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Oxysterols: An emerging class of broad spectrum antiviral effectors

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Keywords Oxysterols; 25-hydroxycholesterol; 27-hydroxycholesterol; enveloped virus; non-enveloped viruses; antiviral; innate immunity

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DEPARTMENT OF CLINICAL AND BIOLOGICAL SCIENCES

Orbassano, 8th April 2016

Dear Editor,

please find herewith attached the manuscript entitled: “**Oxysterols: an emerging class of broad spectrum antiviral effectors**” by Lembo *et al.*, to be considered for publication in *Molecular Aspects of Medicine* as a Review article.

Yours sincerely

A handwritten signature in blue ink, appearing to read 'D. Lembo', with a stylized flourish at the end.

Oxysterols: an emerging class of broad spectrum antiviral effectors

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Abstract

Oxysterols are a family of cholesterol oxidation derivatives that contain an additional hydroxyl, epoxide or ketone group in the sterol nucleus and/or a hydroxyl group in the side chain. The majority of oxysterols in the blood are of endogenous origin, derived from cholesterol via either enzymatic or non-enzymatic mechanisms. A large number of reports demonstrate multiple physiological roles of specific oxysterols. One such role is the inhibition of viral replication. This biochemical/biological property was first characterised against a number of viruses endowed with an external lipid membrane (enveloped viruses), although antiviral activity has since been observed in relation to several non-enveloped viruses. In the present paper, we review the recent findings about the broad antiviral activity of oxysterols against enveloped and non enveloped human viral pathogens, and provide an overview of their putative antiviral mechanism(s).

Keywords

Oxysterols, 25-hydroxycholesterol, 27-hydroxycholesterol, enveloped virus, non-enveloped viruses, antiviral, innate immunity.

List of abbreviations

24OH: 24-hydroxycholesterol; 25OH: 25-hydroxycholesterol; 27OH: 27-hydroxycholesterol; LXRs: liver X receptors; RXRs: retinoid X receptors;

1. Introduction

Oxysterols are a family of cholesterol oxidation derivatives that contain an additional hydroxyl, epoxide or ketone group in the sterol nucleus and/or a hydroxyl group in the side chain of the cholesterol molecule. Normocholesterolemic healthy individuals contain a mixture of these compounds in their peripheral blood, accounting for 1-5% of total cholesterol. A small part of this oxysterol haematic pool is derived from exogenous origins, i.e. oxysterols originating from cholesterol-rich food that have undergone non-enzymatic oxidation. The rest are of endogenous origin. Some endogenous oxysterols are the result of enzymatic hydroxylation of cholesterol at specific sites of the molecule and are almost ubiquitous in the human body; while other endogenous oxysterols result from the non-enzymatic attack of a variety of oxidant species. This latter pathway is greatly enhanced in sites of inflammation compared to non inflamed areas of the body.

In contrast with unoxidized cholesterol, oxysterols exert a wide variety of biochemical effects with potential biological consequences (Schroepfer, 2000; Leonarduzzi et al. 2002; Leonarduzzi et al. 2005). **Figure 1** shows the main oxysterols recognised to date for their potential involvement/role in human pathophysiology; they are subdivided into the following three groups: i) oxysterols of enzymatic origin; ii) oxysterols of non-enzymatic origin; and iii) oxysterols produced via both enzymatic and non-enzymatic pathways.

Even though marked pro-oxidant, pro-inflammatory and pro-apoptotic activities have been reported for a number of oxysterols of biological interest, a large number of studies clearly point to multiple physiological roles of certain oxysterols – mainly those of enzymatic (thus natural) origin.

With regard to the pathological effects of certain oxysterols, it is important to note that their negative effects have only been displayed in *in vitro* model systems in which oxysterols were added at high micromolar concentrations. On the other hand, the fact that the enzymatic production of oxysterols has been maintained over the course of evolution, and even enhanced, supports the supposition that these compounds play relevant roles in human physiology. An important example is

the unanimous recognition that side-chain oxysterols, like 24-, 25- and 27-hydroxycholesterol (24OH, 25OH and 27OH), act as primary ligands of a number of nuclear receptors with key physiological roles; such receptors include: liver X receptors (LXRs), retinoid X receptors, (RXRs) (Janowsky et al., 1999; Bensinger and Tontonoz, P., 2008) and estrogen receptors (Umetani et al., 2007).

The common opinion that the oxysterol 'story' was far from complete was further sustained by a recent study confirming the marked antiviral effect of specific oxysterols (once again of mainly enzymatic origin). **Figure 2** lists all the scientific papers identified by PubMed using the key words: oxysterols, 24OH, 25OH or 27OH; the papers are sorted by their year of publication. Less than 3% of the studies researching oxysterols have focussed on the antiviral properties of these molecules, and approximately two-thirds of these papers were published during the last 4-5 years. Thus, the inhibition of viral replication by specific oxysterols constitutes a very recent field of scientific enquiry. This antiviral property was initially described for a variety of viruses all of which are characterised by an external lipid membrane, the so-called enveloped viruses. Only very recently was antiviral activity also demonstrated against non-enveloped viruses. However, our understanding of the mechanism(s) underlying this important aspect of oxysterol activity is far from complete. The present paper provides the first comprehensive overview of the mechanistic studies published to date.

1. Oxysterols as antiviral factors

Multiple mechanisms are known to control and eventually eliminate virus infections from the host. The innate immune response, also known as nonspecific immunity, provides the first line of defence during the early phase of acute viral infection (involving natural killer cells, interferon, fever, inflammation) acting to limit virus multiplication. Specific or adaptive immune responses, based on humoral or cell-mediated effectors, contribute to clear the virus from the organism at the end of the acute phase and confer resistance to reinfection. In recent years, a growing body of evidence points

to a significant role of some oxysterols in host nonspecific antiviral defences. As shown in Table 1, the bulk of the studies on the antiviral activity of oxysterols has focused on 25OH, which emerged as a wide spectrum antiviral molecule against a large number of pathogenic viruses *in vitro*. In 2013, Blanc and colleagues reported the results of a quantitative metabolome profiling that looked at all the naturally occurring oxysterols; the study revealed that 25OH was markedly increased by interferon (IFN) treatment in bone-marrow-derived macrophages due to IFN-induced transcription of the Ch25h gene encoding cholesterol 25-hydroxylase (CH25H). These findings directly linked the innate antiviral response elicited by IFN to the sterol metabolic network and identified 25OH as the effector. One year later, Chen and colleagues added a new piece to the puzzle by reporting that CH25H itself may inhibit the replication of hepatitis C virus in a way that was independent of its enzymatic activity. In fact, this hydroxylase was shown to interact directly with the viral nonstructural protein 5A (NS5A), disrupting the formation of its dimer – an essential event in viral replication. Therefore, besides converting cholesterol into a broad-spectrum antiviral factor, namely 25OH, CH25H can act as a virus-specific antiviral effector. This discovery opened the way to a new line of investigation.

In addition to 25OH, other side chain oxysterols have been found to exert antiviral activity *in vitro*. Recently, the enzymatically-produced oxysterol 27OH was shown to block the infection of several viruses. Murine cytomegalovirus (Blanc et al., 2013), rhinoviruses, human rotaviruses, human papillomaviruses (Civra et al., 2014) and herpes simplex types 1 and 2 (Cagno et al., submitted paper) were all found to be susceptible to its inhibitory activity with EC_{50} values similar to those calculated for 25OH. In addition to extending the panel of antiviral oxysterols, 27OH was also notable because it was not induced by IFN (Blanc et al., 2013), indicating that the innate-immune response to virus infections involving lipid sterol effectors is only partially dependent on IFN stimulation and might even occur in its absence through 27OH. The emerging role of 27OH as a potential effector of the antiviral innate immunity is of particular interest because it is one of the most common oxysterols in the peripheral blood of healthy individuals and its potential contribution

to immune and inflammatory reactions constitutes a hot research topic. A third oxysterol containing an hydroxyl group in its side chain, namely 24-hydroxycholesterol (24OH), has been reported to inhibit the murine cytomegalovirus (Blanc et al 2013). Unpublished observations from the authors of this present review also extend the antiviral activity of 24OH to herpes simplex virus type 1. Finally, 22(S)-hydroxycholesterol was shown to be active against hepatitis B virus (Iwamoto et al., 2014), but neither 22(S)-hydroxycholesterol nor 22(R)-hydroxycholesterol showed any antiviral activity against vesicular stomatitis virus (Liu et al., 2013).

Oxysterols bearing an additional ketone or hydroxyl group in the sterol ring, namely in the B ring, (e.g. 7-K, 7 α -OH, 7 β -OH) are also endowed with some antiviral activity. Of note, in the few papers where the two classes (i.e. side chain oxysterols vs. oxysterols with an additional ketone or hydroxyl group in the sterol nucleus) have been compared in terms of antiviral potency, the latter have repeatedly been shown to be far less active than 25OH and 27OH or not active at all (Moog et al., 1998; Cibra et al 2014). The higher antiviral potency of side chain oxysterols, along with the fact that they are produced by specific enzymes, may suggest that this subgroup of cholesterol oxidation products had been evolutionarily selected as an early antiviral defence mechanism; the B ring oxysterols, on the other hand, are generated by a non-enzymatic, unscheduled mechanism and do not therefore implicate an evolutionary advantage.

The well-known membrane-modifying effect of 25OH (Lange et al.,1995; Olsen et al., 2011; Gale et al., 2009) has led most studies to focus on viruses characterized by the presence of a lipid envelope, whose entry into the host cell depends on fusion between the viral and cellular membranes. Of note, none of the enveloped viruses tested to date have been found to be resistant to 25OH. Moreover, as non-enveloped viruses can enter the cell directly without undergoing a fusion process, they are less likely to be a target of oxysterol-mediated antiviral activity. In line with this view, two studies recently reported the lack of 25OH antiviral activity against two types of adenovirus (i.e.Adv5 and Adv19), a non-enveloped virus (Blanc et al., 2013; Liu et al., 2013).

However, around the same time Arita and colleagues observed, for the first time, the activity of 25OH against a non-enveloped virus, namely poliovirus. The question of whether the broad antiviral activity of 25OH against enveloped viruses could also extend to non-enveloped viruses remained open until 2014 when two studies provided new pieces of information. Roulin and colleagues reported on the antiviral effect of 25OH against rhinoviruses, while Civra *et al.* showed a marked activity of 25OH and 27OH against rhinovirus, human rotavirus and human papillomavirus-16, all of which are non enveloped. These findings demonstrate that specific oxysterols are endowed with exceptionally broad spectrum antiviral activity *in vitro*. Thus the critical question that remains to be fully understood regards how oxysterols are able to block the infection of such a wide range of viruses characterised by distinct tissue tropism, genome composition, virion architecture and replicative cycles.

3. Antiviral mechanisms of action of oxysterols

In an attempt to answer the above question, we herein present a review of the available literature regarding the step(s) of viral replication inhibited by specific oxysterols (see also **figure 3**) and their underlying molecular mechanisms of action.

3.1. Enveloped viruses

The data gathered so far suggest that multiple mechanisms may contribute to the antiviral activity of 25OH, depending on the virus-host context. In 2013, Liu and colleagues (Liu et al., 2013) reported broad spectrum antiviral activity of 25OH against the following enveloped viruses: vesicular stomatitis virus (VSV), human herpes virus (HSV), human immunodeficiency virus (HIV), murid herpesvirus 68 (MHV68), ebola virus (EBOV), rift valley fever virus (RVFV), Russian spring-summer encephalitis virus (RSSEV) and Nipah virus. This study, demonstrated that 25OH impairs viral entry by inducing cellular membrane changes that alter the virus-cell fusion process; 25OH did not appear to target a specific structural class of fusion proteins or inhibit a particular fusion

mechanism (blocking both pH dependent and pH independent fusion). Considering that 25OH can permeate membranes, Liu and colleagues suggested that 25OH may directly change the membrane properties by actually inserting itself into the cellular membrane, thereby changing its composition and affecting a more basic fusion process involving both the viral and the cellular membrane.

In 2013, Blanc and colleagues confirmed the broad antiviral activity of 25OH against a panel of diverse enveloped viruses, namely Influenza A (H1N1), herpes simplex virus-1 (HSV-1), varicella zoster virus (VZV) and murine gamma herpes virus 68 (MHV-68) (Blanc et al., 2013). In this study, the metabolic profiling of oxysterols showed that the response of activated macrophages is highly focused toward 25OH. Blanc and colleagues demonstrate that IFN regulates the production of 25OH by recruiting the transcriptional factor STAT1 to the promoter proximal region of the Ch25h gene. Data gathered in this study demonstrate that 25OH plays an important role in the innate immune response by promoting an antiviral cellular response that is liver X receptor (LXR)-independent, but that relies on sterol regulatory element-binding proteins (SREBPs), a family of transcription factors that regulate cellular lipid biosynthesis in mammals (Goldstein et al., 2006). SREBPs include two proteins: SREBP-1 (responsible for cholesterol and fatty acid synthesis) and SREBP-2 (that mediates cholesterol synthesis). Blanc and colleagues conclude that the antiviral activity of 25OH relies on its ability to alter the lipid composition of cellular membranes; they propose a double mechanism of action linked to the dose of 25OH: at low doses they suggest a protein interaction mechanism involving the SREBP pathway, while at high doses they propose that cell membranes are altered; they also exclude the involvement of the LXR pathway and suggest blockade of the mevalonate branch of the sterol pathway contributes to its effect, but is not essential.

The viral step inhibited by 25OH is not well defined or common between viruses; Blanc and colleagues (2013) observed a general reduction in the size of plaques for an array of viruses tested, while an increase in plaque number was only observed for HSV-1. In relation to MCMV, they

suggested that 25OH blocks a post entry stage of the virus replicative cycle and they verified a decrease in viral protein expression.

The mechanism of action of 25OH against HCV has been extensively investigated. Several studies demonstrate that 25OH treatment yields an antiviral state within Huh-7 cells by modulating the mevalonate pathway (Sagan et al., 2006; Su et al., 2002, Ye et al., 2003, Wang et al., 2005). Pezacki and colleagues (Pezacki et al., 2009) performed global transcriptional profiling of Huh-7 cells and Huh-7 cells bearing HCV replicons. They demonstrated that 25OH downregulates many key genes involved in the mevalonate pathway, leading to cholesterol depletion and an antiviral state against HCV. Inhibition of host lipid synthesis blocks HCV RNA replication and virus production (Su et al., 2002; Yang et al., 2008; Ye et al., 2003; Olmstead et al., 2012).

Xiang and colleagues (Xiang et al., 2015) have shown that CH25H-mediated SREBP antagonism might be responsible for its antiviral activity, noting that the SREBP pathway may not account for all of the observed antiviral effect, suggesting that CH25H may possess additional anti-HCV activity that is independent of SREBP suppression. It has also been shown that 25OH, as an SREBP inhibitor, can decrease HCV replication within hepatoma cells (Su et al., 2002).

The discovery that the antiviral activity of 25OH relies on its ability to alter host cell lipid metabolism is not surprising. For example, HCV exploits host lipid metabolism during its entire viral life cycle. The virus i) relies on host lipid transport molecules, LDLR and SRB1, for its entry (Agnello et al., 1999; Scarselli et al., 2002), ii) creates a lipid-rich microenvironment for viral genome replication (Hsu et al., 2010), iii) utilizes host lipid storage vesicles lipid droplet for viral particles assembly (Miyanari et al., 2007) and iv) releases viral particles in a very-low-density-lipoprotein-dependent manner (Huang et al., 2007). HCV upregulates host lipid metabolism through a variety of molecular mechanisms (Waris et al., 2007; Oem et al., 2008; Li et al., 2013), which may ultimately contribute to the development of hepatic steatosis, which occurs in almost 50% of HCV-infected patients (Ramalho et al., 2003).

Molina and colleagues (Molina et al., 2007) were the first to demonstrate the ability of 25OH, to control HCV infection in parallel with the ability to modulate LDLr expression and activity; their results suggested that 25OH was able to inhibit the early steps of HCV infection, in contrast with those obtained by Su and colleagues (Su et al., 2002). More recently, Anggakusuma and colleagues (Anggakusuma et al., 2015) confirmed the inhibition of HCV infection in primary hepatocytes and tissues derived from liver biopsies by 25OH. They also reported that 25OH was able to inhibit virus entry by modifying the cellular membrane, although the level of virus inhibition at the post entry stage was much greater, assessed as the block of membranous web formation, which is necessary for HCV replication. In 2014, Chen and colleagues (Chen et al., 2014) suggested that CH25H exerts inhibitory activity against HCV replication via a mechanism that is independent of its hydroxylase activity, but that does involve a direct interaction between CH25H and the HCV NS5A protein.

The mechanism of 25OH-induced HCV inhibition was recently proposed to be mediated by the action of 2 different miRNAs, namely miR-130b and miR-185; although only miR-185 seems to be directly stimulated by the oxysterol (Singaravelu et al., 2015). These miRNAs were also shown to exert antiviral activity independent of 25OH; moreover, HCV infection caused a decrease in their expression, probably in order to oppose the antiviral mechanism of 25OH.

3.2. Non-enveloped viruses

The antiviral activity of 25OH has been reported in relation to various viruses belonging to the picornaviridae family. In 2013, Arita and colleagues (Arita et al., 2013) demonstrated that 25OH suppresses Poliovirus (PV) pseudovirus replication by reducing the accumulation of PI4P at the Golgi apparatus by targeting members of oxysterol binding protein (OSBP) family I.

In mammalian cells, OSBP or its related proteins (ORPs) are well-characterized candidate cholesterol sensors and/or transporters (Olkkonen et al., 2013). This evolutionary conserved family of proteins includes seven members in the budding yeast *Saccharomyces cerevisiae* and twelve in humans (Beh et al., 2001; Lehto et al., 2001). OSBP, the founding member of the family, was first

identified as a high-affinity cytosolic binding protein specific for oxysterols, such as 25OH (Taylor et al., 1984). One model of PV replication suggests that virus protein 3A acts as an indirect tether (3A/GBF1/ARF1) for recruiting phosphatidylinositol 4-kinase III beta (PI4KIII β) to the site of virus RNA replication, and that PI4KIII β provides phosphatidylinositol 4-phosphate (PI4P) for the recruitment of virus RNA-dependent RNA polymerase (virus protein 3D) via direct interaction of PI4P and 3D protein on reorganized membrane vesicles for the formation of virus replication complexes (Hsu et al., 2010).

In particular, Arita and colleagues demonstrated the antiviral activity of 25OH and other compounds to have a mechanism of action linked to the delocalization of OSBP protein to the Golgi vesicles and the reduction of the PI4P at the Golgi apparatus, suggesting a role of its synthesizing enzyme PI4KIII β . Antiviral activity was present when 25OH was added during infection or up to 5 hours post infection, while pre-treatment with 25OH before infection did not enhance its inhibitory activity, suggesting a direct role and not one resulting from transcriptional activation or suppression. In 2014, Roulin and colleagues described a similar mechanism for a second member of the picornaviridae family, namely human rhinovirus (Roulin et al., 2014). The results of this study demonstrated that rhinoviruses build up PI4P-rich Golgi membranes and that they depend on this mechanism for the recruitment of RNA replication machinery. They went on to show that 25OH inhibits the replication of several serotypes of human rhinovirus by binding to OSBP with high affinity and locking it in a lipid exchange inactive state; this led to a blockade of PI4P shuttling between the endoplasmic reticulum, Golgi and replication membranes, thereby halting rhinovirus replication.

The mechanism by which 25OH and 27OH exert antiviral action against human rotaviruses and human papillomaviruses remains to be elucidated (Civra et al., 2014).

4. Conclusions and perspectives

A significant volume of evidence support the role of specific oxysterols (in particular those with a side chain oxidized by enzymatic activity) in innate immune defences against virus infections. However, several issues remain to be addressed. While it is clear that 25OH constitutes one of the many effectors in the antiviral state induced by IFN, less is known about the stimuli activating the production of other oxysterols, like 27OH and 24OH, and in which tissues they are produced in response to infection; moreover their mechanism(s) of action remain to be elucidated. Evidence demonstrating their activity following viral infections *in vivo* is also lacking. Most studies to date have been conducted in cultured cells and very little data has been generated using animal models of viral infection. One study supporting the protective activity of 25OH *in vivo* was published by Liu and coworkers in 2013. They found that 25OH suppressed HIV replication and rescued T-cell depletion in humanized mice, while Ch25h-knockout mice were more susceptible to MHV68 lytic infection (Liu et al., 2013). These findings are concordant with the results obtained *in vitro* in the same study. By contrast, Gold and colleagues (Gold et al., 2014) reported a discrepancy between the *in vitro* and *in vivo* antiviral effects of 25OH against influenza virus. Although they observed an inhibitory activity of 25OH *in vitro*, Ch25h-knockout mice were protected from influenza infection because there was less inflammatory damage, while the viral load was not significantly different. These data can be explained by the fact that the outcome of influenza infection depends, not only on the host's ability to control viral replication, but also on the level of damage caused by the host's immune response to the virus. Taken together, these findings indicate that oxysterols may exert either a protective or a detrimental effect depending on the type of viral infection. Clearly, this issue must be carefully addressed and a great deal of research remains to be done in order to advance our knowledge on the role played by oxysterols during the course of different viral infections *in vivo* and to assess their clinical potential as antiviral therapeutics in humans.

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Table 1. Antiviral activity of oxysterols

Oxysterol	Antiviral activity		References
	Enveloped viruses	Non-enveloped viruses	
25-hydroxycholesterol	HIV VSV MHV68 EboV RVFV RSSEV Nipah virus Influenza virus		Moog et al., 1998
			Liu et al., 2013
			Liu et al., 2013
			Liu et al., 2013
			Liu et al., 2013
			Liu et al., 2013
			Liu et al., 2013
			Liu et al., 2013
			Blanc et al., 2013
			Gold et al., 2014
	HSV-1 VZV MCMV HCV		Liu et al., 2013
			Blanc et al., 2013
			Blanc et al., 2013
			Blanc et al., 2013
			Sagan et al., 2006
27-hydroxycholesterol	HBV	Poliovirus EMCV HPV-16 HRoV HRhV	Su et al., 2002
			Ye et al., 2003
			Wang et al., 2005
			Pezacki et al., 2009
			Yang et al., 2008
			Olmstead et al., 2012
			Anggakusuma et al., 2015
			Singaravelu et al., 2015
			Iwamoto et al., 2014
			Arita et al., 2013
			Dorobantu et al., 2015
			Civra et al., 2014
			Civra et al., 2014
			Civra et al., 2014
			Roulin et al., 2014
24-hydroxycholesterol	MCMV HSV-1	HPV-16 HRoV HRhV	Civra et al., 2014
			Civra et al., 2014
			Civra et al., 2014
7 beta-hydroxycholesterol	HIV HBV		Blanc et al., 2013
			Author's Unpublished results
7 beta, 25-dihydroxycholesterol	HIV		Moog et al., 1998
25-epoxycholesterol	MCMV		Iwamoto et al., 2014
22(S)-hydroxycholesterol	HBV		Blanc et al., 2013
20 α -hydroxycholesterol	HBV		Iwamoto et al., 2014

HIV: human immunodeficiency virus; VSV: vesicular stomatitis virus; MHV68: murid herpes virus 68; EboV: Ebola virus; RVFV: Rift valley fever virus; RSSEV: Russian spring-summer encephalitis virus; HSV: herpes simplex virus (type 1 and 2); VZV: varicella zoster virus; MCMV: murine cytomegalovirus; HCV: hepatitis C virus; HBV: hepatitis B virus; EMCV: encephalomyocarditis virus; HPV-16: human papillomavirus type 16; HRoV: human rotavirus; HRhV: human rhinovirus.

Figure captions

Figure 1 - *Main oxysterols stemming from enzymatic and/or non-enzymatic, ROS-mediated, oxidation of cholesterol.*

ROS: reactive oxygen species; CYP7A: cholesterol 7 alpha-hydroxylase; CYP46A: cholesterol 24-hydroxylase; CYP27A1: cholesterol 27-hydroxylase; Ch25h: cholesterol 25-hydroxylase

Figure 2 - *The steadily growing number of oxysterols-related studies.*

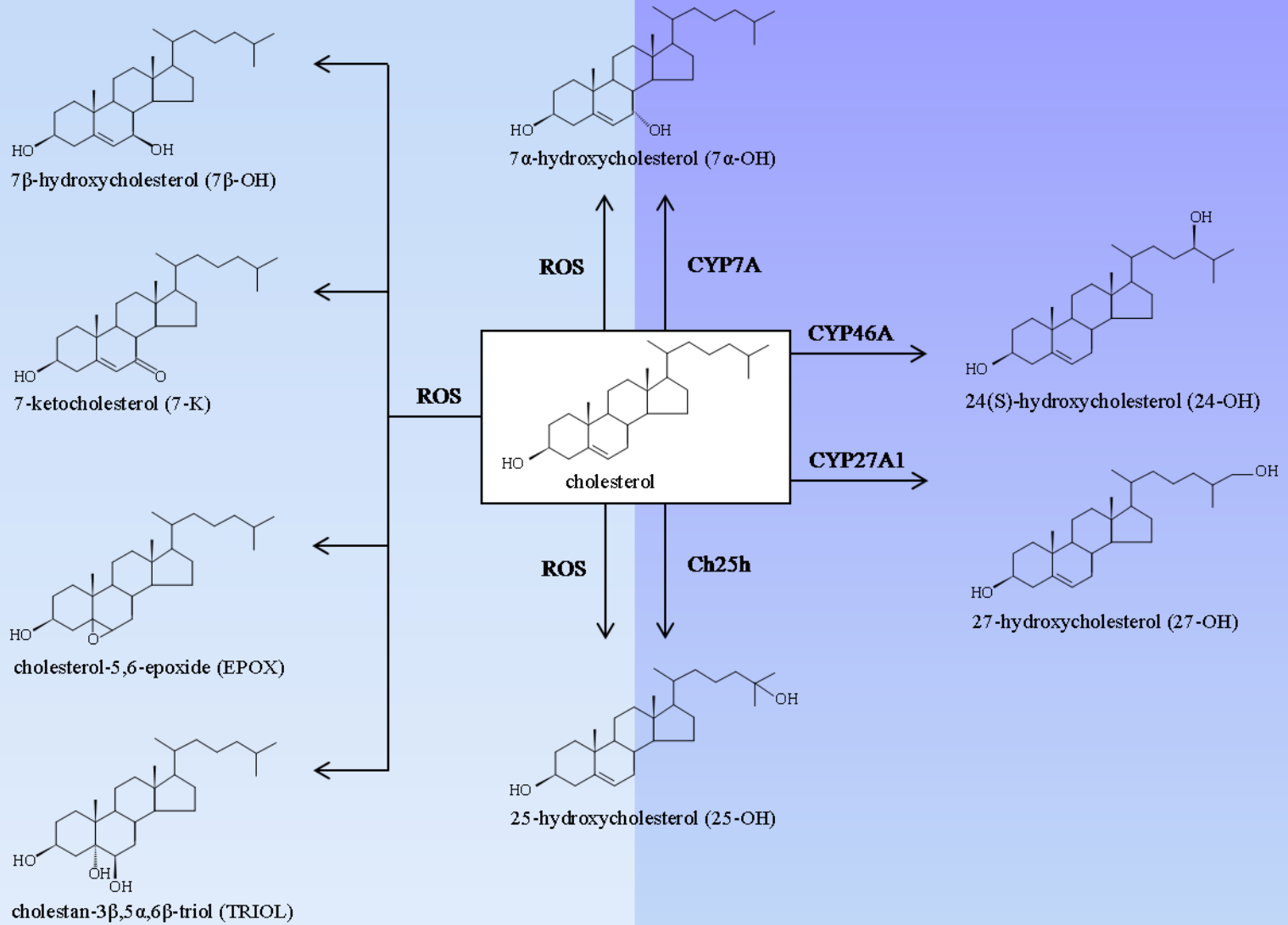
Data were obtained from Pub Med - MEDLINE database, using the keyword "oxysterols".

Figure 3 - *Mechanism of action of 25OH against non-enveloped (panel A) and enveloped (panel B).*

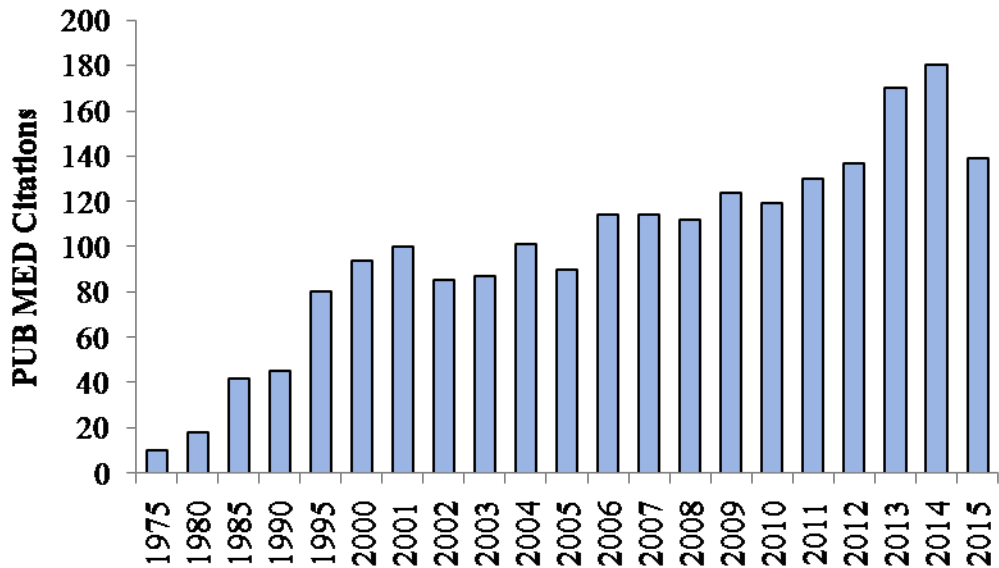
N: nucleus; 25OH: 25-hydroxycholesterol

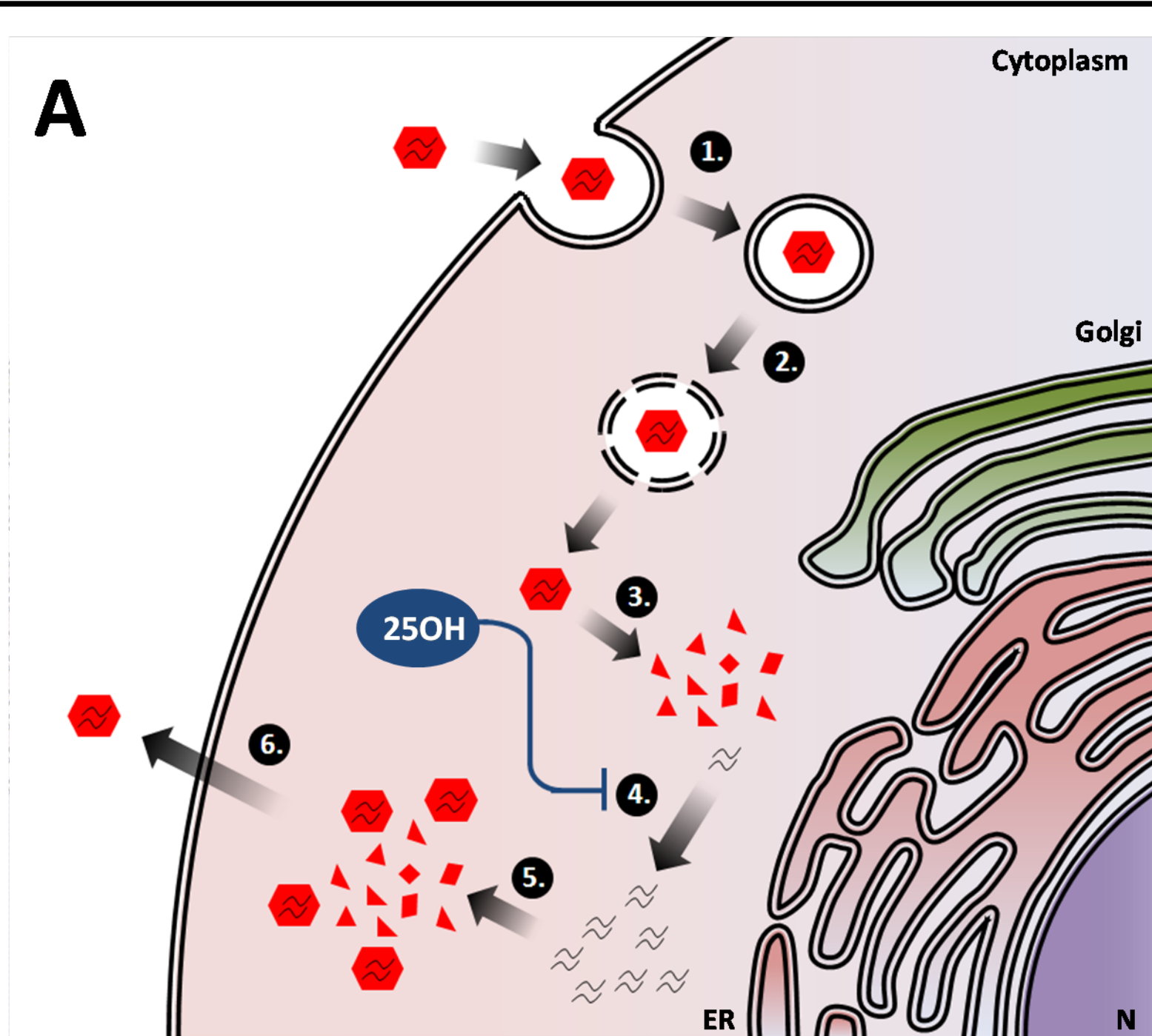
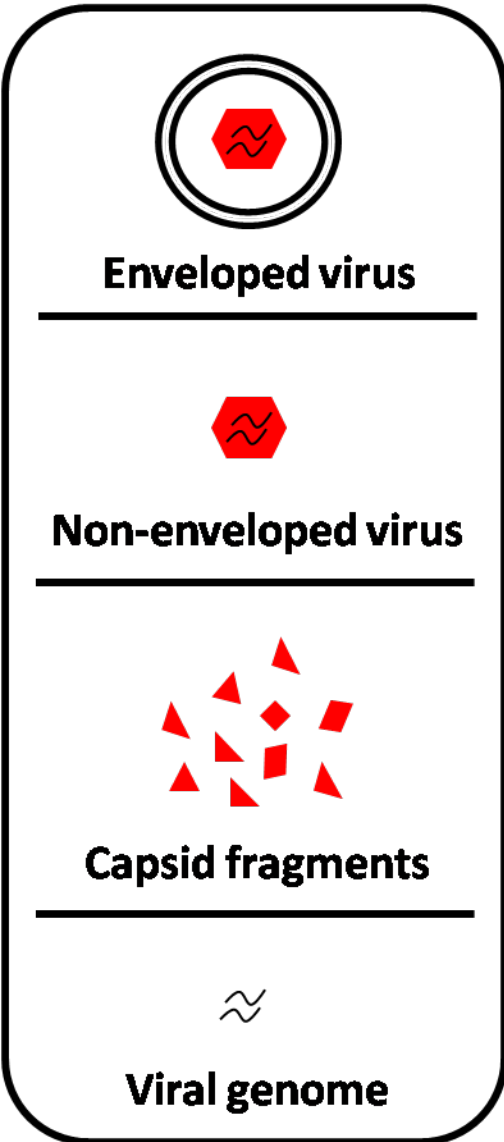
NON-ENZYMATIC OXYSTEROLS

ENZYMATIC OXYSTEROLS

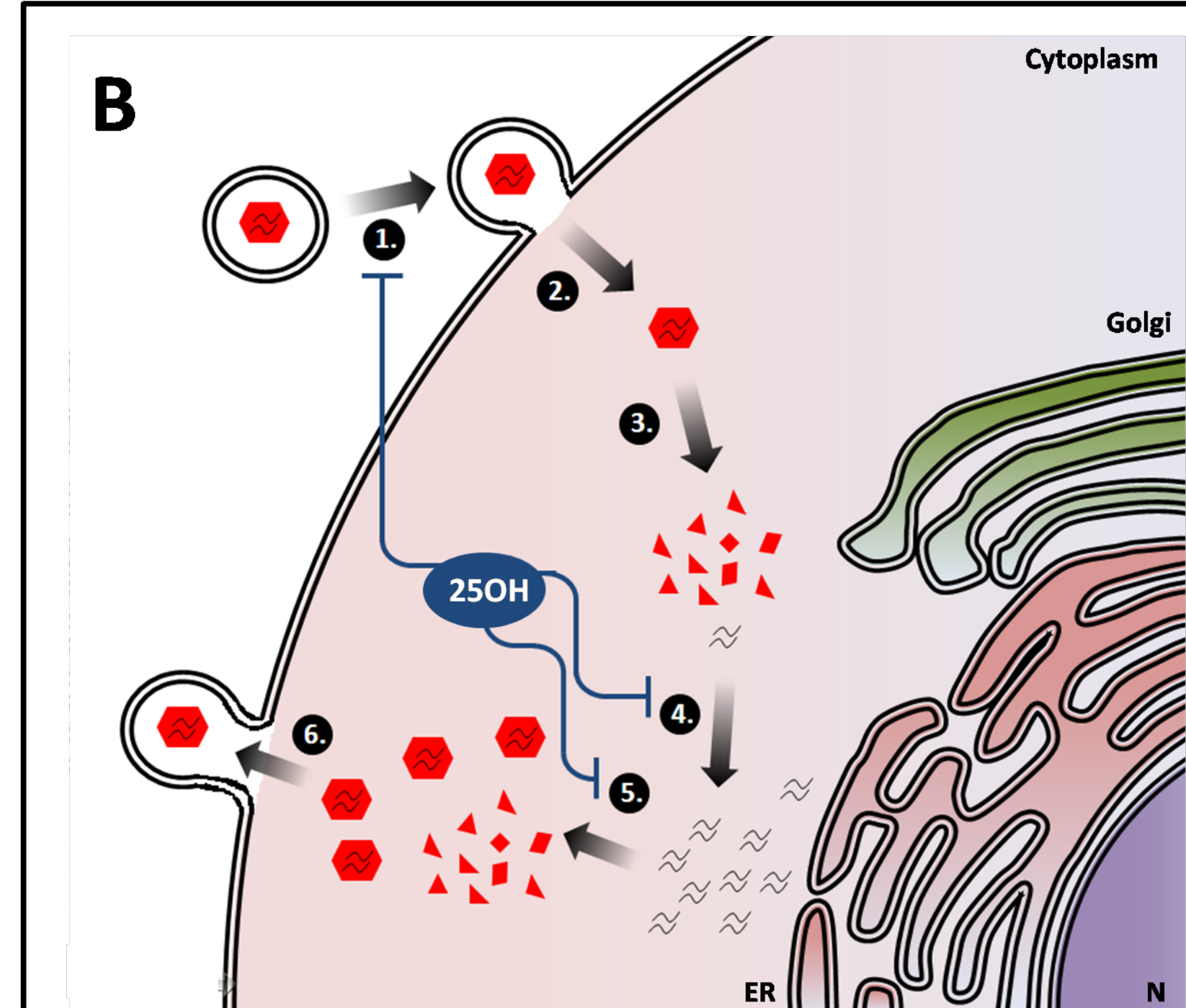


Oxysterols





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|------------------------------|--|
| 1. Virus endocytosis | 4. Genome replication |
| 2. Cell penetration | 5. Protein expression/capsid assembly |
| 3. Capsid disassembly | 6. Cellular egress |



- | | |
|------------------------------|--|
| 1. Fusion | 4. Genome replication |
| 2. Cell penetration | 5. Protein expression/capsid assembly |
| 3. Capsid disassembly | 6. Cellular egress |