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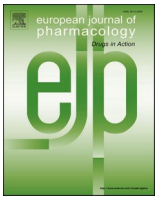

Studies in the antiviral molecular mechanisms of 25-hydroxycholesterol: Disturbing cholesterol homeostasis and post-translational modification of proteins

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ABSTRACT

Efficient antiviral drug discovery has been a pressing issue of global public health concern since the outbreak of coronavirus disease 2019. In recent years, numerous in vitro and in vivo studies have shown that 25-hydroxycholesterol (25HC), a reactive oxysterol catalyzed by cholesterol-25-hydroxylase, exerts broad-spectrum antiviral activity with high efficiency and low toxicity. 25HC restricts viral internalization and disturbs the maturity of viral proteins using multiple mechanisms. First, 25HC reduces lipid rafts and cholesterol in the cytomembrane by inhibiting sterol-regulatory element binding proteins-2, stimulating liver X receptor, and activating Acyl-coenzyme A: cholesterol acyl-transferase. Second, 25HC impairs endosomal pathways by restricting the function of oxysterol-binding protein or Niemann-pick protein C1, causing the virus to fail to release nucleic acid. Third, 25HC disturbs the prenylation of viral proteins by suppressing the sterol-regulatory element binding protein pathway and glycosylation by increasing the sensitivity of glycans to endoglycosidase. This paper reviews previous studies on the antiviral activity of 25HC in order to fully understand its role in innate immunity and how it may contribute to the development of urgently needed broad-spectrum antiviral drugs.

1. Introduction

Frequent outbreaks of deadly viruses have become a global public health crisis, leading to an urgent need for the development of effective antiviral drugs. Viral infection is a complex process involving attachment, entry, synthesis, assembly, and release, and each of these steps relies on the lipidomic reprogramming of host cells to facilitate the formation of viral vesicles, the maturation of viral proteins, and their subsequent assemble (Yuan et al., 2019). Exploration of the molecular mechanisms of drugs contributes to understanding the relationship between cellular metabolism and pharmaceutical activity and provides a foundation for the development of new antiviral targets and methods for drug screening.

Previous studies have shown that lipid rafts, which are microdomains in the cytomembrane, are essential for almost all viral invasions, such as Ebola, influenza, and human immunodeficiency virus (HIV) (Campbell et al., 2001; Jin et al., 2020; Mercer et al., 2010; Takeda, 2004). Most enveloped viruses favor binding to the related receptor using spike proteins to attach to the host cell. The physical binding of these glycoprotein (GP) receptors to lipid rafts is critical for the promotion of viral fusion (Vitiello et al., 2015). Some enveloped and all naked viruses invade the host cell through endocytosis, which requires lipid rafts to recruit clathrin-coated pits for the formation of virion-containing vesicles (Harmon et al., 2012; Parton and Richards, 2003). Most of these virion-containing vesicles fuse with acidic environmental endosomes, such as late endosome (LE) and lysosome (LY), using the endolysosomal system to degrade the capsid and cause the release of viral nucleic acid into the cytoplasm (Sanchez et al., 2017).

Cholesterol accounts for 20%–30% of a membrane lipid and is a critical component of the formation of membrane rafts. The hydrophobic rigid structure of cholesterol promotes the stacking of the membrane, and cytomembrane fluidity is determined by the concentration of cholesterol in the membrane. A cholesterol-rich cytomembrane is required for the formation of lipid rafts that promote signal transduction of the receptor, which contributes to viral entry, replication, and budding (Barman and Nayak, 2007; Yamakawa et al., 2021). However, the formation of rafts and the aggregation of clathrin-coated pits can be inhibited if there is a depletion of cholesterol in the membrane (Barman and Nayak, 2007; Li et al., 2021; Subtil et al., 1999). Cholesterol homeostasis is crucial for maintaining the normal amount of lipid raft in

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the cell membrane and the regular function of the endolysosomal system.

25-Hydroxycholesterol (25HC), an endogenous oxysterol, is generated in macrophages and is induced by the pathway of type I interferons (IFN). 25HC restricts the activity of various viruses using multiple mechanisms (Fig. 1) (Blanc et al., 2013; Liu et al., 2013). As a potent reactive oxysterol, 25HC alters sub-domains of the membrane by interacting with the lipid bilayer (Massey, 2006). In recent years, an increasing number of studies on the antiviral mechanisms of 25HC have focused on its impact on intracellular lipid metabolism (Cao et al., 2020) and have revealed that it perturbs intracellular cholesterol homeostasis by affecting synthesis, metabolism, and transport (Anggakusuma et al., 2015; Civra et al., 2018; Saulle et al., 2020; Wang et al., 2020). While there have been a few studies on the mechanism by which 25HC disturbs post-translational modifications (PTMs), these studies explored the pathway of proteinic glycosylation in general and are only confirmed the effectiveness of the mechanism on a few viruses, such as the Lassa fever virus (LASV) (an adenovirus) (Shrivastava-Ranjan et al., 2016).

Herein, previous studies on the mechanism of how cholesterol-25-hydroxylase (CH25H) and 25HC disturb cholesterol homeostasis and inhibit viruses directly were reviewed to provide basics for the development of antiviral candidates and reference for understanding the related immunology.

2. CH25H is a key enzyme for the production of 25HC

The human CH25H gene lacks an intron and is located in 10q23 of the chromosome-encoding multiple transmembrane GP, CH25H (Lund et al., 1998). During the catalyzation of CH25H, cholesterol is converted to 25HC by the hydroxylation of C_{25} in the endoplasmic reticulum (ER) (Nitta et al., 2018; Russell, 2000). There are three clusters of histidine residues in the CH25H of all vertebrates: cluster 1 (Trp-His-Leu/-Val-Leu/Val-His-His) for residues 142–148, cluster 2 (Phe/Ile--His-Lys-Val/Met/Leu-His-His) for residues 157–162, and cluster 3 (His-His-Asp-Leu/Met-His-His) for residues 238–244 (Holmes et al., 2011). These three clusters are wrapped in an ER membrane and are essential for the enzymatic activity of CH25H that catalyzes cholesterol to 25HC (Fox et al., 1994).

Generally, CH25H cannot be detected in most tissues because of its low content. However, the expression of CH25H is drastically increased by the stimulation of multiple Toll-like receptors ligands or IFN-signaling pathways in various organs, such as the liver, heart, brain, muscle, kidneys, and lungs (Chen et al., 2014). Heightened expression of CH25H has been found in macrophages, particularly in human CD206+ of visceral adipose tissue and in alveolar macrophages (Madenspacher et al., 2020; Russo et al., 2020). It has also been reported that high expression of CH25H in liver tissue produces more 25HC, which improves sensitivity to insulin and enables the level of CH25H in the liver to be a risk marker for unhealthy obesity (Noebauer et al., 2017). Furthermore, as a factor involved in neoplastic metastasis, the expression of CH25H in cells may be a predictor of the metastasis of advanced cancers (Mittempergher et al., 2013).

After viral infection, IFN-stimulated genes are substantially expressed by immune cells to inhibit viral replication. As a protein induced by IFN-stimulated genes, CH25H is a critical endogenous substance that is directly engaged in the inhibition of viral replication

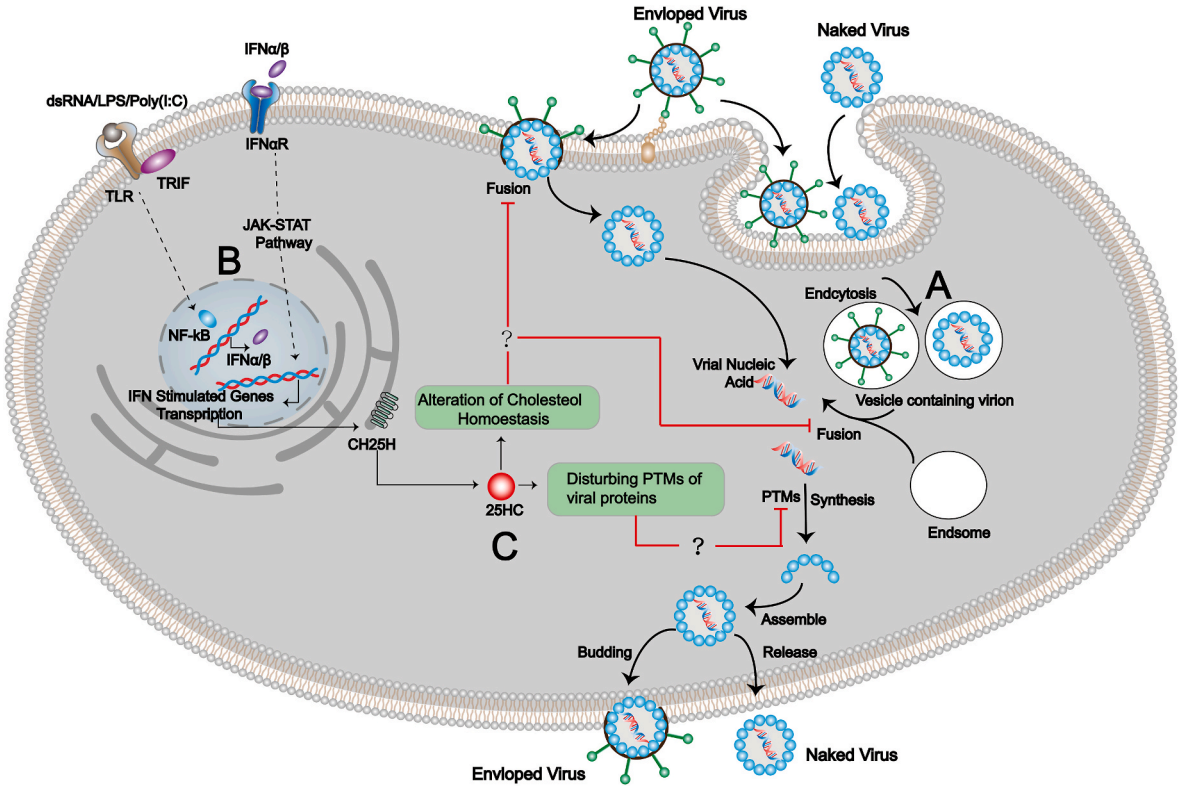

Fig. 1. An overview of the antiviral pathway of 25-hydroxycholesterol (25HC). A: Process of viral infection. Enveloped viruses invade through fusion and endocytosis, whereas naked viruses enter the cell through endocytosis. The vesicles of some viruses rely on the endosomal pathway, which requires fusion with late endosome/lysosome to release viral nucleic acid into the cytoplasm. Subsequently, viral nucleic acid synthesizes proteins to assemble virions, a process in which some proteins mature through post-translational modifications (PTMs). B: Activation of interferon (IFN) stimulated genes-cholesterol-25-hydroxylase (CH25H) antiviral pathway relying on the IFN signaling pathway. After induction by viral dsRNA/LPS/Poly (I:C), Toll-like receptors 3/4 recruits an adaptor protein, Toll/IL-1R domain-containing adaptor-inducing IFN β, to dimerize with it and stimulate expression of IFN α/β. IFN α/β binds to the IFN α receptor and promotes the expression of CH25H via the JAK-STAT pathway to catalyze cholesterol to 25HC. C: Antiviral pathways of 25HC. First, 25HC alters cholesterol homeostasis to impede viral internalization and the release of viral nucleic acids. Second, 25HC disturbs the PTMs of proteins, causing the formation of abnormal viral protein.

(Fig. 1) (Doms et al., 2018; Li et al., 2017). The overexpression of CH25H in cells has been found to have a conspicuous effect on the infection of porcine reproductive and respiratory syndrome virus (PRRSV) (Song et al., 2017). In the same way, a CH25H mutant lacking hydroxylase activity can still inhibit PRRSV infection. In addition to the antiviral role of 25HC, it has been shown that porcine CH25H inhibits PRRSV by degrading non-structural protein-1α via the ubiquitin-proteasome pathway (Ke et al., 2017). In addition, CH25H has been found to selectively interact with and degrade viral 3D protein of RNA-dependent RNA polymerase by acting independently on the association of proteasomes, LY, and caspases in the encephalomyocarditis virus (Li et al., 2020).

3. Regulation of intracellular cholesterol levels by 25HC

3.1. Cholesterol synthesis

Hepatocellular ER is a primary site for cholesterol synthesis in vivo. Acetyl-coenzyme A, a metabolite of some fatty acids, is essential for the synthesis of isoprene units by the mevalonate pathway; the isoprene units are then used to further synthesize sterol and terpene (Tricarico et al., 2015). The rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) greatly impacts intermediates and intracellular cholesterol levels during the process of cholesterol synthesis (Burg and Espenshade, 2011; Hinson et al., 1997). The intermediates, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), are indispensable for the prenylation of intracellular proteins (Fig. 2) (Berndt et al., 2011).

It was firstly reported that the sterol-regulatory element-binding proteins (SREBP)-2 interact with sterol regulatory elements 1 to stimulate expression of the HMGCR gene in 1993 (Hua et al., 1993). SREBP cleavage-activating protein (SCAP) is separated from the complex between insulin-induced genes (INSIGs) proteins and SCAP–SREBP to escort SREBP from the ER to the Golgi apparatus once cholesterol in the ER membrane is less than 5%. Mature SREBP is produced by two key enzymes in the Golgi apparatus—S1P and S2P—and stimulates the expression of HMGCR and low-density lipoprotein (LDL) receptor to restore cholesterol levels. Conversely, cholesterol detains SCAP by binding with it to restrict the expression of HMGCR once cholesterol in the ER membrane is higher than 5% (Fig. 3) (Bielska et al., 2014; Cyster et al., 2014; Zhao et al., 2020).

Like most oxysterols, 25HC can restrict SREBP maturity (Nohturfft et al., 1996; Yang et al., 1995). Furthermore, it has been suggested that 5-Cholesten-3b,25-Diol,3-Sulfate, produced by cholesterol catalyzed by sulfotransferase-2B1b, reduces the expression of SREBP-1, acetyl-coenzyme A carboxylase-1, and fatty acid synthase, while lowering the expression of liver X receptor (LXR) (Figs. 2 and 3) (Ma et al., 2008; Ren et al., 2007).

3.2. Cholesterol metabolism

Under the effect of various enzymes, cholesterol is metabolized to different oxysterols, including 25HC, 24HC, and 27HC, which are ultimately utilized to synthesize bile acid (Cyster et al., 2014; Russell, 2000). Still, significant levels of 25HC have been found in several tissues of *CH25H*$^{-/-}$ mice, which indicates that the formation of 25HC is involved in other critical enzymes, including CYP27A1, CYP3A4, and CYP46A1 (Diczfalusy, 2013; Diczfalusy and Bjorkhem, 2011). Additionally, much 25HC is derived from CYP3A4 in vivo, especially in cell lines from liver and plasma of mouse (Honda et al., 2011; Nitta et al., 2018). Besides cholesterol transforming to 27HC and 24HC through CYP27A1 and CYP46A1, respectively, some 25HC is produced in both pathways (Figs. 2 and 3) (Honda et al., 2011).

Like most oxysterols, 25HC promotes expression of CH25H and CYP3A4 by stimulating LXR to form a heterodimer with retinoic acid receptors (RXR) (Figs. 2 and 3) (Ren and Ning, 2014). Furthermore, acyl-coenzyme A: cholesterol acyl-transferase (ACAT), an ER-resident enzyme responsible for esterification of cholesterol to cholesteryl ester (CE), is activated by 25HC to impact cholesterol levels in the ER (Fig. 3). Normally, esterification contributes to the preservation of CE in lipid droplets to avoid the cytotoxicity caused by cholesterol accumulation (Du et al., 2004).

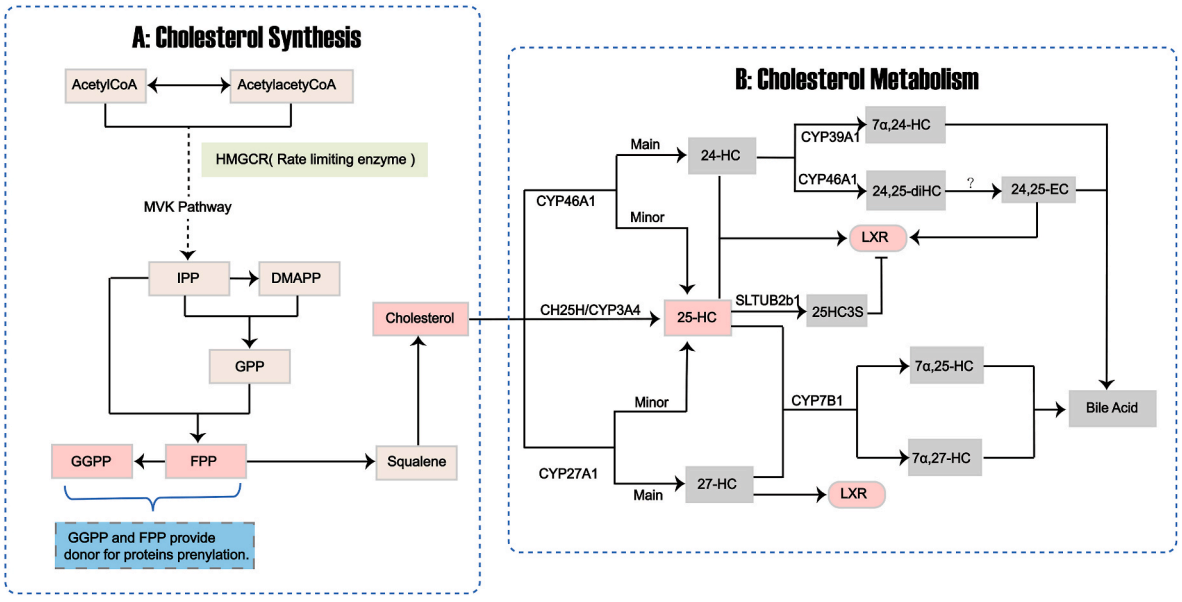

Fig. 2. The synthesis and metabolism of cholesterol. A: Cholesterol synthesis. Fatty acid metabolites coenzyme A synthesizes isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) in the mevalonate pathway. Geranyl pyrophosphate (GPP), synthesized by the condensation of IPP and DMAPP, combines with IPP to synthesize farnesyl pyrophosphate (FPP) further. Bimolecular FPP is condensed into squalene to synthesize more cholesterol and is also converted to geranylgeranyl pyrophosphate (GGPP) in the presence of a relevant enzyme. B: Cholesterol metabolism. There are four primary enzymes involved in the generation of 25-hydroxycholesterol (25HC), including CYP46A1, cholesterol-25-hydroxylase (CH25H), CYP3A4, and CTP27A1. 25HC is produced mainly by CH25H and CYP3A4. In addition to producing some 25HC, CYP46A1 and CYP27A1 primarily generate 24HC and 27HC, respectively. 25HC, 24HC, 27HC, and 24(S), 25-epoxycholesterol (24,25-EC) are all liver X receptor (LXR) agonists, while 25HC3S converted from 25HC inhibits the expression of LXR.

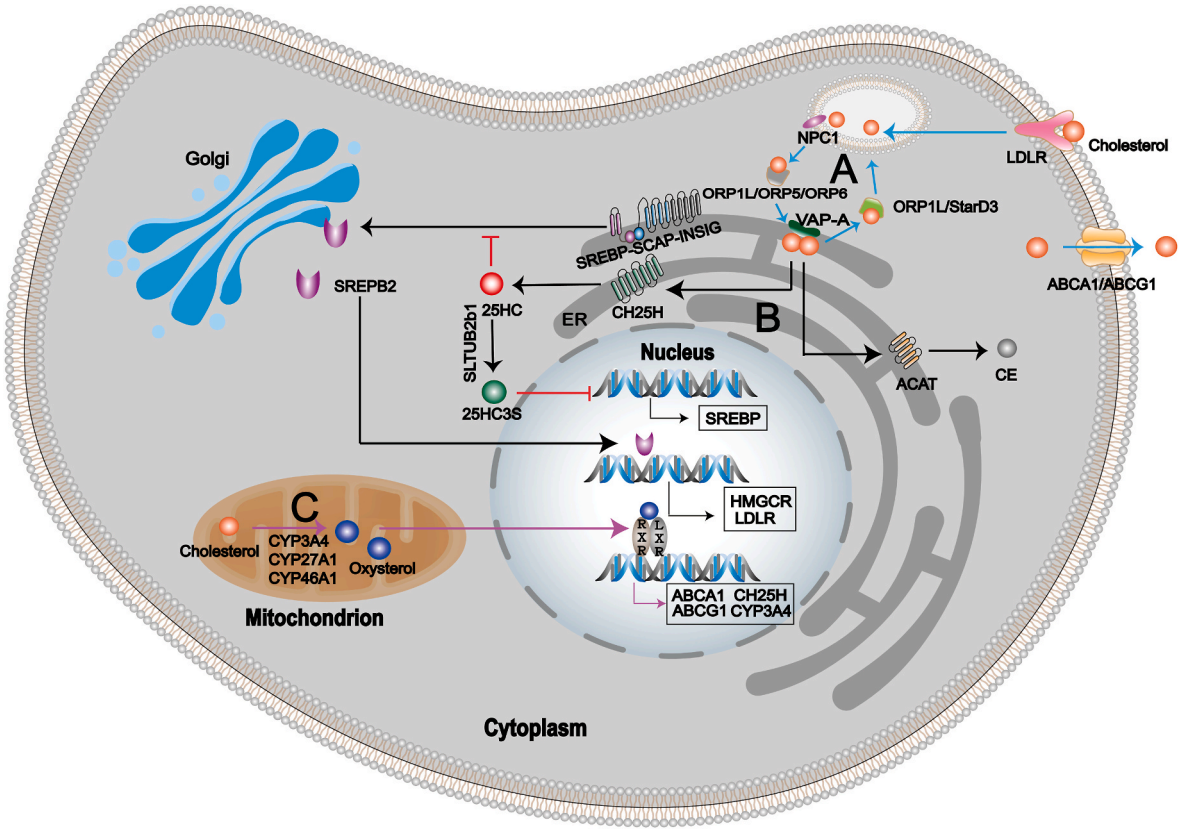

Fig. 3. The transport of cholesterol in cell. A: Extracellular cholesterol entry into cells by combination with low-density lipoprotein receptor (LDLR). Transport of cholesterol from late endosome (LE)/lysosome (LY) to the endoplasmic reticulum (ER) requires ORP1L, ORP5, or ORP6 as sterol transfer proteins to combine with vesicle-associated membrane protein-associated protein (VAP-A). The cholesterol in the ER is transported to LE/LY by ORP1L or StarD3. Niemann–Pick protein C1 (NPC1) is responsible for the exportation of cholesterol from LE/LY, and adenosine triphosphate-binding cassette (ABC)A1/ABCG1 is responsible for the efflux of intracellular cholesterol (blue lines). B: The cholesterol in the ER is transformed into 25-hydroxycholesterol (25HC) by cholesterol-25-hydroxylase (CH25H) and then inhibits the maturity of sterol-regulatory element-binding proteins (SREBP) and reduces the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) and LDLR by hindering the separation of SREBP cleavage-activating protein (SCAP) and insulin-induced genes (INSIGs) protein. 25HC is catalyzed by SLTUB2b1 into 25HC3S to further inhibit the expression of SREBP. The cholesterol in the ER is also transformed into cholesteryl ester (CE) by acyl-coenzyme A: cholesterol acyl-transferase (ACAT) (black and red lines). C: Oxysterols oxidized from cholesterol in mitochondrion enter into the nucleus to stimulate expression of ABCA1/ABCG1, CH25H, and CYP3A4 by activating liver X receptor (LXR) (magenta lines).

3.3. Transport of cholesterol

Intracellular cholesterols distribute unevenly in organelles and are transported by vesicular and non-vesicular pathways. Extracellular cholesterol transfers into cells through the formation of LDL vesicles after binding with LDL receptor (Fig. 3) (Du et al., 2015). As opposed to vesicular trafficking, where cholesterol is conveyed as a membrane component of transport vesicles, non-vesicular trafficking requires cholesterol binds with sterol transfer proteins (STPs) as an important way for cholesterol transport between organelles, such as from LY/LE to ER (Luo et al., 2019). When the cholesterol levels in LE/LY are low, the cholesterol in the ER is transferred to LY/LE through STPs, including ORP1L and StarD3, for the vesicle-structural formation of LY/LE (Fig. 3) (Ridgway and Zhao, 2018). In the reverse direction, the transport of cholesterol from several organelles or LY/LE to the ER is dependent on the combination of vesicle-associated membrane protein-associated protein A (VAP-A) on the ER membrane and diverse oxysterol-binding proteins (OSBP), including ORP1L, ORP5, and ORP6 (Wyles et al., 2002). In addition, the exportation of cholesterol in LE/LY is the responsibility of a membrane-bound protein, Niemann–Pick protein C1 (NPC1) (Luo et al., 2019). In the presence of excessive intracellular cholesterol, cholesterol is transported out of the cell by adenosine triphosphate-binding cassette (ABC) transporters on the cytomembrane, such as ABCA1 and ABCG1 (Fig. 3) (Schmitz and Kaminski, 2001).

It has been reported that 25HC hinders cholesterol transport from LY/LE to the ER by blocking the association of OSBP and VAP-A (Civra et al., 2018) and is capable of stimulating the expression of ABCA1/ABCG1 by activating LXR (Aye et al., 2010; Sparrow et al., 2002). Furthermore, 25HC competes with cholesterol to bind with NPC1 to reduce the exportation of cholesterol in LE/LY (Kwon et al., 2009).

4. 25HC inhibits viruses using multiple molecular mechanisms

A majority of enveloped and non-enveloped viruses are inhibited by 25HC. It has been reported that 25HC significantly inhibits the invasion of Zika virus (ZIKV) and alleviates inflammatory responses to it, such as viremia and infection in cortical tissue, resulting in no obvious cytotoxicity with a concentration of 25HC of up to 10 μM (Li et al., 2017). In addition, the use of 25HC in combination with C34 (a peptide-inhibiting fusion between virus and cell) may suppress the infection of drug-resistant viruses, which may be a result of the altered properties of the cytomembrane after interacting with the membrane's lipid rafts (Gomes et al., 2019). It has also been reported that 25HC affects the biogenesis or integrity of the membrane web of the host cell, inhibiting the membrane rearrangements caused by hepatitis C virus (HCV) infection, whereas the molecular mechanism of how 25HC disturbs structure of the membrane has yet to be demonstrated (Anggakusuma et al., 2015). In recent years, many studies have provided evidence of the antiviral function of 25HC for other viruses, such as avian leukosis virus subgroup J (Xie et al., 2019), bovine parainfluenza virus type 3 (Lv et al.,

2019), PRRSV (Ke et al., 2017), HIV (Gomes et al., 2018), and severe fever with thrombocytopenia syndrome virus (Tani et al., 2016).

Multiple antiviral molecular mechanisms of 25HC synergistically function during the viral infection process. Herein, the mechanisms of 25HC are classified into two categories in accordance with the alteration of different molecular metabolisms in the cell: alteration of cholesterol homeostasis and disturbance of proteinic PTMs (Cao et al., 2020; Raniga and Liang, 2018).

4.1. Alteration of cholesterol homeostasis

The first category of antiviral mechanisms of 25HC are those that affect cholesterol levels, which include those that reduce cholesterol and the amount of membrane raft (a microdomain constituted of cholesterols and sphingolipids) in the cytomembrane and those that lead to an accumulation of cholesterol in LE/LY. First, 25HC affects the lipid composition of the membrane through three mechanisms: reduction of cholesterol synthesis by inhibiting the maturity of HMGCR, augmentation of cholesterol efflux by stimulating the expression of ABCA1/ABCG1, and promotion of cholesterol esterification by activating ACAT. Second, 25HC leads to the accumulation of cholesterol in LE/LY by restricting the function of the two translocators responsible for cholesterol: OSBP and NPC1 (Fig. 4).

4.1.1. Inhibition of cholesterol synthesis

INSIGs proteins are ER-resident sterol sensor responsible for intracellular sterol levels and can be divided into two subtypes, INSIGs-1 and INSIGs-2 proteins, both of which exist as a homotrimer. Each protomer consists of six transmembrane segments (TM). INSIGs-1 proteins destroy HGMCR by recruiting protein-degradation machinery to reduce cholesterol synthesis (Ren et al., 2015). 25-HC binding to INSIGs-2 proteins, promotes complex formation between INSIGs proteins and SCAP–SREBP, and blocks transport of SCAP-SREBP to the Golgi (Cyster et al., 2014; Radhakrishnan et al., 2007). There is a distinct hydrophobic pocket enclosed by TM1, 2, 3, and 6 of INSIG-2 proteins and located between INSIGs-2 proteins and SCAP, the size of which is suitable for the binding of 25HC but not for cholesterol. Binding sites of the pocket are comprised of several hydrophobic residues on TM3 and TM4 of INSIGs-2 proteins and S4, S5 and S6 segments of SCAP, of which the Phe_{115} on TM3 is essential for the recognition of 25HC. 25HC is likely to lie in the interspace formed by the S4 to S6 segments in SCAP and TM3 and TM4 in INSIGs-2 proteins, which provides a reasonable explanation for the role of 25HC in promoting INSIGs-2 protein's association with SCAP (Yan et al., 2021).

The SREBP pathway for the synthesis of intracellular lipids is critical for the formation of viral vesicles and the modification of viral proteins. In an infection of Middle East respiratory syndrome coronavirus, the levels of intracellular enzymes associated with lipid synthesis, such as acetyl-coenzyme A carboxylase, fatty acid synthase and HMGCR, significantly increase, which indicates that Middle East respiratory syndrome coronavirus infection is heavily dependent on intracellular lipid biosynthesis. It has been revealed that SREBP control lipid synthesis by transactivating the gene encoding fatty acid synthase (Yuan et al., 2019). A reliance on the SREBP pathway has been verified for hantavirus and SARS-CoV-2 (Kleinfelter et al., 2015; Lee et al., 2020). Treatment with 25HC has been shown to significantly reduce the

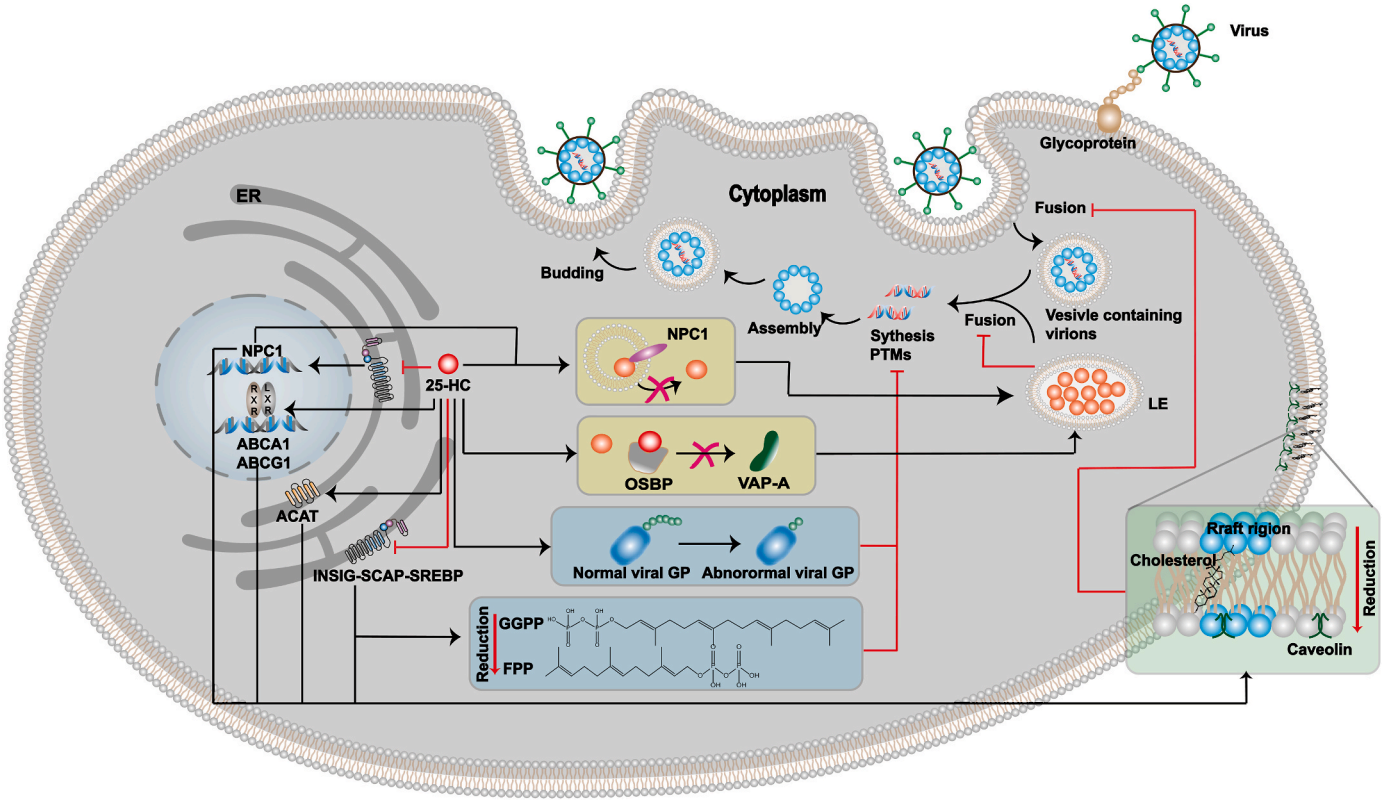

Fig. 4. The antiviral mechanisms of 25-hydroxycholesterol (25HC). Yellow box: 25HC inhibits cholesterol transport by reducing the expression of Niemann–Pick protein C1 (NPC1) or interacting with oxysterol-binding proteins (OSBP) and NPC1 directly, which both result in accumulation of cholesterol in late endosome (LE) that impedes viral vesicle fusing with LE. Blue box: 25HC disturbs prenylation of viral proteins by reducing production of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) in the dependent pathway of sterol-regulatory element-binding proteins (SREBP)-2; 25HC promotes production of abnormal viral glycoprotein by increasing the sensitivity of the N-linked glycan to endoglycosidase. Green box: 25HC impairs lipid raft in the cytomembrane by stimulating expression of adenosine triphosphate-binding cassette (ABC) A1/ABCG1 in the dependent pathway of liver X receptor (LXR) and may reduce clathrin-coated pits by inhibiting NPC1 in order to suppress viral entry; 25HC depletes the level of cholesterol in the membrane and disturbs the synthesis of intracellular lipids required for viral invasion by activating LXR and cholesteryl ester by acyl-coenzyme A: cholesterol acyl-transferase (ACAT) and inhibiting the SREBP pathway.

maturation of SREBP-2 in hepatic cells (Huh7 and Huh7.5.1) infected with HCV while decreasing the infection rate (Xiang et al., 2015).

4.1.2. Augmentation of cholesterol efflux

LXR forms a heterodimer with RXR after activation by oxysterols such as 25HC, 27HC, and 24(S),25-epoxycholesterol, then combines with LXR-responsive elements consisting of repeated 5′-AGGTCA-3′ sequences to enhance the expression of ABCA1/ABCG1, which results in reduced intracellular cholesterol levels (Gabbi et al., 2014). It has been demonstrated that the mRNA levels of ABCA1 and ABCG1 are upregulated 3.7-fold and 7-fold, respectively, while their protein levels are increased to approximate 2-fold and 3-fold in placental cells, respectively, after treatment with 25HC. With pre-treatment of GGPP, an LXR antagonist, there was no impact on the upregulations of expression of ABCA1/ABCG1 by 25HC, while pre-treatment of another agonist, 22 (R)-HC, weakened the upregulation by half (Aye et al., 2010). Based on this discovery, we speculate that the retained ability of 25HC in this upregulation may be caused by its competitive combination with the site occupied by GGPP at LXR or caused by stimulation of other genes responsible for the expression of ABCA1/ABCG1.

Formation of membrane rafts can be destroyed by stimulating expression of ABAC1/ABCG1 through 4-Cholesten-3-one, a LXR activator, which may provide an explanation for the dysfunction of a membrane (Elia et al., 2019). Treatment with 25HC causes HIV-1-infected monocyte-derived macrophages to increase the expression of genes related to LXR, especially ABCA1/ABCG1, and reduces the infection rate significantly (Saulle et al., 2020). In HIV infection, 25HC first blocks viral entry by reducing the membrane raft via an LXR-dependent pathway. Second, low cholesterol levels caused by the augmentation of cholesterol efflux significantly impede viral assembly (Mukhamedova et al., 2016). Clearly, further exploration of ABCA1/ABCG1 inhibitors and LXR-selective activators can contribute to the development of effective antiviral drugs.

4.1.3. Restriction of cholesterol transport

For most viral entities, virion-filled vesicle fusing with LE/LY is a critical precondition for the release of viral nucleic acid into the cytoplasm (Mercer et al., 2020). Once inside LE/LY, capsid—the outer protein of the virion—is striped in a degradative manner caused by the acidic environment of LE/LY and then transformed into viral nucleic acid or inner capsid particles to be released into the cytoplasm (Civra et al., 2018). The accumulation of cholesterol in LE has been demonstrated to restrict the fusion of intralumenal vesicles with the limiting membrane of LE (Amini-Bavil-Olyaee et al., 2013; Sobo et al., 2007).

It has been found that 25HC significantly inhibits human rotavirus by impeding the fusion of vesicles containing virions with LE/LY and then locking the viral particles in the vesicles. 25HC blocks the association of OSBP and VAP-A to cause failed cholesterol transport from LE to ER, which results in a substantial accumulation of cholesterol in LE (Fig. 4) (Civra et al., 2018). In addition, IFN-inducible transmembrane protein 3, a molecule with the ability to interact with VAP-A, has been shown to inhibit viruses through the accumulation of cholesterol in LE (Amini--Bavil-Olyaee et al., 2013). Impaired intra-endosomal trafficking induced by cholesterol accumulation in LE is fatal to viruses. Nevertheless, the mechanism behind this has not been revealed, although some suspect this damage to be related to the increased compartment size of LE (Sobo et al., 2007).

Recent studies have demonstrated that the accumulation of cholesterol in LE/LY may also be caused by the inhibition of NPC1 by 25HC. The infection rate of cells without NPC1 expression is significantly lower than that of normal cells (Zang et al., 2020). The invasion of several viruses, such as Ebola (Carette et al., 2011), has been shown to depend on the function of NPC1. 25HC inhibits NPC1 in two ways: by competing with cholesterol for the NPC1 binding pocket (Fig. 4) (Kwon et al., 2009) and by inhibiting the activation of NPC1 promoter by inhibiting SREBP-2 (Alrefai et al., 2007). The formation of clathrin-coated pits is also regulated by NPC1 to promote viral entry (Li et al., 2021). These studies indicate that NPC1 may be a highly viable antiviral target.

4.1.4. Promotion of cholesterol esterification

The degree of cholesterol esterified by ACAT reflects the status of cholesterol recycled by the regulatory pool of cholesterol located in the ER (Du et al., 2004). There is much evidence that 25HC is an effective agent for the allosteric activation of ACAT, triggering the rapid esterification of cholesterol, which causes peripheral cholesterol to be recycled into the regulatory pool of cholesterol (Fig. 4) (Abrams et al., 2020; Lathe et al., 2014). With the global outbreak of coronavirus disease 2019, recent studies have shown that 25HC inhibits the entry of SARS-CoV-2 into human lung epithelial cells by causing ACAT to deplete the cholesterol in the cell membrane (Wang et al., 2020). However, the molecular mechanism of 25HC that stimulates ACAT remains undefined.

4.2. Disturbance of PTMs

4.2.1. Inhibition of glycosylation of viral protein

In the normal glycosylation of intracellular proteins, the common N-linked oligosaccharide at the asparagine of the conserved region (Asn-X-Ser/Thr) is a high-mannose glycan, which is processed in the Golgi apparatus by remolding the oligosaccharide. The maturity of viral GP is also necessary for their replication. First, N-linked glycosylation of protein on the viral envelope is necessary for the virus to seize the receptors on the cytomembrane in order to fuse with the host cell or to be endocytosed; the same can be said of the budding of the virus. Second, during the variation of the virus, a small change in viral GP may allow the virus to effectively evade detection by the host immune system by reducing recognition of its antibody (Vigerust and Shepherd, 2007). In the case of arenaviruses, tripartite mature GP complex consists of a stable signal peptide and GP1/GP2 produced by the cleavage of the GP precursor (GPC), in which GP1 is responsible for recognition of the receptor on the host cytomembrane, whereas GP2 is responsible for fusion with the host cell (Burri et al., 2012). The spike protein S of SARS-CoV-2 is a GP trimer assembled in the ER and initially modified by high-mannose glycans; it is critical for it is critical for the infectivity. During an invasion of SARS-CoV-2, the S1 subunit of spike S is responsible for combining with angiotensin-converting enzyme 2, and the S2 subunit is processed by the host proteases to induce viral fusion with the host cell through extensive conformational changes (Zhang et al., 2021).

When cells infected with LASV (an adenovirus) were treated with 25HC, it was found that the LASV particles contained a large amount of abnormal GP1 and lost the ability to bind with alpha-dystroglycan, the recognition elements of the host cell (Fig. 4). Glycosylation of GPC is required for it to correctly fold such that it can be cleaved by SKI-1/S1P, and glycosylation of GP1 and GP2 is a requirement of viral budding and antigenicity. Treated with 25HC, abnormal GP1 is produced, caused by the increasing sensitivity of N-linked glycan to endoglycosidase, but GP2 and GPC are not disturbed (Shrivastava-Ranjan et al., 2016).

4.2.2. Inhibition of the prenylation of protein

By inhibiting the SREBP-2 pathway, 25HC impedes the production of two hydrophobic molecules, FPP and GDPP, which provide farnesyl and geranylgeranyl for the prenylation of proteins, respectively (Figs. 2 and 4). Generally, the prenylation of protein includes farnesylation and geranylgeranylation, which are implemented by the attachment of farnesyl or geranylgeranyl to the sulfhydryl of proteinic Cys in covalent bonds (Berndt et al., 2011). Upon viral infection, protein geranylgeranylation subsequent palmitoylation promote Rac1 (a Rho family small guanosine triphosphatase) translocation into the mitochondria-associated ER membranes to limit immune signaling mediated by the mitochondrial antiviral signaling protein. (Yang et al., 2019). Prenylated viral proteins are also critical and are different in diverse viruses, as has been found in E1A of human adenovirus 1, large delta antigens of hepatitis delta virus, the non-structural 5A protein of

HCV, uncharacterized protein IRL9 of human herpesvirus 5, PB2 protein of influenza A virus, hemagglutinin-neuraminidase of molluscum contagiosum virus subtype 1, nef protein of HIV-1, UL32 protein of human herpesvirus -1 and 2, Us2 of pseudorabies virus, and host RhoA of respiratory syncytial virus (Pronin et al., 2021). In the case of hepatitis delta virus, the prenylation of Cys^{211} in the large antigen encoded by L genomes is crucial for maturity and assembly of virions (Glenn et al., 1992). 25HC reduces synthesis of the prenyl donors, GGPP and FPP, by inhibiting expression of HMGCR, which may efficiently disturb prenylation of viral protein.

In previous studies, the intracellular levels of GGPP and FPP, as prenyl donors, are significantly decreased in presence of 25HC (Casella et al., 2014), the same could be said for the expression of FPP synthase (Le Jossic-Corcos et al., 2004). After treatment with 25HC, the titer of murine cytomegalovirus is remarkably reduced, and a supplement of geranylgeraniol (a geranylgeranylation inhibitor) can rescue the declining antiviral effect caused by lowering the concentration of 25HC (Blanc et al., 2013) in mouse embryo fibroblasts cells. It has been reported that Kaposi's sarcoma-associated herpesvirus reduces expression of farnesyl-diphosphate farnesyltransferase-1 in the host cell by its miRNAs to cut off the transform of FPP to squalene, which proves that excessive FPP is needed for farnesylation of viral proteins (Serquina et al., 2017).

4.3. Effects of the structure activity of 25HC on the properties of the biomembrane

In comparison with the structure of cholesterol, the hydroxylation of C_{25} of 25HC located in the non-polar regions is the crucial difference with cholesterol. In simulations of molecular dynamics, it can be seen that 25HC is more easily inserted into the biomembrane horizontally because of the polar group at both molecular ends. Furthermore, 25HC forms clusters of hydroxy at the hydrophobic region of the phospholipid bilayer to allow several polar molecules to penetrate the biomembrane, which changes the structure and dynamics of the biomembrane to promote the membrane's interactions with particular biomolecules, especially with proteins (Bielska et al., 2014; Olkkonen and Hynynen, 2009; Olsen et al., 2009). The dimer, trimer, and high polymer of 25HC aggregate in the biomembrane and are generated by forming hydrogen bonds spontaneously at the oxygen atoms of C_3 and C_{25} (Galiano and Villalain, 2020), thereby potentially altering the means of interaction between the proteins related to viral fusion and the membrane; they may even change the region of the proteins. The results of these molecular dynamic simulations suggest that this special interaction between 25HC and the membrane may be an additional way in which 25HC suppresses viral fusion with the host cell.

5. Antiviral study of 25HC in vivo

Recent studies have considered the antiviral activity of 25HC in vivo using various animals. With treatment of 50 mg/kg 25HC, the viremia of mice infected with ZIKV was found to improve, and embryonic mice were protected from infection and microcephaly induced by the virus. The study also found that the dosage had almost no adverse effects on pregnant mice. Likewise, treatment of rhesus monkeys with 25HC (1.5 mg/kg/day) was found to inhibit ZIKV infection in vivo (Li et al., 2017). Another study reported that the therapeutic regimen of 25HC combined with antiretroviral therapy inhibited simian immunodeficiency virus infection in rhesus monkeys and that restoring the ratio of $CD4^+/CD8^+$ induced by 25HC further contributed to the inhibition of simian immunodeficiency virus. The improvement in the number of viremias caused by infection has been attributed to the reduction of several proinflammatory factors (IL-2 and TNF-α) in vivo by treatment with 25HC (Wu et al., 2021).

6. The promising antiviral targets

A large number of studies in vitro and in vivo had let us understand more about the antiviral molecular mechanism of 25HC. There are some key steps in the process of viruses invading cells, such as SREBP-dependent lipid reprogramming of viral invasion, NPC1-regulated viral entry, and OSBP-mediated release of the virion from LE. The key molecules of these steps are promising antiviral targets. SREBP-mediated lipid metabolism has been a concern in the field of cardiovascular disease for a long time. It is also critical for lipid reprogramming after the viral invasion (Lee et al., 2020). Virion-release could be suppressed once the cholesterol-transport function of OSBP is damaged (Civra et al., 2018). Likewise, virion would be locked the in vesicular compartment when vesicular trafficking is impaired by inhibiting the function of NPC1 to flux cholesterol out of LE (Luquain-Costaz et al., 2020). Viral entry might be obstructed by LXR-mediated depletion of cholesterol and destruction of lipid raft as well (Sviridov et al., 2020).

After viral entry, for the sequent steps, including replication and assembly, there are still several attention-worthy targets that could be interfered with. The generation of lipid droplets is dominated by ACAT-1, which is a benefit for the assembly of several viruses, such as HCV (Meyers et al., 2016), ZIKV (Shang et al., 2018), and hepatitis B virus (Schmidt et al., 2021). The activity of intracellular glycosidase, for instance, mannosidase-α, is a key factor in the glycosylation of viral proteins (Yang et al., 2020). Two molecules, FPP and GGPP, are crucial donors for the prenylation of viral proteins (Pronin et al., 2021).

By now, several specific inhibitors or agonists with high efficiency have been developed, particularly, NPC1 inhibitor (U18666A, (3S,8R,9S,10R,13S,14S)-3-[2-(diethylamino)ethoxy]-10,13-dimethyl-1,2,3,4,7,8,9,11,12,14,15,16-dodecahydrocyclopenta[a]phenanthren-17-one, hydrochloride) (Li et al., 2021), OSBP antagonist (IFN-induced transmembrane protein 3) (Amini-Bavil-Olyaee et al., 2013), LXR agonist (LXR-623, 2-(2-chloro-4-fluorobenzyl)-3-(4-fluorophenyl)-7-(trifluoromethyl)-2H-indazole) (Hwang et al., 2019) and mannosidase-I alkaloid inhibitor (kifunensine, (5R,6R,7S,8R,8aS)-6,7,8-trihydroxy-5-(hydroxymethyl) hexahydroimidazo [1,2-a]pyridine-2,3-dione) (Yang et al., 2020). For ACAT-1, FPP/GGPP and SREBP, no efficient inhibitor for antiviral therapy has been reported yet. The continued explorations would facilitate to design a clinical therapeutic strategy of viral infection in future.

7. Conclusion and prospects

Based on the multi-function viral inhibition of 25HC, in particular the ability to restrict viral fusion by affecting intracellular lipid metabolism, exploration of its antiviral mechanism and derivatives are important for designing antiviral therapy strategies. Multiple antiviral molecular mechanisms of 25HC in humans have been identified to date, which have different effects on molecular metabolism that inhibit viral replication, including disturbance of cholesterol and several viral proteins. It is, however, likely that additional mechanisms for suppressing viral infection can be identified, such as whether 25HC disturbs the palmitoylation of viral proteins.

Thus far, numerous studies have shown the potent broad-spectrum antiviral effects of 25HC in vitro; however, it is difficult to generalize these results to other viruses. In vivo, the effect has been identified in a few animals, and there has yet to be a pharmacokinetic evaluation of 25HC. Therefore, future studies should consider the antiviral effect of 25HC in vivo, its pharmacokinetic characteristics, and the differences faced by its dominant mechanism in dealing with various viruses. Such studies will greatly further the design of clinical therapeutic strategies.

In conclusion, there is a need to continue to focus on the antiviral mechanisms of 25HC in terms of disturbing lipid metabolism, PTMs of viral proteins, or others in order to develop new chemical antiviral treatments with high efficiency and low toxicity. Such studies can also act as references for exploring the pathology and therapeutic targets of

other disorders. For example, the role of 25HC in inhibiting HMGCR may contribute to the development of drugs for the treatment of hyperlipemia. The reduction of interleukin caused by suppressing SREBP-1 by 25HC may also help in the development of anti-inflammatory drugs. Thus, to defeat the catastrophic threat of viral infection in this era, future studies exploring the mechanisms of multifunctional antiviral drugs such as 25HC are essential.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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