing regions in a way that Cys pairs that are potentially in contact should have a sequence separation of at least four amino acids. The first Cys-containing region includes C270, the second C284 and 287, the third C300, C301 and C302, and the last one C310 and C312 (Figure 3A). We applied secondary and tertiary structure prediction methods together with the contact prediction method RaptorX (Ma et al., 2015)(Wang et al., 2017), based on coevolution signals, to predict disulfide connectivity in the "a" determinant, which may identify which Cys forms disulfide bonds. Notably, RaptorX predicted structural contacts between either region (data not shown) and we highlighted pairs of residues in contact in the four Cys-regions, with the strongest signal detected between the third and fourth regions (Figure 3B). Next, applying the JPred secondary structure prediction method (Cole et al., 2008), we predicted two β-strands in the Cys-rich regions delimited by the S segments 298-303 and 310-313 (Figure 3A). Then, considering the secondary structure

prediction and the contact prediction, we built a three-dimensional model for the region 294-317 (Figure 3C), which indicated that this sequence is compatible with a β-hairpin structural motif containing a cross-strand disulfide (CSD) between C301 and C310. Finally, through the analysis of its five χ dihedral angles (data not shown), this disulfide bond was classified in a "-HStaple conformation", which is a particular type of disulfide geometry associated with allosteric functions by triggering a conformational change upon switching between the reduced and oxidized states (Chiu and Hogg, 2019; Hogg, 2003).

We therefore hypothesized that the redox state of this disulfide may act as an allosteric switch that controls conformational rearrangements of the S protein. Thus, we used our structural model of the C301-C310 disulfide bond to design a mutant of S that could disrupt this hypothetical allosteric function, *i.e.*, the T303C/G308C double mutant which induces an additional C303-C308 disulfide bond (Figure 3C). Further molecular dynamics (MD) simulations (1000 frames per MD trajectory) carried out to differentiate between allosteric and structurally stabilizing disulfides, where the disulfides can be classified based to their angles (data not shown), showed that both mutants predominantly form structural disulfide bonds.

Aiming to validate our prediction that an additional disulfide bond between the two β-strands could, by stabilizing the 298-313 β-hairpin motif, prevent membrane fusion from occurring, we produced HDV particles carrying the individual (T303C or G308C) and double (T303C/G308C) mutations in HBV GPs. By measuring HDV RNAs in cell supernatants, we found that all mutants could produce similar levels of viral particles as compared to wt virus (data not shown), suggesting absence of gross alterations of HBV GP conformation that would otherwise preclude virion assembly (Abou-Jaoudé and Sureau, 2007). Importantly, we found that while HDV particles harboring GPs with the individual mutations were as infectious as wt, those that incorporated the T303C/G308C double mutation and the putative additional C303-C308 CSD bond were not infectious (data not shown). Moreover, we found that HDV particles harboring GPs with the T303C/G308C mutation had similar binding levels on Huh7 cells than those generated with wt GPs (Figure 4A), underscoring a post-binding defect. Then, to address this possibility, we performed cell-cell fusion assays with either HBV GP mutant, which were readily expressed at the cell surface (data not shown). We found that whereas the single mutations displayed similar fusion activity as compared to wt HBV GPs, the T303C/G308C double mutation completely prevented HBV GP-induced cell-cell fusion activity (data not shown).

Altogether, these results suggested that the putative C303-C308 additional disulfide bond stabilizing the loop containing the CSD bond inhibited HBV entry and fusion, perhaps by

preventing conformational rearrangements of HBV GPs that are required for promoting membrane fusion.

**ERp57 is a protein disulfide isomerase that promotes HBV entry and infectivity *in vivo***. Assuming that C301 and C310 form a CSD with allosteric functions (Figure 3), we reasoned that its isomerization could facilitate some conformational rearrangements required to promote membrane fusion. We therefore hypothesized that such an isomerization could be induced by a host factor from the Protein Disulfide Isomerase (PDI) family, which are enzymes that can both reduce and oxidize disulfide bonds. To address if PDIs are involved in HBV entry, we tested the effect of inhibitors (NTZ, EGCG, Rutin, Bacitracin, PX-12) that target different PDI species (PDIA1, ERp5, ERp57, TMX1) for their effect in cell entry of viral particles.

First, through binding assays of viral particles to Huh7 or Huh7-NTCP cells performed in the presence of either inhibitor, we found that none of these inhibitors affected binding of HDV particles generated with either wt or T303C/G308C mutant GPs (Figure 4A). Second, using infection assays with Huh7-NTCP cells pre-incubated with either inhibitor, we found that HDV particles had strongly reduced infectivity in presence of NTZ and EGCG inhibitors that both target ERp57 (Figure 4B). Third, we confirmed these results using infection assays with authentic HBV particles (Figure 4C). Finally, to demonstrate that the inhibitors acted at the level of membrane fusion, we performed cell-cell fusion assays, as above-described, whereby either inhibitor was added at the onset of co-cultures of HBV GP-expressing Huh7 donor and Huh7-NTCP-Tat indicator cells, and kept throughout the assay period. Remarkably, we found a strong reduction of the levels of cell-cell fusion with the same drugs that inhibited HDV infection (Figure 4D). Hence, these results suggested a potential role of ERp57 in HBV membrane fusion.

Next, aiming to confirm and extend these findings, we selected a subset of the above PDIs, i.e., ERp46, ERp57 and ERp72, that display low but significant expression at the cell surface (Figure 5A), in agreement with a previous study (Turano et al., 2002). We down-regulated either PDI in target cells *via* transduction of Huh7-NTCP cells with shRNA-expressing lentiviral vectors (Figure 7A, B, C). We found that down-regulation of ERp57, though not ERp46 or ERp72, strongly reduced the levels of HDV (Figure 5A) and HBV infection (Figure 5B) and of cell-cell fusion (Figure 5C). Altogether, these results indicated that ERp57 is a protein disulfide isomerase that promotes HBV entry at a membrane fusion step.

Finally, we sought to demonstrate that ERp57 inhibition may prevent HBV propagation *in vivo* using NTZ, which has a short half-life of about 1.5h (Ruiz-Olmedo et al., 2017; Stockis et al., 1996). We generated a cohort of liver humanized mice (HuHep-mice) derived from the

NFRG mouse model (Azuma et al., 2007) (Figure 6A). We retained the animals that displayed >15 mg/mL of human serum albumin (HSA), which corresponds to 40-70% of human hepatocytes in the liver (Calattini et al., 2015). In agreement with previous reports (Perez-Vargas et al., 2019), these animals supported HBV infection (Group#1) for several weeks/months (Figure 6B). The second group of HuHep mice was treated with NTZ 30min prior to inoculation with HBV, and then, treated again with NTZ 1h later. We found that viremia in this group was delayed by about 4 weeks, as compared to Group#1 for which HBV could disseminate immediately after inoculation. This indicated that the blocking of ERp57 can prevent HBV infection *in vivo*.

**Discussion**

The entry process of enveloped viruses into cells is the series of all events that take place, from the attachment of the virus to the host cell until the release of its genome in the cytoplasm. It is a finely regulated and complex process with several steps, in which many viral and cellular factors are involved. The first interaction, which may lack specificity, serves to give a virus an initial catch hold from which it can recruit specific receptors and entry co-factors that drive the reactions leading to entry. Fusion is the last step of enveloped virus entry and allows the release of the viral capsid in the cytoplasm following the merging of the viral membrane with a membrane of the infected cell. The interactions with the target cell that trigger conformational changes of the viral surface glycoproteins, ultimately leading to the insertion of their fusion peptide into the cell membrane, vary widely for enveloped viruses and can be divided into different scenarios. In a first one (*e.g.*, HIV), fusion is triggered directly by the interaction of the viral glycoprotein with its cellular receptor, through allosteric conformational rearrangements. In some cases, a sequential interaction with additional host factors is required to trigger the conformational change required for fusion. In a second scenario (*e.g.*, influenza virus), the interactions with the receptor at the cell surface leads to the endocytosis of viral particles, which is followed by GP protonation in the low pH environment of the intracellular endosomal organelles that triggers the fusogenic conformational change. In a third scenario (*e.g.*, Ebola virus), the initial interactions of the virion with the cell surface trigger its endocytosis followed by, a second interaction with an internal receptor, often found in late endosomes, which is preceded by proteolytic cleavage of the fusion protein by an endosomal protease, leading to fusion activation (Harrison, 2015; White and Whittaker, 2016). Finally, for certain viruses (*e.g.*, Sindbis virus), the fusion protein requires an activating redox reaction

involving disulfide bonds of their glycoprotein to induce membrane fusion (Key et al., 2015; Rey and Lok, 2018).

Using a cell-cell fusion assay, we found that HBV fusion activity reached similar levels whether indicator cells expressed or not HSPG and/or NTCP but was not increased when the cell co-cultures were exposed at low pH, in contrast to *bona fide* pH-dependent GPs such as VSV-G or CCHFV Gn/Gc (Figure 1). That both HSPG and NTCP, which are respectively HBV virion membrane capture (Leistner CM, et al Cell Microbiol 2008;10:122–133 and Schulze A, et al. Hepatology2007;46:1759–1768) and specific entry factors (Yan H, et al. Elife 2012;1 e00049 and Ni Y, et al Gastroenterology 2014;146:1070–1083), are not required for cell-cell fusion highlights that this assay reveals late entry events, such as those occurring after virus interaction with either factor. Similarly, for other viruses such as e.g., influenza virus or hepatitis C virus (HCV), binding to their cell-free entry receptor is not a requirement for both cell-cell fusion (Lin and Cannon, 2002) and liposome fusion (Lavillette et al., 2006) assays triggered by low pH treatment. Thus, while it is clear that cell-cell fusion does not recapitulate per se all the events required to promote cell entry of viral particles since it bypasses the step of internalization that subsequently allows membrane fusion in endosomes that are required for entry of the above-mentioned viruses and as proposed for HBV (Macovei et al., 2013; Iwamoto et al., 2019), it is a good reflect of the events that are required for fusion triggering for many enveloped viruses (Earp et al., 2004). Accordingly, our results indicate that the trigger for the HBV membrane fusion mechanism not only is independent of an allosteric interaction so its GPs with the NTCP receptor but also is independent of GPs protonation that is induced by the low pH environment of endosomes. That low pH does not increase HBV cell-cell fusion agrees with previous results indicating that pharmacological agents that raise or neutralize the pH of the endocytic pathway had no effect on HBV infection (Macovei et al., 2010b, 2013b; Rigg and Schaller, 1992b). Previous results from the group of Camille Sureau showed that cysteine residues of the HBV antigenic loop (AGL) are essential for HDV infectivity and that viral entry is blocked by inhibitors of thiol/disulfide exchange reaction (Abou-Jaoudé and Sureau, 2007). Our results extend this notion as they indicate that such reactions seem to be necessary to mediate a critical early post-binding event but not at a later stage of the infection process since no effect in virus infectivity could be detected when DTNB was added at 4h post-infection (Figure 2A). Since isomerization of some disulfide bonds has been shown to be crucial for conformational rearrangements of GP from another enveloped viruses leading to fusion (Fenouillet et al., 2007), we sought to investigate if and how such reactions could be implicated during a membrane fusion step of HBV entry. Here, using our cell-cell fusion assay, we found

that DTNB blocked HBV GP-mediated membrane fusion (Figure 2B). Altogether, these results indicated a role of disulfide bond network of S GP during HBV membrane fusion.

Capitalizing on the above-mentioned experimental information that oxidizing reagents alter virus entry, we sought to examine how disulfide bonds of the HBV GPs, or rather, how a potential reshuffling of its disulfide bonds profile, could be important for HBV entry. Indeed, cross-strand disulfides occurring in some viral surface GPs are believed to play a role in virus entry (Wouters et al., 2004; Barbouche et al., 2003; Jain et al., 2007; Rosenthal et al., 1998). Particularly, allosteric disulfide bonds can modulate the activity of the proteins in which they reside by mediating a structural change when they are reduced or oxidized (Hogg, 2003; Schmidt et al., 2006). Allosteric control of protein function is defined as a change in one site (the allosteric site) that influences another site by exploiting the protein's flexibility; an allosteric disulfide bond represents the "allosteric site" and the conformational change triggered by cleavage of such bonds alters protein function. For the HBV S protein, we used the contact prediction method RaptorX (Ma et al., 2015)(Wang et al., 2017) to predict contacts between four Cys-rich regions of the AGL determinant (Figure 3), which highlighted that two of these regions may likely interact: *i.e.*, the Cys-rich regions III and IV (data not shown). Using the secondary structure prediction method JPred (Cole et al., 2008), we proposed that these regions organize in two β-strands and we constructed a three-dimensional model of the region 294-317 of the HBV S GP, which indicated that this sequence is compatible with a β-hairpin structural motif containing a CSD bond between C301 and C310 (Figure 3C). Interestingly, the analysis of the signs of the five χ dihedral angles defined by the Cys residues allowed to classify this particular disulfide bond in a -HStaple conformation, which is a particular type of disulfide geometry associated with allosteric functions that is known to trigger conformational changes upon switching between the reduced and oxidized states (Chiu and Hogg, 2019; Schmidt et al., 2006). Hence, we hypothesized that the redox state of the C301-C310 disulfide bond may act as an allosteric switch controlling conformational rearrangements of the HBV GP leading, ultimately, to exposure of the fusion peptide. Of note, the β-hairpin region with the predicted CSD lies at the surface of the S protein according to a three-dimensional *in silico* model (van Hemert et al., 2008), which may allow interactions with other HBs subunits and/or cellular factors. Yet, to test our structural and dynamic model involving a C301-C310 CSD bond in S GP (Figure 3), we reasoned that creating an additional, neighboring disulfide bond between positions 303 and 308 may stabilize the β-hairpin motif (Figure 3C), which may prevent molecular rearrangements and thus, membrane fusion to occur. The *in silico* analysis indicates that the T303C/G308C double mutant generate predominantly two structural CSD according to

our JPred-based (Cole et al., 2008) structural modelling, which affects the structural conformation of the C301-C310 CSD that is no longer classified as an allosteric bond. When we tested the T303C/G308C mutation in functional assays, we found that the mutant HBV GPs induced an almost complete loss of infection and fusion activity (data not shown), hence suggesting that by stabilizing cross-strand disulfide exchange, the putative additional disulfide bond prevented conformational rearrangements of HBV GPs that are required for promoting membrane fusion.

Assuming that the isomerization of the C301-C310 allosteric CSD or of other thiol/disulfide of the AGL determinant could facilitate the conformational rearrangements of HBs required to promote HBV membrane fusion, we hypothesized that such isomerization could be induced by a host factor from the PDI family, which are enzymes that can both reduce and oxidize disulfide bonds. Protein disulfide isomerases consist of a family of 21 structurally-related proteins with a thioredoxin-like domain. Most of these isomerases have CXXC motif that catalyzes formation, reduction and rearrangement of the disulfide bonds in proteins (Abell and Brown, 1993). These isomerases are primarily involved in the folding of proteins in the endoplasmic reticulum (ER), catalyzing formation of their disulfide bonds, and most of these isomerases have ER retention signals. However, some isomerases from the PDI family have also been shown to be present at the cell surface, both in functional and in biochemical assays (Turano et al., 2002). Accordingly, cell surface-localized PDIs are involved in processes such as cell adhesion, nitric oxide signalling, and in cell entry of different viruses (Diwaker et al., 2013; Fenouillet et al., 2007). In support of the notion that PDIs are involved in HBV entry, we found that inhibitors that target different PDI members could block infection and cell-cell fusion though not the binding of viral particles to the cell surface. Of note, we found that bacitracin, which targets PDIA1, did not inhibit HBV entry and membrane fusion, in line with a previous study showing that it could not inhibit HDV entry (Abou-Jaoudé and Sureau, 2007). While the above ruled out PDIA1 as an entry co-factor of HBV, we found a strong reduction of the levels of HBV and HDV infection as well as of HBV-induced cell-cell fusion when we used the NTZ and EGCG inhibitors (Figure 4), which target ERp57 (Müller et al., 2008; Pacello et al., 2016). Consistently, we detected a low but significant expression of ERp57 as well as ERp46 and ERp72 at the cell surface (Figure 5), in agreement with a previous study (Turano et al., 2002). Using a gene silencing approach, we confirmed that down-regulation of ERp57 but not of these alternative PDIs could decrease the levels of HDV and HBV infection as well as of cell-cell fusion (Figure 5). Importantly, we showed that a short time treatment of liver humanized mice with NTZ could delay HBV infection by *ca.* 2-4 weeks (Figure 6). Since NTZ has a short half-

life of about 1.5h *in vivo* (Ruiz-Olmedo et al., 2017; Stockis et al., 1996) and since NTZ was administrated at very short times before and after HBV inoculation, we calculated that less than 10% of the drug was still present in those mice at 7h post-infection, which likely precludes an effect on HBV post-entry steps (Korba et al., 2008). Altogether, these results support the role of ERp57 at early steps of HBV infection and validate this PDI as a therapeutic target. Note that our results did not discard the possibility that some other PDI could also play a role during HBV entry into cells.

The fusion-mediating GPs of enveloped viruses contain a sequence, termed fusion peptide, that interacts with and destabilizes the cellular target membrane. Such an event is finely controlled so as to occur at the appropriate time and location and to prevent fortuitous inactivation of GP fusion activity and virus infectivity. Hence, a conformational change in these GPs is a requirement to induce the accessibility and function of the fusion peptide segment. Candidate fusion peptides are generally identified as hydrophobic sequences, of *ca.* 16 to 26 residues in length, that are conserved within a virus family and that may adopt $\alpha$-helical conformation with strongly hydrophobic faces. They can be internal or located at the amino-terminus of fusion GP subunits (Apellániz et al., 2014; Epand, 2003; Martin et al., 1999). There are a number of criteria that characterize fusion peptide segments and, while none of these criteria taken individually are absolute to define a fusion peptide segment, they are sufficiently restrictive to predict if a given region of a protein present features of a fusion peptide segment (Delos and White, 2000; Delos et al., 2000).

Previously, a conserved peptide comprising 23 amino acids at N-terminal end of HBV S protein and overlapping its TM1 sequence was shown to interact with model membranes, causing liposome destabilization in a pH-dependent manner (Rodriguez 1994, 1995, 1999). However, it was also demonstrated that hydrophobic residues in TM1 were critical for S protein expression as well as for infectivity (Siegler VD, 2013). An essential role of TM1 in fusion mechanism, albeit in a pH-independent manner, could be shown for the duck hepatitis B virus (DHBV) (Chojnacki J 2005), although there is also evidence for the involvement of the preS domain of DHBV at an early step of infection, likely during the fusion process (Delgado 2012). Here, through a computational hydropathy analysis of the HBV GP, we identified two potential short sequences within the preS1 and preS2 regions that may potentially interact with membrane bilayers. To validate these predictions, we characterize in both infection and cell-cell fusion assays HBV GP mutants in key positions of either sequence. We found that while none of the mutations in the preS2 segment altered infection or membrane fusion activities, mutations in the preS1 sequence induced an almost complete loss of infectivity and cell-cell

fusion (data not shown). Note that these mutants had similar if not identical levels of cell surface expressed L, M and S proteins and/or capacity to induce the formation of HDV particles. These results suggested that the preS1 region harbors a fusion peptide in addition to a NTCP binding determinant (Meier et al., 2013).

Overall, our study characterizes some crucial determinants of HBV entry and membrane fusion. The mechanism by which fusion proteins are activated and undergo conformational rearrangements or fusion intermediates is a particularly complex process involving several regions of viral surface GPs. Our results suggest that for HBV, this mechanism could be triggered by ERp57, allowing a thiol/disulfide exchange reaction to occur and regulate isomerization of a critical CSD, which ultimately results in the exposition of the fusion peptide located within the preS1 region.

**Table section**

**Table 1: Useful nucleotide sequences for practicing the invention**

| SEQ ID NO | Nucleotide or amino acid sequence |
|---|---|
| **1) ERp57 amino acid human sequence** | MRLRRLALFPGVALLLAAARLAAASDVLELTDDNFESRISDTG<br>SAGLMLVEFFAPWCGHCKRLAPEYEAAATRLKGIVPLAKVDC<br>TANTNTCNKYGVSGYPTLKIFRDGEEAGAYDGPRTADGIVSH<br>LKKQAGPASVPLRTEEEFKKFISDKDASIVGFFDDSFSEAHSEF<br>LKAASNLRDNYRFAHTNVESLVNEYDDNGEGIILFRPSHLTNK<br>FEDKTVAYTEQKMTSGKIKKFIQENIFGICPHMTEDNKDLIQG<br>KDLLIAYYDVDYEKNAKGSNYWRNRVMMVAKKFLDAGHKL<br>NFAVASRKTFSHELSDFGLESTAGEIPVVAIRTAKGEKFVMQE<br>EFSRDGKALERFLQDYFDGNLKRYLKSEPIPESNDGPVKVVVA<br>ENFDEIVNNENKDVLIEFYAPWCGHCKNLEPKYKELGEKLSK<br>DPNIVIAKMDATANDVPSPYEVRGFPTIYFSPANKKLNPKKYE<br>GGRELSDFISYLQREATNPPVIQEEKPKKKKKAQEDL |
| **2) ERp57 nucleic acid human sequence** | agacgcgcgagcgcaagcagcgggttagtggtcgcgcgcccgacctccgcagtcccagccga<br>gccgcgacccttccggccgtccccaccccacctcgccgccatgcgcctccgccgcctagcgctg<br>ttcccgggtgtggcgctgcttcttgccgcggcccgcctcgccgctgcctccgacgtgctagaactc<br>acggacgacaacttcgagagtcgcatctccgacacgggctctgcgggcctcatgctcgtcgagtt<br>cttcgccccctggtgtggacactgcaagagacttgcacctgagtatgaagctgcagctaccagatt<br>aaaaggaatagtcccattagcaaaggttgattgcactgccaacactaacacctgtaataaatatgga<br>gtcagtggatatccaaccctgaagatatttagagatggtgaagaagcaggtgcttatgatggaccta<br>ggactgctgatggaattgtcagccacttgaagaagcaggcaggaccagcttcagtgcctctcagg<br>actgaggaagaatttaagaaattcattagtgataaagatgcctctatagtaggttttttcgatgattcatt<br>cagtgaggctcactccgagttcctaaaagcagccagcaacttgagggataactaccgatttgcaca<br>tacgaatgttgagtctctggtgaacgagtatgatgataatggagagggtatcatcttatttcgtccttca<br>catctcactaacaagtttgaggacaagactgtggcatatacagagcaaaaaatgaccagtggcaaa<br>attaaaaagtttatccaggaaaacatttttggtatctgccctcacatgacagaagacaataaagatttg<br>atacagggcaaggacttacttattgcttactatgatgtggactatgaaaagaacgctaaaggttccaa<br>ctactggagaaacagggtaatgatggtggcaaagaaattcctggatgctgggcacaaactcaactt<br>tgctgtagctagccgcaaaaccttagccatgaactttctgattttggcttggagagcactgctggag<br>agattcctgttgttgctatcagaactgctaaaggagagaagtttgtcatgcaggaggagttctcgcgt<br>gatgggaaggctctggagaggttcctgcaggattactttgatggcaatctgaagagatacctgaagt |

| | |
|---|---|
| | ctgaacctatcccagagagcaatgatgggcctgtgaaggtagtggtagcagagaattttgatgaaa<br>tagtgaataatgaaaataaagatgtgctgattgaatttatgccccttggtgtggtcactgtaagaacct<br>ggagcccaagtataaagaacttggcgagaagctcagcaaagacccaaatatcgtcatagccaag<br>atggatgccacagccaatgatgtgccttctccatatgaagtcagaggttttcctaccatatacttctctc<br>cagccaacaagaagctaaatccaaagaaatatgaaggtggccgtgaattaagtgattttattagctat<br>ctacaaagagaagctacaaacccccctgtaattcaagaagaaaaacccaagaagaagaagaagg<br>cacaggaggatctctaaagcagtagccaaacaccactttgtaaaaggactcttccatcagagatgg<br>gaaaaccattggggaggactaggacccatatgggaattattacctctcagggccgagaggacaga<br>atggatataatctgaatcctgttaaattttctctaaactgtttcttagctgcactgtttatggaaataccag<br>gaccagtttatgtttgtggttttgggaaaaattatttgtgttgggggaaatgttgtgggggtggggttg<br>agttgggggtattttctaatttttttttgtacatttggaacagtgacaataaatgagacccctttaaactgtc<br>ttattttccaccagattgagaaccagatgttctctacacactatcactgttcaatagagctttcttcagtg<br>atggaaatgctctgtaatctacactgttcagtacaggtagctacggagcatctgaaatatggctagaa<br>actacatttttgttttgattaatttaaatagccatacagttgctaccatactggtcacggcagctgtagac<br>tgactgggtccatagttcatcacctcaaaattctttcaaaatttattatctctttctctccttacatgtttattt<br>cccaggcctaccctggtgattagaacagctgaagggcctttcttgttaggctgtccatgccctaagg<br>atgggttcctgtttatccttgccacgcagctgagcttactgcatgtttatatctcccaaggactgttctct<br>gctcagaaatgccctgtcaagggtgtggcatcacgcagtttcatccaagttgtttcaggaattgctga<br>cactgctgggtgcagttctatcccctaaagcctagggtgtggccctttaacttccccttcagtagaact<br>gggaaaggcagtaccattcctagttatgagtgaagcatccactttcttttgtaacaatgaggaacaga<br>aaggataagactgaagagtgatcttttgtccaactaaaccatttatctccttttgtaggtaaattcaaatc<br>ctgcccagttatagttttcagtcactggagaattccaggtaggagccctactttaggtgatcctagga<br>actcctatgttcagaaaagaattttcttcctactatataattacagtatttagctgtcaattttaagatgaat<br>ttggtagagccttatagtaaagtatgtatcttggtcacacacaaagcttggaaaaagtaaaagatgtct<br>aaaccataatcttgtaactcataacatctgggctggggcccggggatctaattgtttagagagcccc<br>aaaagtggctcagatgtaaagccagggttaagaaccatctgccttggaagatttaagggaaacata<br>taaacttgtacaaaggacacagaagcagtcagttttaatttttagccatgttggtaaaagttcattttca<br>gtacatgggtaacacccaggccctttcccattatatccaggtatgctacaagttcttttaactcttatca<br>gaagttattattactgtttccttagagaggctaccaggctaaaattcacttagtttggtttgtctaatgtcc<br>tcattattttatcctgaagatgatgtcatttctcaggacttgaaaatgacttggctgaactaaaggtaaa<br>aaagccaagcctctgtcacttttcctagactcctaggcacagctatggagtctttgcacagtgcccat<br>accctaaaaattaataatgaaaaccaaacctcaagaacctagagcagctctctacttgccaccatgg<br>actccagtggtcagcataagaaaagcagatagttgcattctatttagtttatagctgctttgttcctttgt |

| | |
|---|---|
| | gtttcactaagcagaggctcaaaaattcccttgataacttcagctgcccctgttcttttcctcaaactcc aggatgagacctttaatgtgggacaatttctggtgaaggtactcacagcgacgcttttcttctctgtaa cttgggtactgctgcagagaaaaagcatccatgtcaaaaagtaaaaattctcattctaccttg |
| **3) shRNA ERp57 N°3** | ccgggctgcactgtttatggaaatactcgagtatttccataaacagtgcagctttttg |
| **4) shRNA ERp57 N°4** | ccggcgatttgcacatacgaatgttctcgagaacattcgtatgtgcaaatcgttttttg |
| **5) 48-66 (pre S1) candidate peptide fusion** | GAGAFGLGFTPPHGGLLGW |
| **6) 127-66 (pre S2) candidate peptide fusion** | GLYFPAGGSSSGTVNPVLT |

**REFERENCES:**

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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## CLAIMS:

1. An inhibitor of ERp57 for use in the treatment of Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV) infection wherein the said inhibitor of ERp57 prevents or reduces the virus entry into hepatocytes at membrane fusion step and wherein said antagonist is an inhibitor of ERp57 activity and/or an inhibitor of ERp57 gene expression.

2. The inhibitor for use according to claim 1, wherein the HBV and/or HDV infection is a chronic infection.

3. The inhibitor of ERp57 according to claim 1 for use in the treatment of Hepatitis B virus (HBV) and Hepatitis D virus (HDV) infection wherein the said inhibitor of ERp57 prevents or reduces the virus entry into hepatocytes at membrane fusion step and wherein said antagonist is an inhibitor of ERp57 activity and/or an inhibitor of ERp57 gene expression.

4. The inhibitor of ERp57 for use according to claim 1 to 3 wherein said inhibitor of ERp57 activity is selected from the list consisting of a small organic molecule, an anti- ERp57 neutralizing antibody, or an aptamer.

5. The inhibitor of ERp57 for use according to claim 1 to 3 wherein the inhibitor of ERp57 gene expression is selected from the list consisting of antisense oligonucleotide, nuclease, siRNA, shRNA or ribozyme nucleic acid sequence.

6. A method for screening a plurality of candidate compounds useful for treating Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV) infection comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit ERp57 and (b) and positively selecting the candidate compounds capable of inhibiting said ERp57.

7. The method according to claim 6 for screening a plurality of candidate compounds useful for treating Hepatitis B virus (HBV) and Hepatitis D virus (HDV) infection comprising the steps consisting of (a) testing each of the candidate compounds for its ability to inhibit ERp57 and (b) and positively selecting the candidate compounds capable of inhibiting said ERp57.

8. A pharmaceutical composition for use in a method for treating Hepatitis B virus (HBV) and/or Hepatitis D virus (HDV) infection in a subject in need thereof, comprising the inhibitor of ERp57 according to any claim 1 to 5.

9. The pharmaceutical composition according to claim 8 for use in a method for treating Hepatitis B virus (HBV) and Hepatitis D virus (HDV) infection in a subject in need thereof, comprising the inhibitor of ERp57 according to any claim 1 to 5.

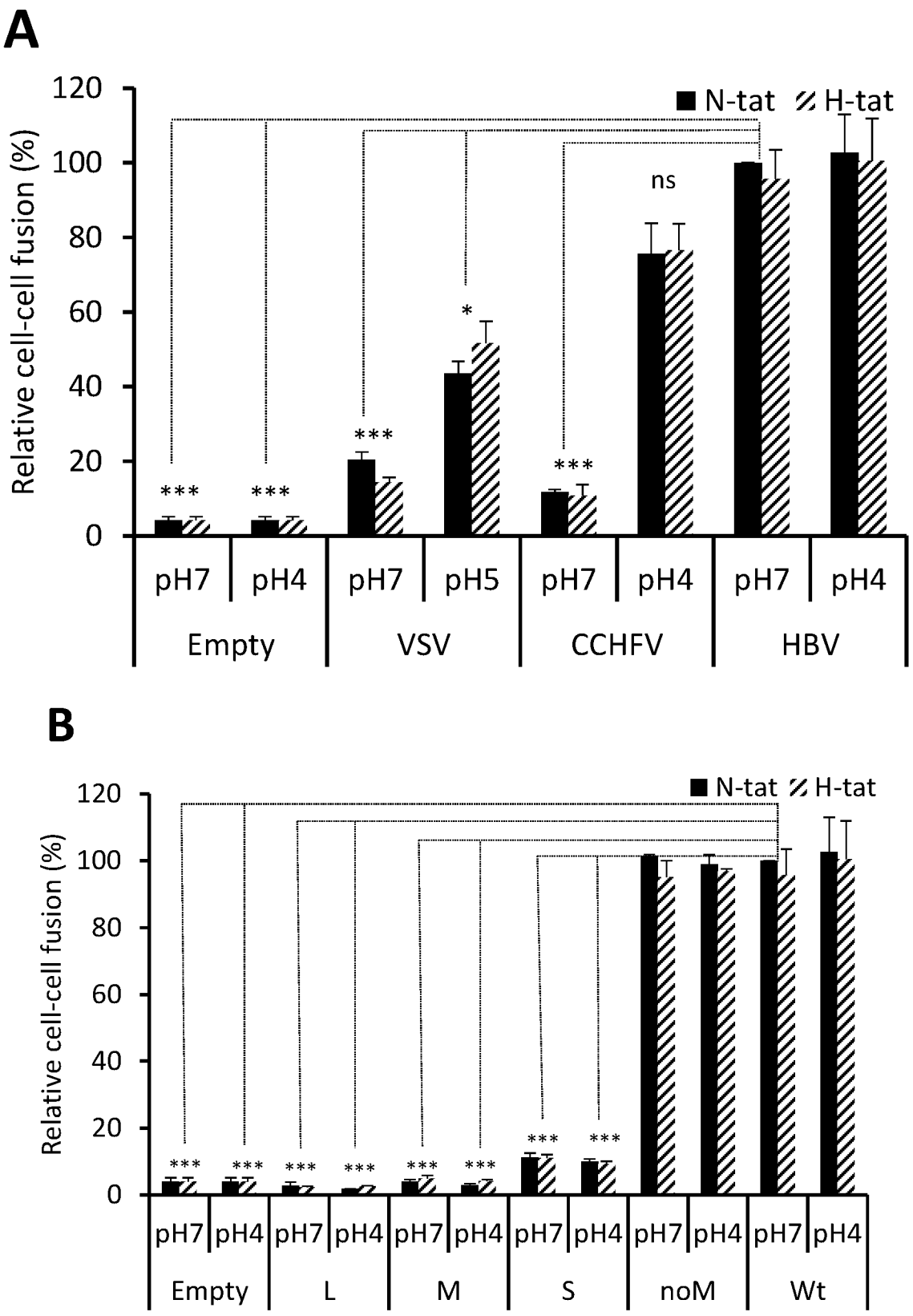



**Figure 1A and B**

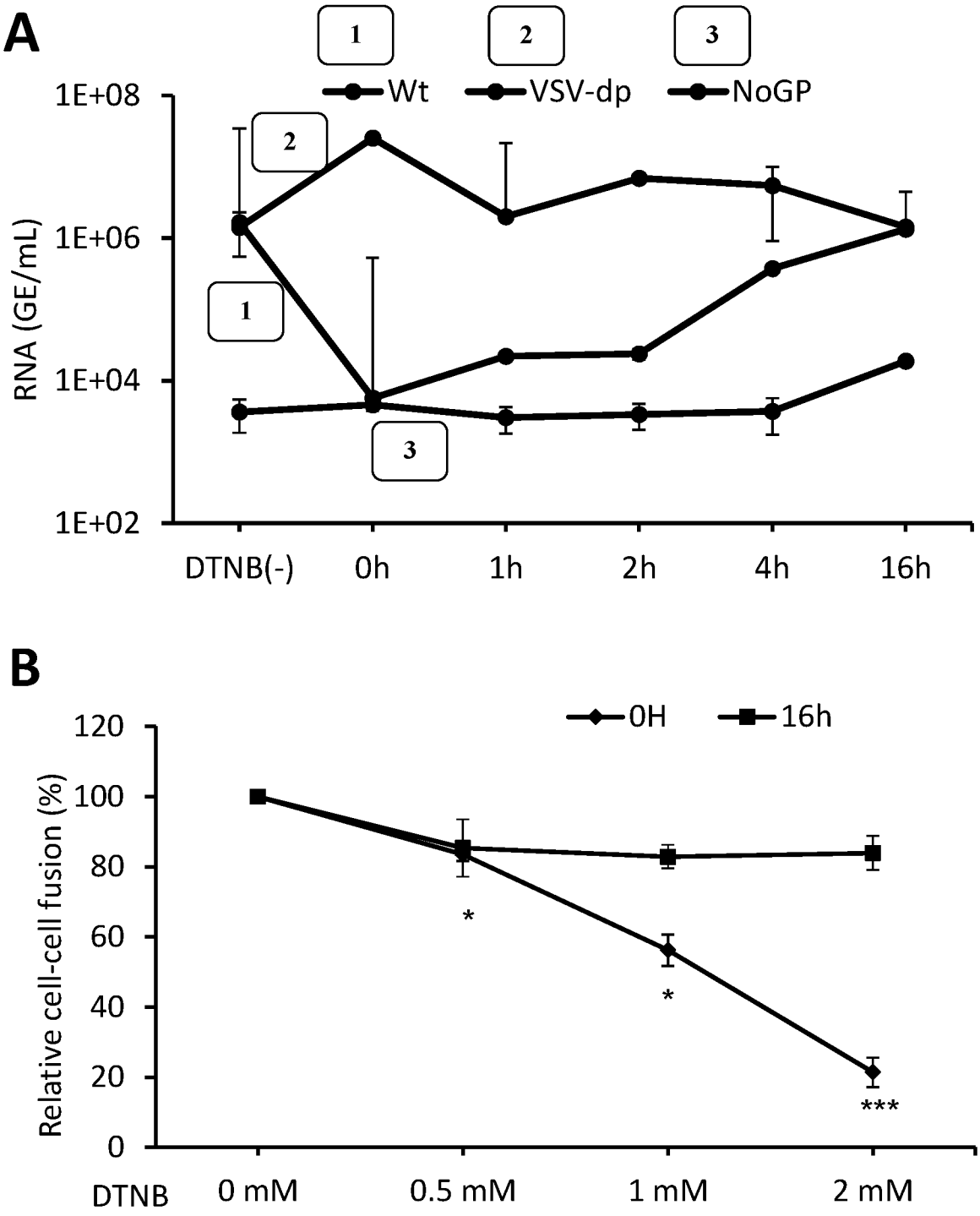
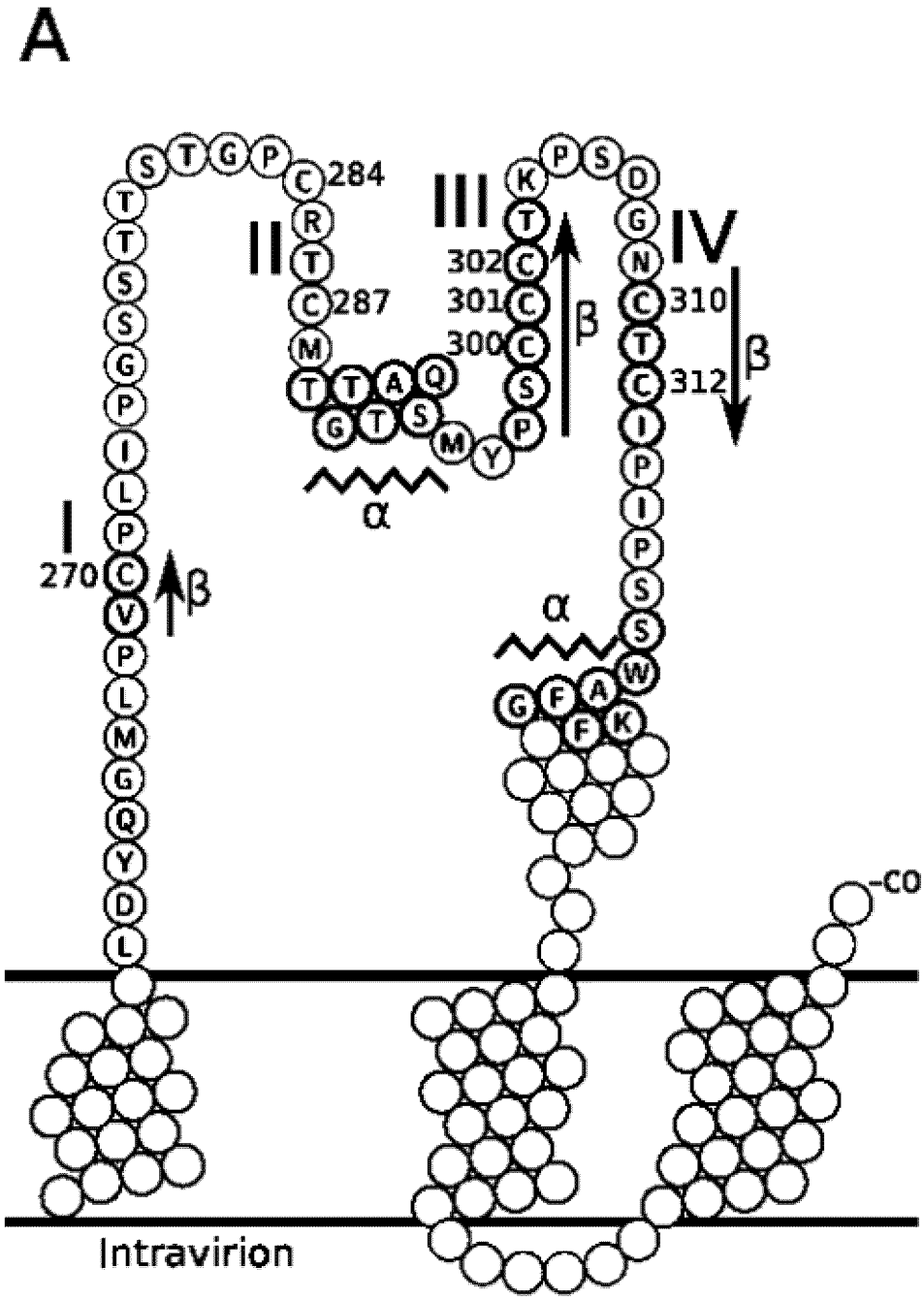



**Figure 2A and B**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β
II
284
287
α
III
302
301
300
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IV
310
312
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α
-CO
Intravirion

Figure 3A

B

| | | II | | | | III | | | IV | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 284 | 285 | 286 | 287 | 300 | 301 | 302 | 310 | 311 | 312 |
| I | 269 | 0.30 | 0.18 | 0.19 | 0.28 | 0.38 | 0.39 | 0.30 | 0.38 | 0.39 | 0.45 |
| | C270 | 0.47 | 0.23 | 0.26 | 0.43 | 0.53 | 0.58 | 0.47 | 0.57 | 0.40 | 0.57 |
| | 271 | 0.29 | 0.20 | 0.22 | 0.31 | 0.35 | 0.35 | 0.29 | 0.35 | 0.34 | 0.35 |
| II | C284 | | | | | 0.60 | 0.58 | 0.52 | 0.64 | 0.45 | 0.62 |
| | 285 | | | | | 0.40 | 0.38 | 0.30 | 0.36 | 0.27 | 0.34 |
| | 286 | | | | | 0.44 | 0.36 | 0.28 | 0.38 | 0.35 | 0.39 |
| | C287 | | | | | 0.58 | 0.50 | 0.40 | 0.49 | 0.35 | 0.58 |
| III | C300 | | | | | | | | 0.49 | 0.43 | **0.83** |
| | C301 | | | | | | | | 0.62 | 0.54 | **0.84** |
| | C302 | | | | | | | | **0.87** | **0.78** | **0.84** |

C

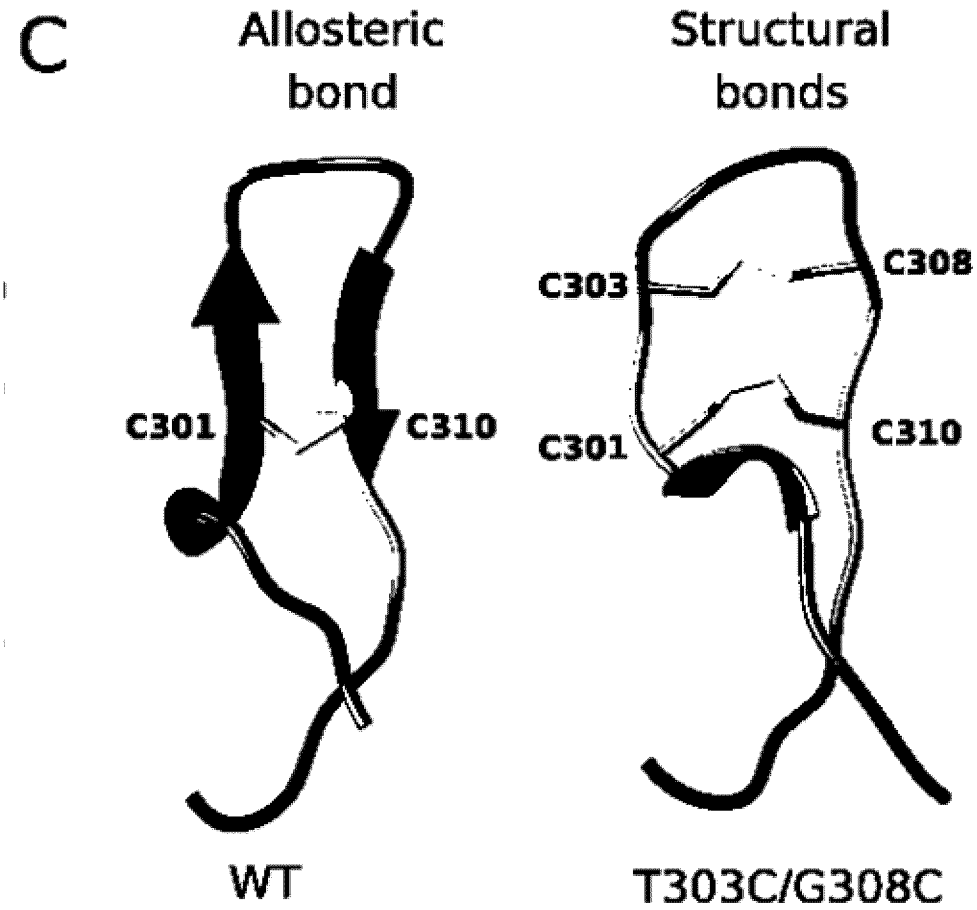



**Figure 3B and C**

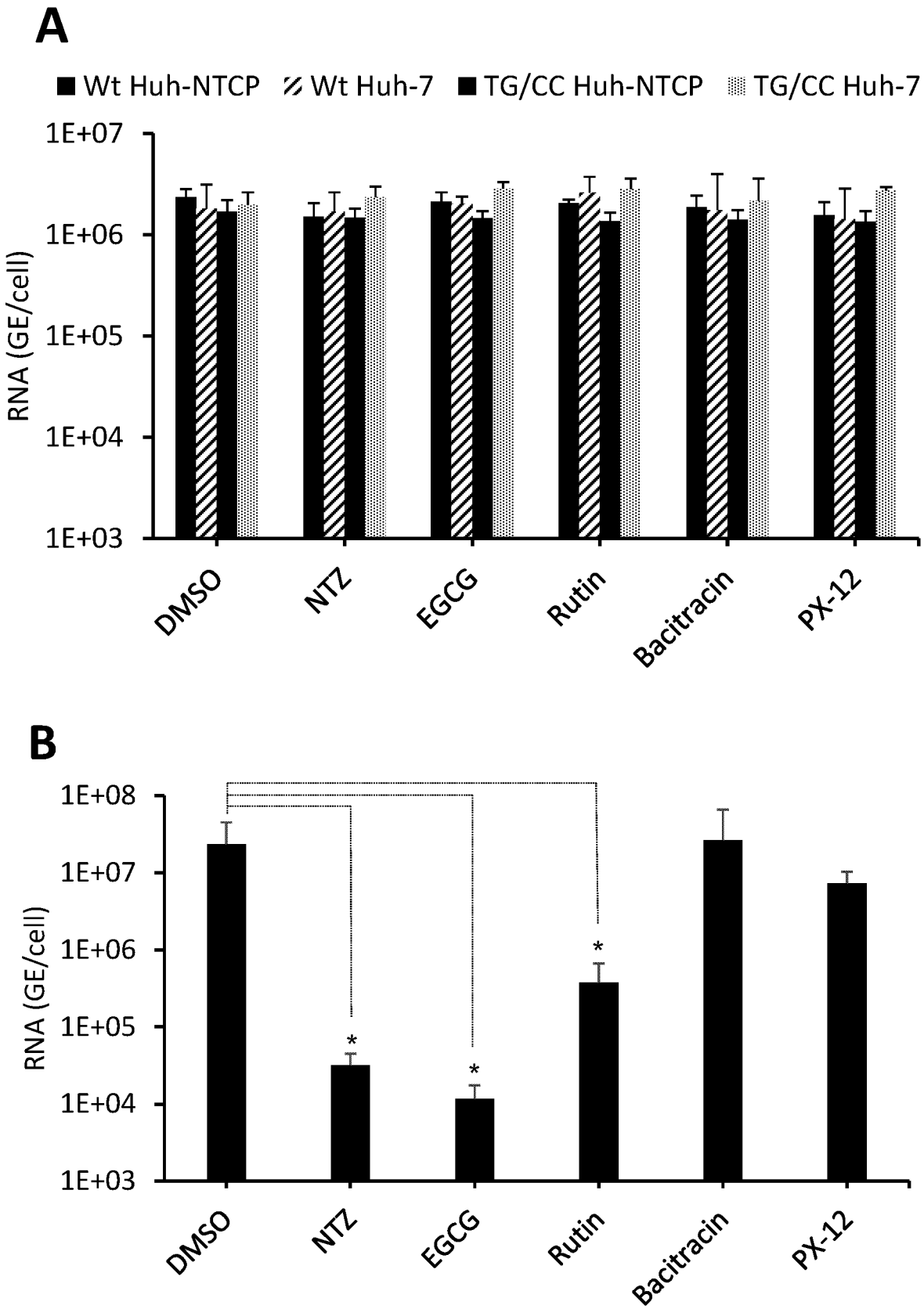



**Figure 4A and B**

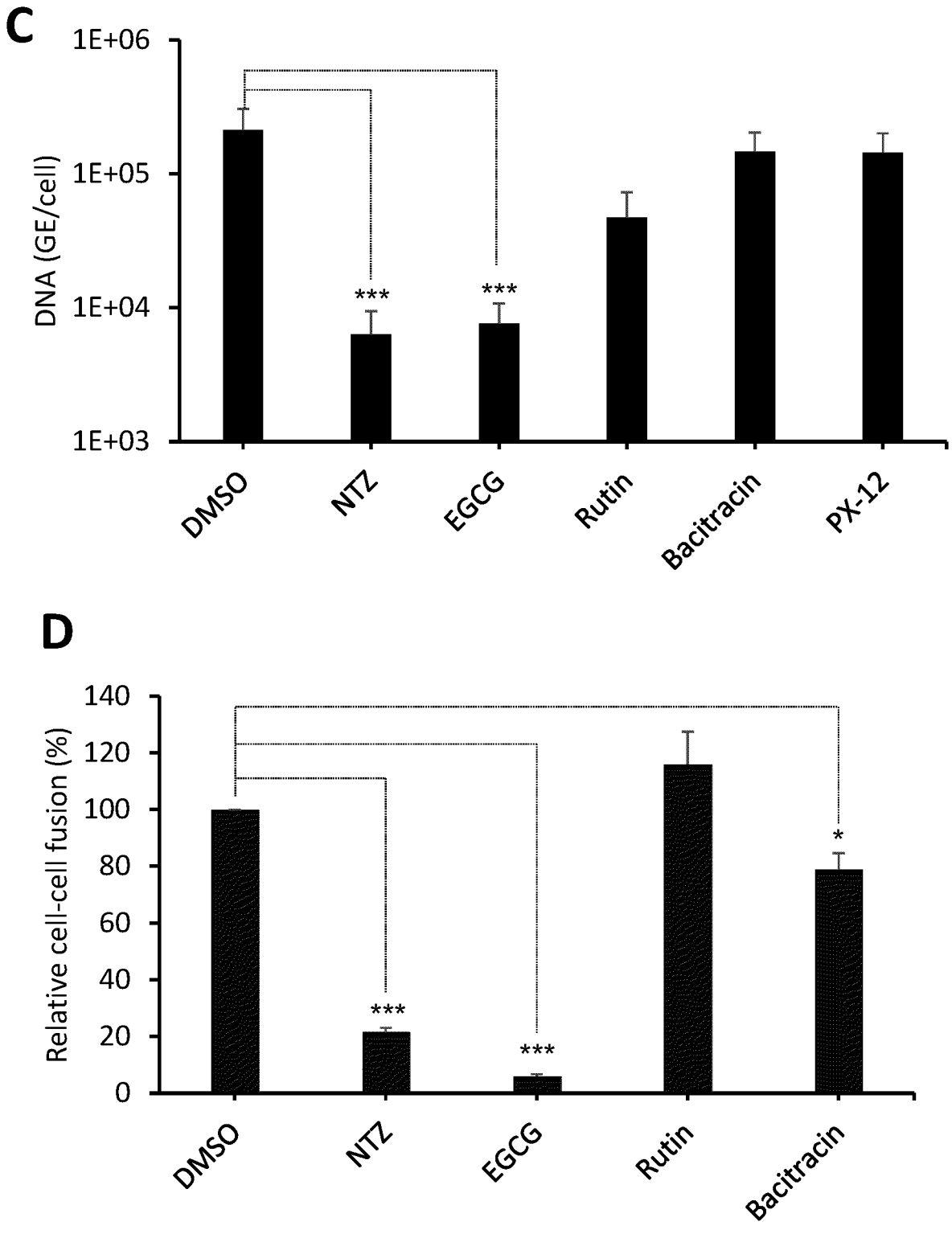



**Figure 4C and D**

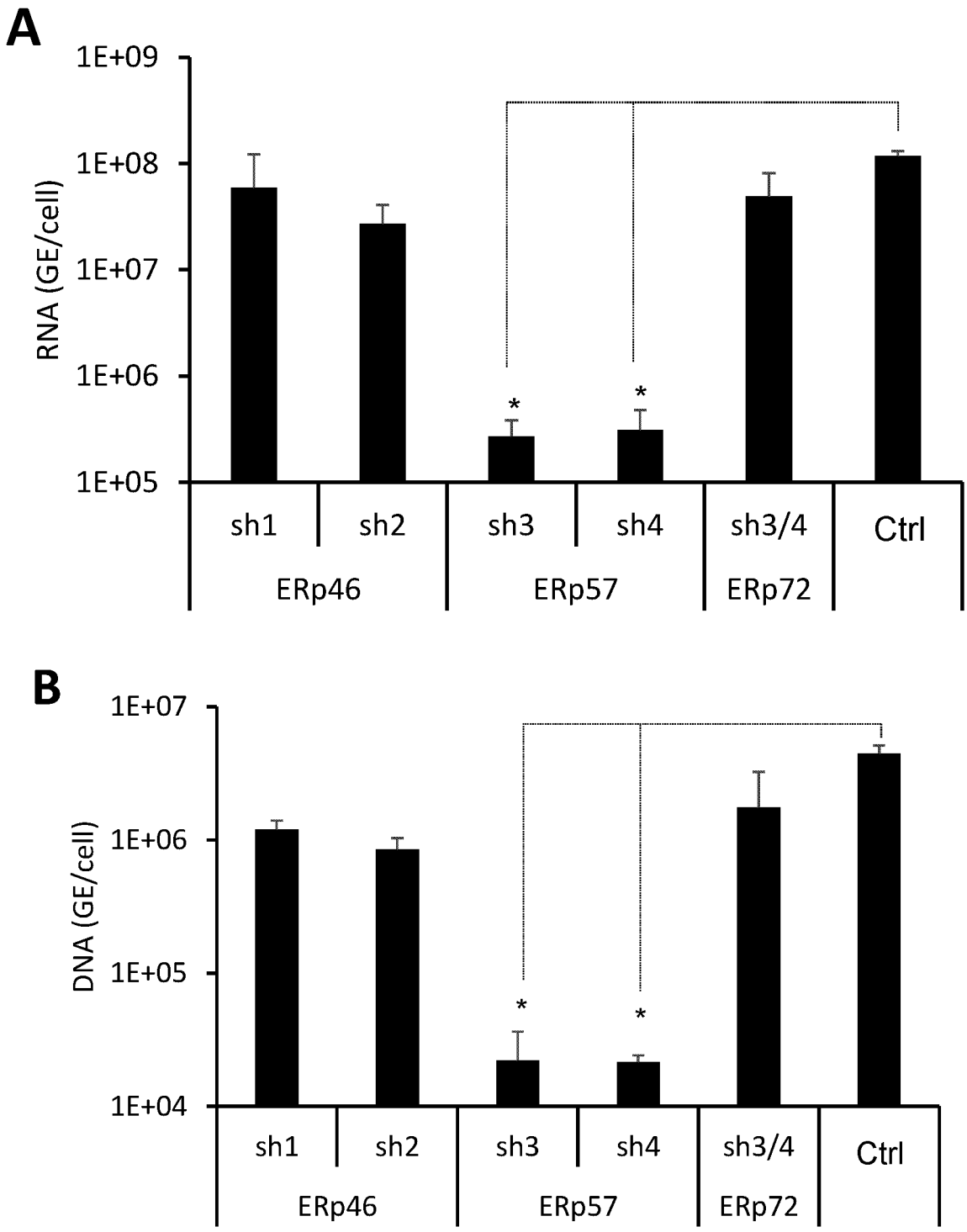
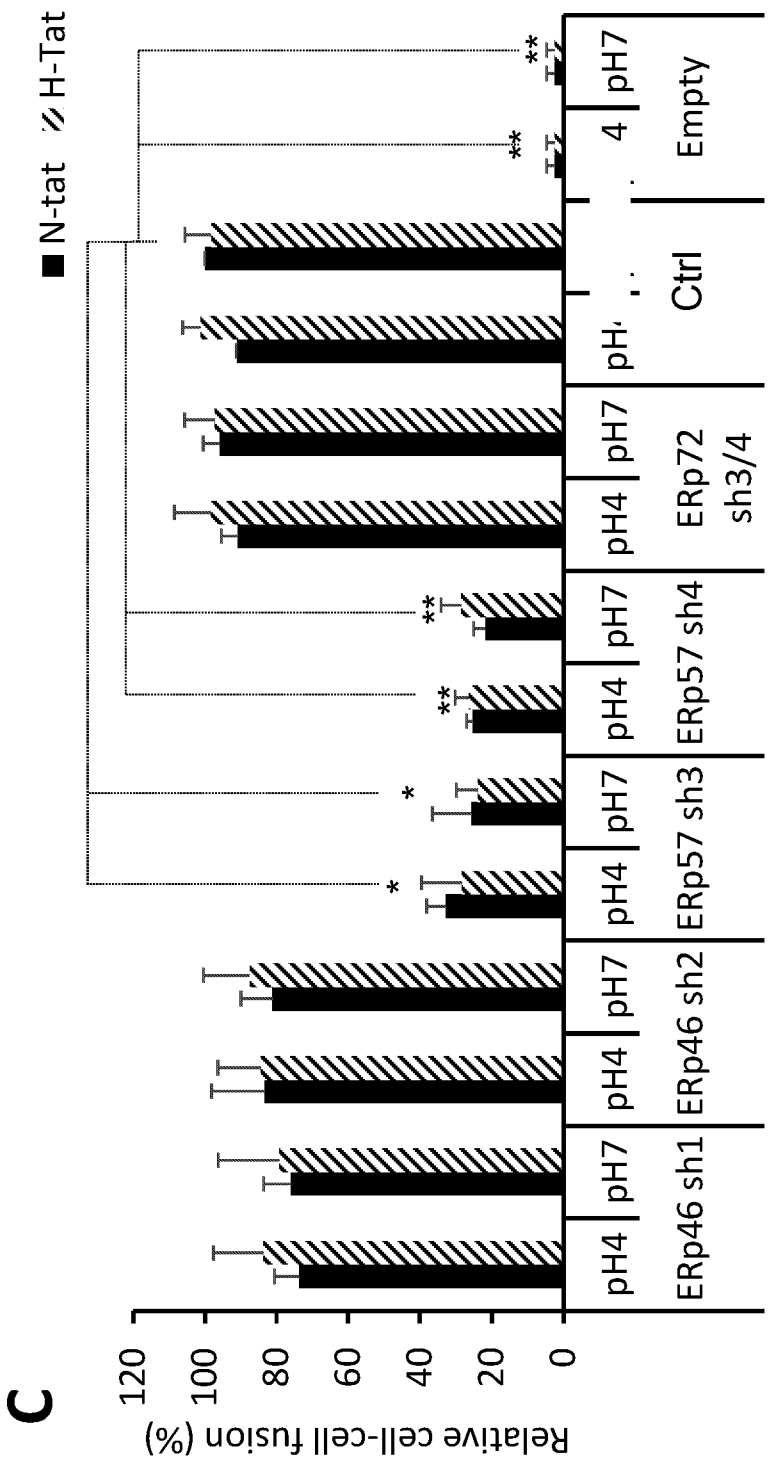



**Figure 5A and B**


C
Relative cell-cell fusion (%)
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100
80
60
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pH4
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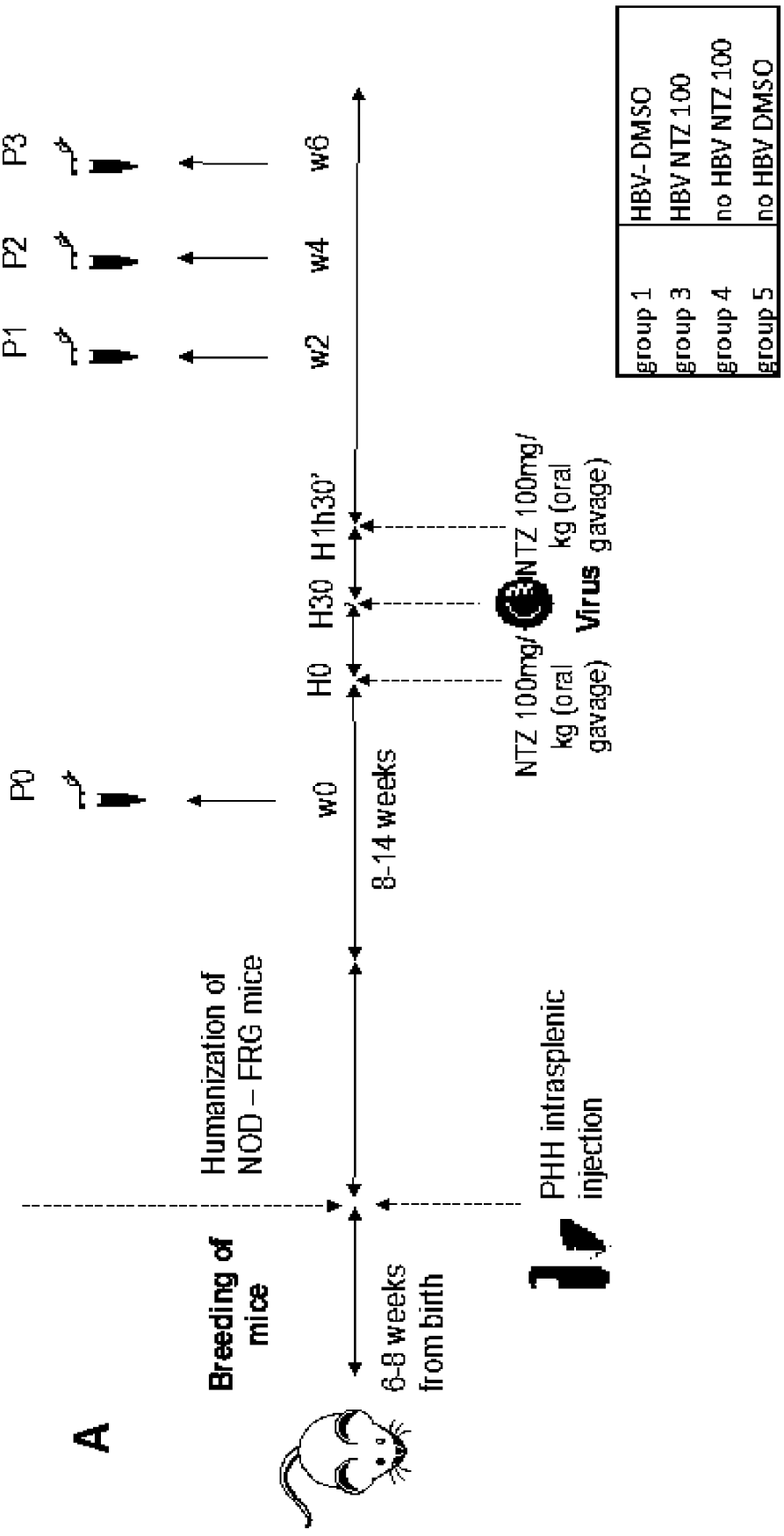
Figure 5C


A
Breeding of mice
6-8 weeks from birth
Humanization of NOD – FRG mice
PHH intrasplenic injection
8-14 weeks
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H0
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H1h30'
w2
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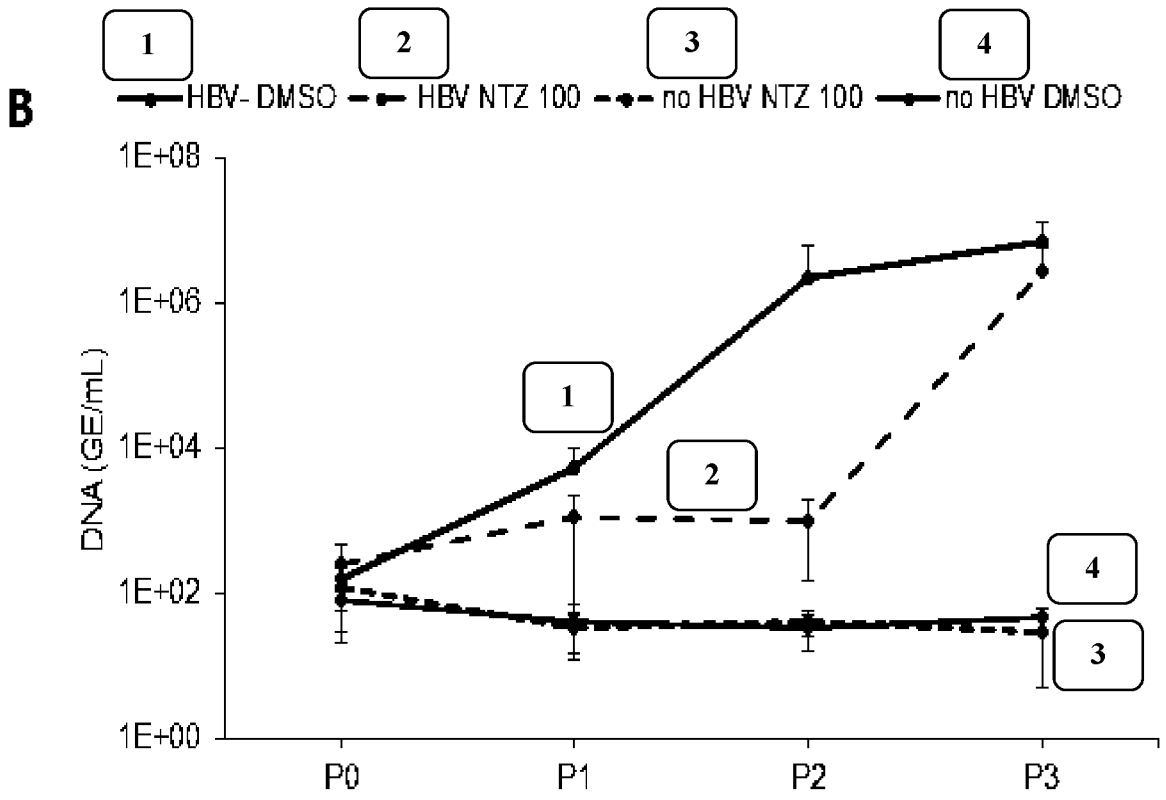
w6
P0
P1
P2
P3
NTZ 100mg/ kg (oral gavage)
NTZ 100mg/ kg (oral gavage)
Virus
group 1 HBV- DMSO
group 3 HBV NTZ 100
group 4 no HBV NTZ 100
group 5 no HBV DMSO

Figure 6A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DNA (GE/mL)
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**Figure 6B**

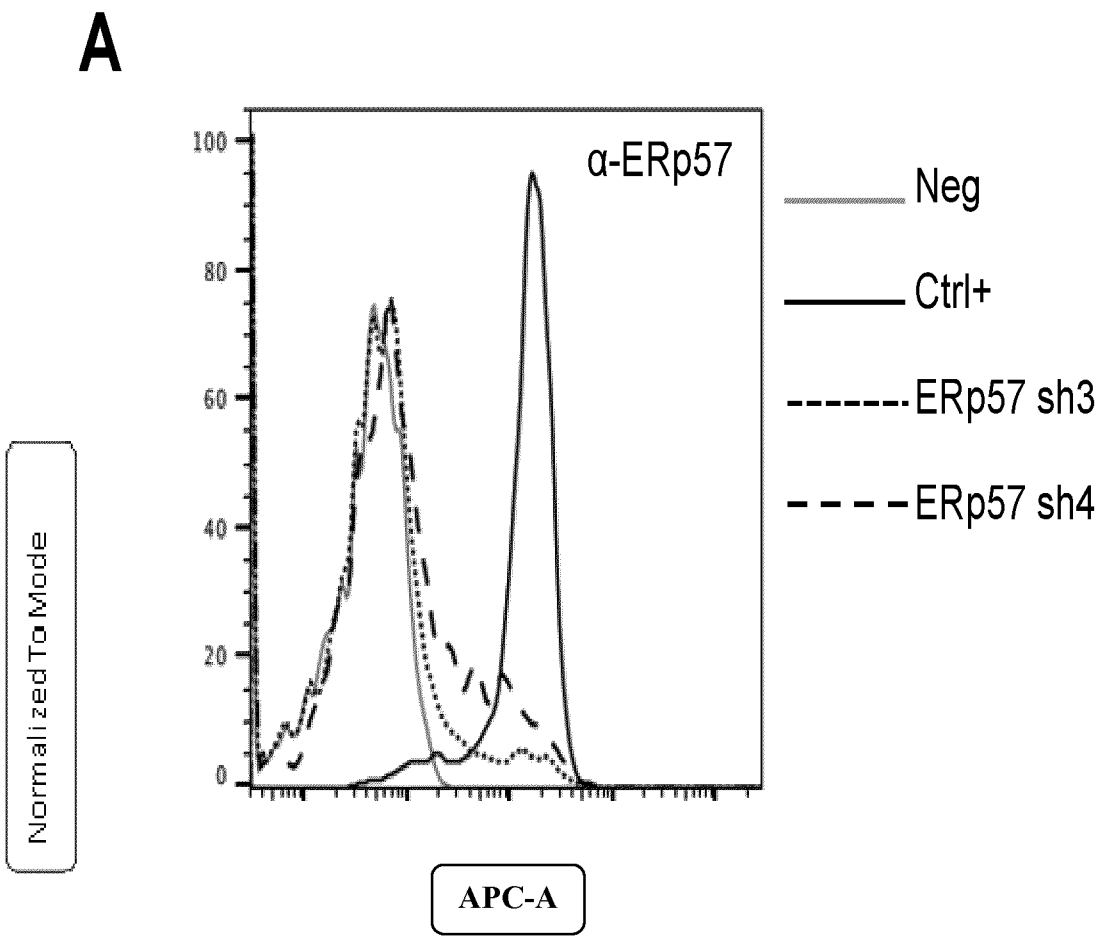
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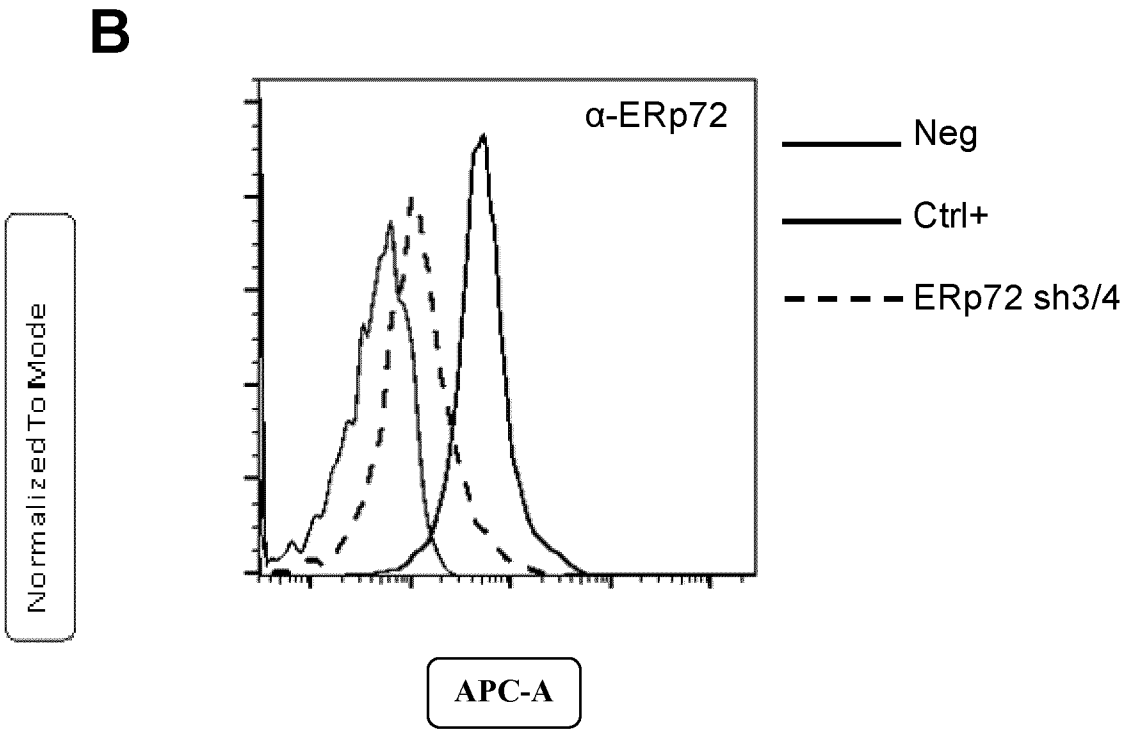
Figure 7A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Normalized To Mode
APC-A

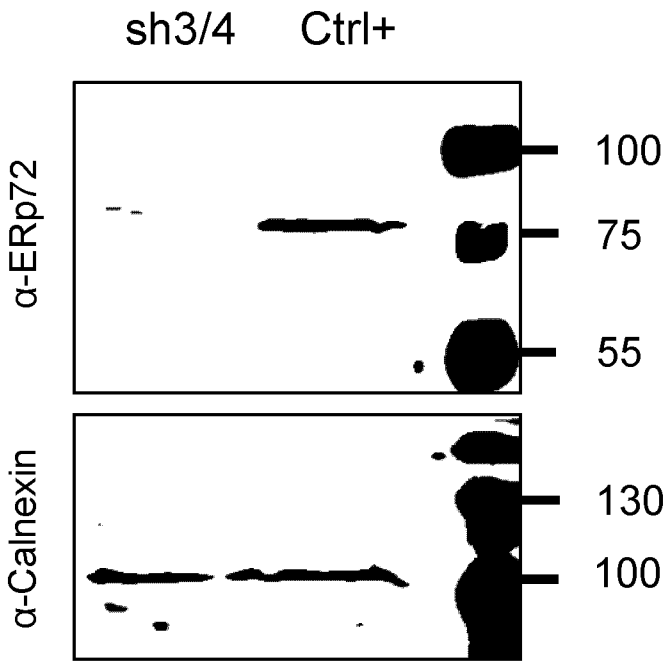
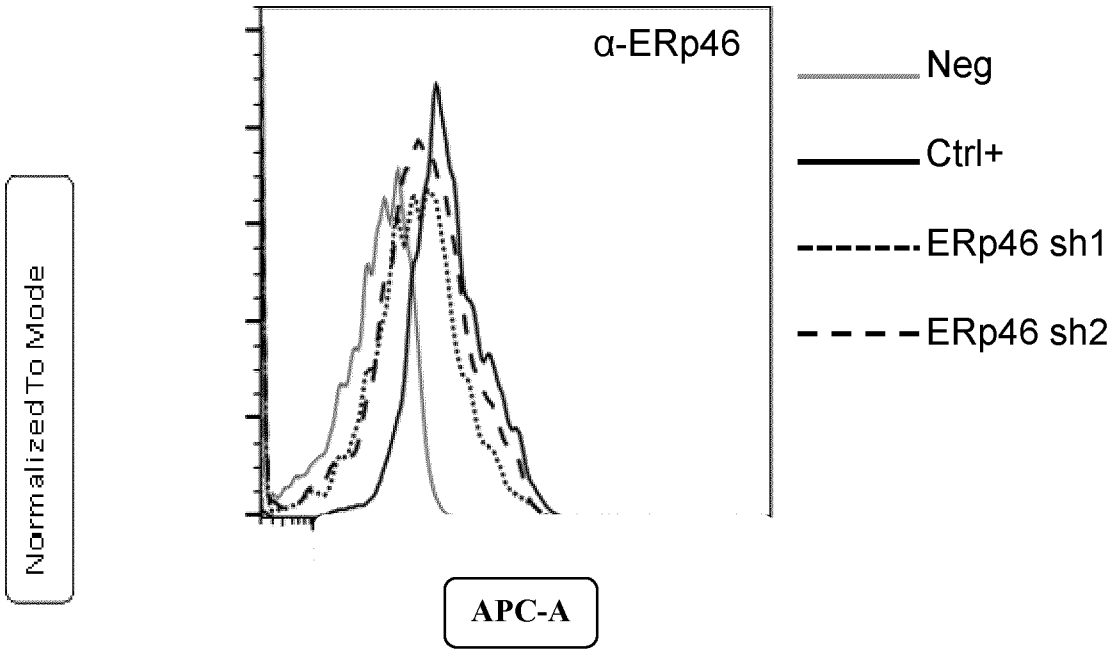

sh3/4
Ctrl+
α-ERp72
100
75
55
α-Calnexin
130
100

Figure 7B

C


α-ERp46
Neg
Ctrl+
ERp46 sh1
ERp46 sh2
Normalized To Mode
APC-A

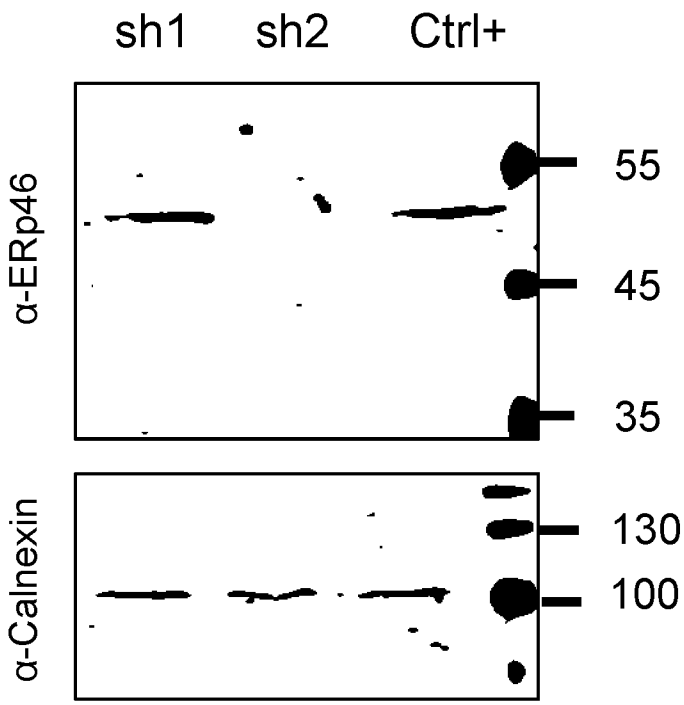

sh1
sh2
Ctrl+
α-ERp46
55
45
35
α-Calnexin
130
100

Figure 7C

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2022/059790

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      [ ] on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2022/059790

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P31/20 C12N15/113
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61P C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | YU-HENG LAI ET AL: "Epigallocatechin gallate inhibits hepatitis B virus infection in human liver chimeric mice", BMC COMPLEMENTARY AND ALTERNATIVE MEDICINE, BIOMED CENTRAL LTD, LONDON, UK, vol. 18, no. 1, 6 September 2018 (2018-09-06), pages 1-7, XP021260353, DOI: 10.1186/S12906-018-2316-4 | 1,4,5,8 |
| Y | the whole document | 3,5-7,9 |

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-/--

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

* Special categories of cited documents :

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

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 8 July 2022 | 21/07/2022 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Wiame, Ilse |

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KORBA ET AL: "Nitazoxanide, tizoxanide and other thiazolides are potent inhibitors of hepatitis B virus and hepatitis C virus replication", ANTIVIRAL RESEARCH, ELSEVIER BV, NL, vol. 77, no. 1, 1 January 2008 (2008-01-01), pages 56-63, XP022405848, ISSN: 0166-3542, DOI: 10.1016/J.ANTIVIRAL.2007.08.005 cited in the application the whole document<br>-----<br>LIU CHUNLAN ET AL: "Identification of hydrolyzable tannins (punicalagin, punicalin and geraniin) as novel inhibitors of hepatitis B virus covalently closed circular DNA", ANTIVIRAL RESEARCH, vol. 134, 1 October 2016 (2016-10-01), pages 97-107, XP055839619, NL ISSN: 0166-3542, DOI: 10.1016/j.antiviral.2016.08.026 the whole document<br>-----<br>PACELLO FRANCESCA ET AL: "An ERp57-mediated disulphide exchange promotes the interaction between Burkholderia cenocepacia and epithelial respiratory cells", SCIENTIFIC REPORTS , vol. 6, no. 1 1 August 2016 (2016-08-01), XP055839628, DOI: 10.1038/srep21140 Retrieved from the Internet: URL:https://www.nature.com/articles/srep21140.pdf cited in the application the whole document<br>-----<br>-/-- | 1,2,4,5,8<br>1,3-5,8<br>5,6 |

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | LIU MIAO ET AL: "Increased ERp57 Expression in HBV-Related Hepatocellular Carcinoma: Possible Correlation and Prognosis", BIOMED RESEARCH INTERNATIONAL , vol. 2017 1 January 2017 (2017-01-01), pages 1-8, XP055839613, ISSN: 2314-6133, DOI: 10.1155/2017/1252647 Retrieved from the Internet: URL:https://downloads.hindawi.com/journals/bmri/2017/1252647.pdf the whole document<br>-----<br>ABOU-JAOUDÉ GEORGES ET AL: "Entry of Hepatitis Delta Virus Requires the Conserved Cysteine Residues of the Hepatitis B Virus Envelope Protein Antigenic Loop and Is Blocked by Inhibitors of Thiol-Disulfide Exchange", JOURNAL OF VIROLOGY, vol. 81, no. 23, 26 September 2007 (2007-09-26), pages 13057-13066, XP055940415, US ISSN: 0022-538X, DOI: 10.1128/JVI.01495-07 Retrieved from the Internet: URL:https://journals.asm.org/doi/pdf/10.1128/JVI.01495-07> cited in the application the whole document<br>----- | 1-9<br><br>3,7,9 |