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# Endosomal acidification and cathepsin L activity is required for calicivirus replication

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ABSTRACT

The role of cellular proteases and endosome maturation in the entry of caliciviruses including porcine enteric calicivirus (PEC), murine norovirus (MNV)-1 and feline calicivirus (FCV) were investigated. Treatment with chloroquine or cathepsin L inhibitors, but not cathepsin B inhibitors, significantly reduced the replication of PEC, MNV and FCV. When concentrated PEC, MNV or FCV were incubated with recombinant cathepsin L, the minor capsid protein VP2 of PEC and the major capsid protein VP1 of MNV and FCV were cleaved by the protease based on the Western blot analysis. Confocal microscopy analysis of PEC and MNV-1 showed that viral capsid proteins were retained in the endosomes in the presence of a cathepsin L inhibitor or chloroquine during virus entry. The results of this study suggest the important role of endosome maturation and cathepsin L in the entry of caliciviruses, and cathepsin L as a potential therapeutic target for calicivirus infection.



## Introduction

Caliciviruses are nonenveloped viruses of 35–40 nm in diameter and possess a single-stranded, positive-sense RNA genome of approximately 7–8 kb (Green, 2007). Caliciviruses have a $T=3$ icosahedral capsid assembled with 90 dimers of VP1 which is composed of three domains; the N-terminal arm (NTA), the S (shell) and the P (protruding) domains (Ng and Parra, 2010; Prasad et al., 1999). The P domain of VP1 forms the spike on the virion and is composed of P1 and P2 subdomains. The P2 subdomain is located within the P1 subdomain and contains a conserved region flanked by the hypervariable domains (Prasad et al., 1999). The hypervariable domains are mostly involved in receptor binding (Bhella et al., 2008; Bhella and Goodfellow, 2011; Chen et al., 2006). The function of VP2, a minor structural protein, is not well known but it was reported that VP2 is required for the production of infectious feline calicivirus (FCV) (Sosnovtsev et al., 2005), interacts with VP1 to increase stability of norovirus capsid and is likely to be associated with packaging of RNA genome (Bertolotti-Ciarlet et al., 2003, 2002.

Caliciviruses belong to the family *Caliciviridae* which comprises at least five genera including Norovirus, Sapovirus, Lagovirus, Vesivirus, and Nebovirus (Green, 2007). Noroviruses and sapoviruses cause enteric infections in humans and animals (Green et al., 2001). Lagoviruses and vesiviruses mainly cause oral, respiratory and sometimes systemic infections in animals (Green et al., 2001). Noroviruses account for about 58% of foodborne human illnesses causing about 21 million cases of gastroenteritis and 800 deaths annually in the United States alone (http://www.cdc.gov/norovirus/about/overview.html). Despite the importance of norovirus in public health, research on understanding norovirus biology and development of antiviral drugs has been greatly hindered due to the inability to grow human noroviruses in cell culture. Therefore, easily cultivable murine norovirus (MNV) (Wobus et al., 2004) and FCV (Luttermann and Meyers, 2010), in the family *Caliciviridae*, have been used as surrogate viruses for studying noroviruses.

Virus entry is a multistep process that involves consecutive interactions of cellular and viral factors including binding of virus to cellular receptors, virus uncoating and release of viral genome to initiate virus replication (Grove and Marsh, 2011; Marsh and Helenius, 2006). Virus uncoating may occur at the plasma membrane for direct penetration, or at the endosomes or other cellular compartments (Marsh and Helenius, 2006). Viruses utilize a number of different entry processes, including clathrin-dependent endocytic pathway, clathrin-independent endocytic pathway that involves caveolar or lipid, or other yet poorly defined entry pathways (Mercer et al., 2010). In clathrin-dependent endocytosis, viruses taken up by clathrin-coated vesicles travel through the endocytic

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compartments from where virus must escape before the increasingly harsher environment of maturing endosomes irreversibly degrade viruses. The journey of viruses following virus uptake to virus uncoating and genome release is less well understood for viruses that utilize clathrin-independent endocytosis (Grove and Marsh, 2011; Marsh and Helenius, 2006). However, there are reports that subsequent sorting of endosomal cargo such as viruses in different endocytic pathways may overlap in the early or late endosomes (Naslavsky et al., 2004; Sharma et al., 2003).

In the endosomal compartments, host enzymes including cathepsins are reported to be involved in virus fusion and/or uncoating of virus capsid for some viruses (Grove and Marsh, 2011). The cathepsin family of proteolytic enzymes contains several diverse classes of proteases including cysteine (cathepsins B, L, H, K, S, and O), aspartyl (cathepsin D and E) and serine (cathepsin G) proteases (Vasiljeva et al., 2007). Among them, cathepsin L, B, or S have been reported to be associated with entry and replication of some viruses including severe acute respiratory syndrome (SARS) coronavirus, murine hepatitis virus, reovirus and Ebola virus (Bosch et al., 2008; Brecher et al., 2012; Ebert et al., 2002; Mainou and Dermody, 2012; Qiu et al., 2006; Schornberg et al., 2006). Cathepsin L and B are shown to cleave Ebola virus glycoprotein, leading to exposure of putative fusion domain required for fusion of viral and endosomal membranes (Schornberg et al., 2006). Cathepsin L is also reported to cleave the spike protein of SARS coronavirus for fusion competence (Bosch et al., 2008). In addition to the enveloped viruses that require cathepsin for virus fusion and uncoating, some non-enveloped viruses are also shown to rely on host cell proteases residing in the endosomes for uncoating of virus capsid. Reovirus disassembly in the endosomes is reported to be mediated predominantly by cathepsin L and less efficiently by cathepsin B, generating infectious subvirion particles that are capable of penetrating membranes and deliver core particles into cytoplasm (Ebert et al., 2002; Mainou and Dermody, 2012). Virus entry mechanisms for caliciviruses including MNV-1, and porcine enteric calicivirus (PEC) or human norovirus are not well understood to date, although it has been shown that clathrin-dependent endocytosis and pH-dependent entry is important in FCV replication (Kreutz and Seal, 1995; Stuart and Brown, 2006). For MNV-1, it was reported that MNV-1 entry is not mediated by clathrin or caveolae, but dependent on dynamin and cholesterol (Gerondopoulos et al., 2010; Perry et al., 2009; Perry and Wobus, 2010), but detailed entry mechanism still needs further elucidation.

Here we demonstrated the importance of cathepsin L activity and endosome maturation during the entry stage of caliciviruses using PEC, FCV and MNV-1. We found that cathepsin L inhibitors, but not a B inhibitor, and chloroquine significantly reduced the replication of PEC, FCV and MNV-1. We also found that recombinant cathepsin L cleaved VP1 of FCV and MNV-1, and VP2 of PEC based on the Western blot analysis. Confocal microscopy analysis of PEC and MNV-1 showed that virus was retained in the endosomes in the presence of a cathepsin L inhibitor or chloroquine during virus entry. Our results suggest a crucial role of cathepsin L in the replication of caliciviruses, and cathepsin L as a potential therapeutic target for calicivirus infections.

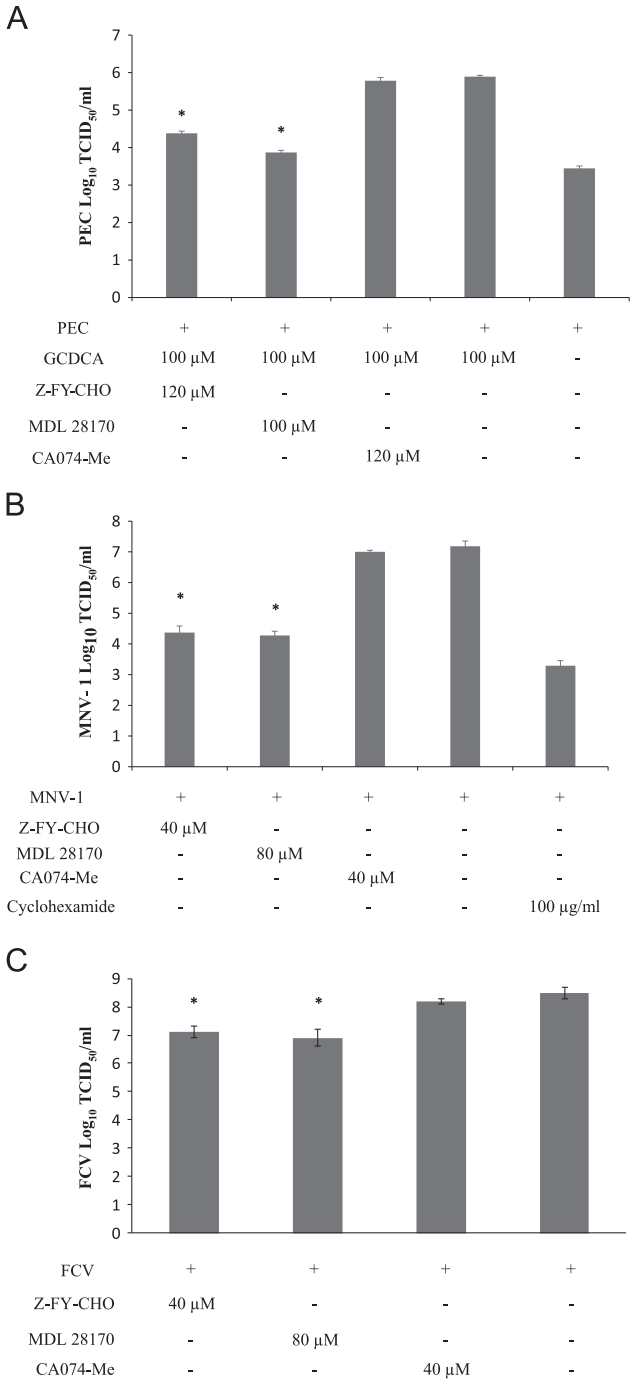


**Fig. 1.** Effects of cathepsin inhibitors in the replication of PEC, MNV-1 or FCV. LLC-PK, RAW267.4 or CRFK cells were incubated with cathepsin L inhibitors, Z-FY-CHO and MDL 28170, or a cathepsin B inhibitor, CA074-Me, for 1 h, then infected with (A) PEC (MOI 50) (B) MNV-1 (MOI 10) or (C) FCV (MOI 50) in the presence of each inhibitor. For PEC, GCDCA (100 μM) was present in the media to support virus replication during virus infection. Following virus infection for 1 h, cells were washed and the media was replaced with fresh media containing an inhibitor. Cells were further incubated at 37 °C and collected at 12 h PI. Viral replication was quantified by the $TCID_{50}$ assay. Asterisk indicates that virus titer was significantly reduced by an inhibitor compared to the control ($P < 0.05$).

## Results

### *Inhibitors of cathepsin L, but not cathepsin B, significantly reduced the replication of caliciviruses*

The role of cathepsin B and L in PEC, MNV-1 and FCV replication was studied using inhibitors of cathepsin L and cathepsin B. Our results showed that treatment of cells with inhibitors of cathepsin L (Z-FY-CHO and MDL 28170) reduced PEC titers by 32.6–106-fold, MNV-1 titers by 649.4–801.2-fold, and FCV titers by 25.1–39.8-fold (Fig. 1A–C). However, cathepsin B inhibitor CA074-Me at up to 120 μM did not lead to a significant reduction of viral replication (Fig. 1A–C). Glycochenodeoxycholic acid (GCDCA), a bile acid, was added to culture media for PEC replication, since PEC