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Research paper

25-Hydroxycholesterol and 27-hydroxycholesterol inhibit human rotavirus infection by sequestering viral particles into late endosomes

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A B S T R A C T

A novel innate immune strategy, involving specific cholesterol oxidation products as effectors, has begun to reveal connections between cholesterol metabolism and immune response against viral infections. Indeed, 25-hydroxycholesterol (25HC) and 27-hydroxycholesterol (27HC), physiologically produced by enzymatic oxidation of cholesterol, act as inhibitors of a wide spectrum of enveloped and non-enveloped human viruses. However, the mechanisms underlying their protective effects against non-enveloped viruses are almost completely unexplored. To get insight into this field, we investigated the antiviral activity of 25HC and 27HC against a non-enveloped virus causing acute gastroenteritis in children, the human rotavirus (HRV). We found that 25HC and 27HC block the infectivity of several HRV strains at 50% inhibitory concentrations in the low micromolar range in the absence of cell toxicity. Both molecules affect the final step of virus penetration into cells by preventing the association of two cellular proteins: the oxysterol binding protein (OSBP) and the vesicle-associated membrane protein-associated protein-A (VAP-A). By altering the activity of these cellular mediators, 25HC and 27HC disturb the recycling of cholesterol between the endoplasmic reticulum and the late endosomes which are exploited by HRV to penetrate into the cell. The substantial accumulation of cholesterol in the late endosomal compartment results in sequestering viral particles inside these vesicles thereby preventing cytoplasmic virus replication. These findings suggest that cholesterol oxidation products of enzymatic origin might be primary effectors of host restriction strategies to counteract HRV infection and point to redox active lipids involvement in viral infections as a research area of focus to better focus in order to identify novel antiviral agents targets.

Introduction

Innate immune response is the first line of defense during the earliest hours of exposure to a novel pathogen. Its mechanisms are non-specific and rely on a group of proteins and phagocytic cells that quickly activate to help destroy invaders. Alongside these pathways, a novel innate immune strategy has begun to reveal connections between cholesterol metabolism and immune response against viral infections. [1–3].

The most widely studied effector of this branch of innate immunity is an oxysterol, 25-hydroxycholesterol (25HC) [1,2]. Oxysterols contain 27 carbon atoms per molecule and are derived from cholesterol by both enzymatic and nonenzymatic oxidation [4–6]. Several among the various cholesterol oxidation products of enzymatic origin contribute to physiological functions: they are intermediates of pregnenolone and steroid hormone synthesis [7] and target nuclear receptors (e.g., the liver X receptor [LXR] and the estrogen receptor α [ER α]), cellular membrane receptors (e.g., C-X-C motif chemokine receptor 2 [CXCR2]) and transport proteins (e.g., insulin induced gene protein [INSIG], Niemann-Pick protein 1 [NPC1], oxysterol binding protein [OSBP] and its related proteins [ORPs]) [8–13]. In contrast, the oxysterols derived from cholesterol autoxidation, a not-regulated and therefore potentially harmful biochemical reaction, appear to be more likely involved in pathophysiological processes associated with inflammation and oxidative stress [4,5,14].

In 2013 Blanc and colleagues provided in vitro findings indicating that 25HC acts as a physiological interferon (IFN)-induced effector of innate immunity against viral infections [1]. They reported that 25HC was the only oxysterol synthesized and secreted by macrophages upon IFN treatment or virus infection and that transcription factor Stat1 directly couples IFN-stimulated signaling to regulation of the cholesterol hydroxylase gene (Ch25h) encoding the 25HC-synthesizing enzyme.

Unlike known antiviral small molecules that target highly specific viral determinants, thus showing a restricted spectrum of activity, 25HC inhibits the replication of a wide spectrum of pathogenic viruses. This includes both enveloped viruses, i.e., those with a phospholipidic bilayer outside the proteic capsid such as human immunodeficiency virus (HIV), herpes simplex virus type 1 (HSV-1), varicella zoster virus (VZV) murine cytomegalovirus (MCMV), vesicular stomatitis virus (VSV), Ebola virus (EBOV), Zika virus, hepatitis C virus, and

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Table 1
Antirotavirus activity of oxysterols (MA104 cells).

Oxysterols	HRV strain	EC_{50}[a] (μM) – 95% CI[b]	EC_{90}[c] (μM) – 95% CI	CC_{50}[d](μM) –95% CI	SI[e]
25HC	Wa	0.16 (0.12–0.20)	1.37 (0.75–2.40)	> 150	> 938
	WI61	0.09 (0.08–0.12)	0.28 (0.16–0.46)	> 150	> 1667
	HRV408	0.12 (0.09–0.16)	0.42 (0.23–0.74)	> 150	> 1250
	HRV248	0.11 (0.07–0.18)	1.26 (0.47–3.38)	> 150	> 1364
	DS-1	0.27 (0.14–0.53)	5.77 (1.15–28.89)	> 150	> 556
27HC	Wa	0.26 (0.22–0.31)	1.73 (1.21–2.47)	> 150	> 577
	WI61	0.19 (0.14–0.27)	0.74 (0.35–1.55)	> 150	> 790
	HRV408	0.40 (0.26–0.62)	4.56(1.62–12.82)	> 150	> 375
	HRV248	0.23 (0.12–0.46)	3.84 (0.80–18.39)	> 150	> 652
	DS-1	0.72 (0.49–1.06)	5.85 (2.43–14.06)	> 150	> 208

n.a. not assessable.
[a] EC_{50} half-maximal effective concentration.
[b] CI confidence interval.
[c] EC_{90}90% effective concentration.
[d] CC_{50} half maximal cytotoxic concentration
[e] SI selectivity index.

orthomixovirus [1,2,15–19], and non-enveloped viruses such as human rhinovirus (HRhV) [20–22], human papillomavirus (HPV), and human rotavirus (HRV) [21].

This unprecedented range of antiviral properties is ascribable to the ability of 25HC to modulate cellular lipid metabolism and transport, thereby modifying the composition and structure of cellular and subcellular membranes [23,24]. Since viral pathogens have to pass through cellular lipid membranes or hijack them to assemble their replicative machinery, regulation of the lipid composition of cellular and subcellular membranes looks like a particularly smart and effective strategy to counteract viral invasion from a strictly evolutionary point of view.

The mechanisms underlying the antiviral activity of 25HC have been extensively investigated for a number of enveloped viruses: 25HC might alter the lipid composition of cellular membranes, thus hampering the fusion between the viral envelope and the cytoplasmic lipid bilayer that allows some enveloped viruses to penetrate into the host cell [1]. This oxysterol directly changes cell membrane properties by inserting itself into the lipid bilayer [2]. Moreover, various studies on 25HC-induced host response to HCV infection clearly correlated this protective action with an imbalance in the mevalonate pathway by the oxysterol [17,25–27], implying the inhibition of cholesterol synthesis and depletion of non-sterol isoprenoid products, in this way impairing cholesterol membrane content and protein prenylation [17,25–28]. Of note, our group showed that 25HC but also a second enzymatically synthesized oxysterol, 27-hydroxycholesterol (27HC) can stimulate the release of interleukin 6 (IL6) and are endowed with an anti-HSV-1 activity which is apparently non-unrelated to the viral entry inhibition [19].

By contrast, the mechanisms underlying the protective effects of cholesterol oxidation products against non-enveloped viruses are largely unexplored, with the unique exception of HRhV [22] and 25HC as the only oxysterol examined so far [22]. In fact, this oxysterol has been shown to markedly reduce the accumulation of phosphatidylinositol 4-phosphate (PI4P) in the Golgi apparatus, a crucial event for the assembly of HRhV replicative machinery, by targeting members of the OSBP family I [20,22].

With regard to non-enveloped viruses, we previously described the antiviral activity exerted by 25HC and 27HC, particularly against HRV [21]. We investigated the mechanism of action of 25HC and 27HC against HRV to shed a light on an almost totally unexplored scenario, which may hold promise to reveal novel antiviral mechanisms and targets, likely exploitable for a new generation of antiviral molecules.

HRV belongs to the reoviridae family and represents one of the leading causes of infective gastroenteritis in children [29]. Its genome consists of 11 segments of double-stranded RNA encoding six structural proteins (VP1–VP4,VP6, and VP7) and six non-structural proteins (NSP1–NSP6). The mature virion is a triple-layered particle (TLP) about 100 nm in diameter and consists of an inner layer (the core) composed of the viral proteins VP1 and VP2, an intermediate layer (the inner capsid made of VP6), and an outer layer (the outer capsid consisting of VP7 and projections of VP4) [30,31], with VP4 being the major determinant of tropism and receptor binding [32–35].

HRVs exploit the endocytic route to enter cells. Various human strains, such as HRV Wa, WI61, and DS-1, travel along this intracellular path to reach the late endosomes (LEs) [36]. Once inside the LEs, a sequence of molecular transformations in the outer-layer proteins VP7 and VP4 strips the proteins from the virion (i.e., the TLP) and delivers into the cytosol an inner capsid particle, known as the double-layered particle or DLP [37].

Here we report on an original mechanism of antiviral action by oxysterols against HRV. We found that both 25HC and 27HC block HRV cell entry and replication by inducing an accumulation of cholesterol in the LEs, thereby sequestering viral particles inside these vesicles. These findings represent a step forward in our understanding the role oxysterols play as effectors of innate immunity against viral infections.

Results

Anti-HRV effect of 25HC and 27HC and their spectrum of activity

In a previous study, we reported on the inhibitory activity of 25HC and 27HC against the Wa strain of HRV [21]. To explore the spectrum of anti-HRV activity, 25HC and 27HC were tested against four additional HRV strains (WI61, HRV 408, HRV 248, DS-1) in MA104 cells. As shown in Table 1, the antiviral activity of both 25HC and 27HC is not strain-restricted, with EC_{50} values falling in the low micromolar range and favorable selectivity index (SI) for all the HRV strains tested. Both oxysterols inhibited HRV infection in a dose-dependent fashion (Fig. 1). The antiviral potency of 25HC was significantly ($p_{Ftest} < 0.001$) higher than that of 27HC against each strain tested. Since most of the assays of the mechanism of action described below were performed by testing 25HC and 27HC against high viruses/target cell ratios (MOI, multiplicity of action), we verified that both molecules were able to inhibit HRV infectivity also under these conditions. Indeed, they significantly inhibited HRV infectivity to a maximum of 90% even at high MOI (MOI 10) (Fig. 3). Finally, 25HC and 27HC were also tested against HRV infection of human intestinal Caco2 cell line to determine whether their antiviral activity was influenced by the cellular model. The two oxysterols displayed a strong antiviral effect in both MA104 and Caco2 cells, two quite different cellular models (Tables 1 and 2; Figs. 1 and 2), thus indicating that their activity is not cell line dependent.