Natural compounds as inhibitors of SARS-CoV-2 endocytosis: A promising approach against COVID-19

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Abstract. *Background and aim:* The recent COVID-19 pandemic caused by SARS-CoV-2 affected more than six million people and caused thousands of deaths. The lack of effective drugs or vaccines against SARS-CoV-2 further worsened the situation. This review is focused on the identification of molecules that may inhibit viral entry into host cells by endocytosis. *Methods:* We performed the literature search for these natural compounds in the articles indexed in PubMed. *Results:* Natural products against viral infections have been gaining importance in recent years. Specific natural compounds like phytosterols, polyphenols, flavonoids, citrus, galangal, curcuma and hydroxytyrosol are being analyzed to understand whether they could inhibit SARS-CoV-2. *Conclusions:* We reviewed natural compounds with potential antiviral activity against SARS-CoV-2 that could be used as a treatment for COVID-19. (www.actabiomedica.it)

Key words: SARS-CoV-2, COVID-19, natural antiviral compounds, endocytosis

Introduction

Coronaviruses (CoVs) belong to the *Coronaviridae* family and *Nidovirales* order. They are enveloped viruses with a positive, single-stranded RNA genome (1). Coronaviruses are divided into three sub-groups: α-CoVs, β-CoVs and γ-CoVs (2). The pandemic that arose late in 2019 in Wuhan (China) was caused by a new β-CoV strain. The virus was called severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) and the associated disease was called coronavirus disease-19 (COVID-19). The most common clinical features of COVID-19 include cough, fever and pneumonia (3). According to WHO reports, SARS-CoV-2 has currently infected more than six million people worldwide and almost 400,000 people have died in the pandemic (WHO website - www.who.int; accessed on May 30, 2020).

The genome of SARS-CoV-2 is almost 29 kb long and has 10 open reading frames. Its 3' terminal region encodes structural proteins like spike, envelope and nucleocapsid proteins. The 5' terminal region encodes two replicase polyproteins, pp1a and pp1b. The spike glycoprotein of SARS-CoV-2 plays a significant role in viral infectivity (4). It consists of a receptor-binding domain that identifies the target receptor of SARS-CoV-2, i.e. angiotensin-converting enzyme 2 (ACE2), and plays a significant role in the fusion of membranes during endocytosis. In view of its importance, the spike glycoprotein may be a good target for preventing entry of SARS-CoV-2 into host cells (5).

Protease inhibitors have also been proposed as possible therapeutic targets for inhibition of the viral life cycle. β-CoV uses these proteases to cleave the structural proteins required for viral reformation and packaging in host cells (6).

The identification and development of effective antiviral compounds is of fundamental importance to combat COVID-19 (7). The aim of this review is to discuss the implications of the endocytic pathway in the pathogenicity of SARS-CoV-2 and the therapeutic potential of targeting this process (8).

The endocytic pathway and its role in SARS-CoV-2 infection

Coronaviruses require fusion of the plasma membrane via endocytosis to enter the host cell. Cholesterol and lipid rafts are major contributors to endocytosis (9). The genome of coronaviruses mostly encodes four major structural proteins: the spike (S) glycoprotein, the membrane glycoprotein, the nucleocapsid protein and the envelope protein (10). The spike glycoprotein is mostly involved in the process of viral entry into host cells by proteolytic cleavage of spike protein into two subunits (S1 and S2) (11). The S1 subunit is involved in receptor-binding, while the S2 subunit is required for membrane fusion (11).

The spike glycoprotein of SARS-CoV-2 binds the ACE2 receptor of human respiratory epithelial cells. At the time of attachment, the spike glycoprotein is cleaved into S1 and S2 subunits. The S1 subunit includes the receptor-binding domain that facilitates viral binding to the ACE2 receptor peptidase domain, while the S2 subunit mediates plasma membrane fusion (9).

Considering the significance of the endocytic pathway for viral entry into host cells, therapeutic strategies that target the endocytosis process may offer incredible opportunities for the development of treatments for COVID-19 (8).

Soluble ACE2 as a main candidate for SARS-CoV-2 inhibition

ACE2 is predominantly expressed in heart, lungs, testes and kidneys and works as a negative regulator of the renin-angiotensin-aldosterone pathway. It also binds to the amino-acid transporters and plays a significant role in amino-acid absorption in the gut and kidneys (12).

Soluble ACE2 (sACE) is a variant of ACE2 that lacks the transmembrane domain but retains enzyme activity and binds the SARS-CoV spike glycoprotein (13). Thus sACE2 can antagonize binding of transmembrane ACE2. Since the mechanism of infection of SARS-CoV is identical to that of SARS-CoV-2, it is reasonable to think that sACE2 can also inhibit

SARS-CoV-2 infection. Soluble ACE2 could also be used as an effective treatment for patients with pneumonia and respiratory distress syndrome due to SARS-CoV-2 infection (14).

Interestingly, the conjugation of sACE2 with cyclodextrins (cyclic oligosaccharides with a macrocyclic pyranose ring of glucose subunits attached by α-1,4 glycoside bonds) significantly increases the water solubility of sACE2 (15). Nasal and eye drops containing cyclodextrin-sACE2 could therefore block coronavirus infection directly in the nasal cavity and conjunctiva (12).

Lipid rafts as target for SARS-CoV-2 inhibition

Lipid rafts are distinct lipid domains in the external leaflet of the plasma membrane and are rich in glycosphingolipids, cholesterol, glycosylphosphatidylinositol-anchored proteins and signaling proteins (16). They take part actively in a variety of molecular processes like disruption of parasitic bacterial and viral infections, signal transduction, cell to cell communication, immune response initiation, membrane transport and apoptosis (17). Numerous studies have also reported that lipid rafts are required at many stages in viral life cycles. For example, human immunodeficiency virus type-1 (HIV-1), Semliki Forest virus and various human enteroviruses need lipid rafts for binding and internalization into their host cells (18). Associations of certain viral receptors and co-receptors with lipid rafts have been reported on cell membranes like CD4 receptor for HIV-1, heat shock protein-70 and -90 receptors for Dengue virus and integrin avb3 co-receptor for human cytomegalovirus. The microenvironments of lipid rafts are also used for viral assembly and release. For instance, in the influenza virus, the cytoplasmic tail and transmembrane domain of the viral protein hemagglutinin help the binding of lipid rafts. In the same way, it has been reported that lipid rafts are necessary for HIV-1 and Sendai virus assembly. In HIV-1, the Nef protein is reported to increase HIV-1 infectivity through lipid rafts. It is therefore well known that lipid rafts may contribute significantly to the replication and infectivity of viruses (18).

Another study analyzed the significance of lipid

rafts for SARS-CoV replication in the Vero-E6 cell line. It examined the significance of cell membrane lipid rafts in the early stages of SARS-CoV replication (18). Since cholesterol-rich microdomains of lipid rafts facilitate the interaction of spike glycoprotein of coronaviruses with the ACE2 receptor of the membrane, lipid rafts are major cell membrane microdomains involved in the coronavirus internalization process. Other studies confirmed these findings, establishing that the homeostatic regulation of cholesterol and the regulation of fatty acid metabolism are involved in coronavirus infectivity (9).

Endocytosis inhibition by methyl-β-cyclodextrin

Methyl-β-cyclodextrin (MβCD) is a macromolecule with cholesterol-depleting activity involved in the inhibition of coronavirus attachment to host cells. The hydrophobic cavity of the macromolecule enables its interaction with lipid rafts. Numerous viruses like HIV, murine leukemia virus, herpes simplex virus, murine hepatitis virus and infectious bronchitis virus, as well as human coronaviruses like SARS-CoV and 229E, are sensitive to reductions in cell membrane cholesterol content (19), so depletion of cholesterol in cell membranes decreases viral infectivity (20).

Higher concentrations and prolonged exposure of MβCD may lead to cholesterol redistribution among raft and non-raft cell membrane regions. *In silico* cell modeling of ACE2 membrane protein showed that MβCD-mediated cholesterol depletion resulted in fewer bonds between ACE and viral spike glycoproteins. Besides decreasing cholesterol levels, this macromolecule also affects ACE2 receptor expression. Thus, the reduction in cell membrane ACE2 expression due to treatment with MβCD also results in a reduction of coronavirus infectivity (21).

Natural compounds as inhibitors of SARS-CoV-2

Natural compounds have gained importance as potent anti-viral agents in recent years. Considering the immediate need for therapeutics for SARS-CoV-2 infection, we reviewed possible natural compounds.

Phytosterols

Phytosterols or plant sterols are natural plant sterol molecules with cholesterol-like structure. They mostly interact with lipid raft components of the cell membrane. This interaction results in a lowering of cell membrane cholesterol or in destabilization of lipid raft structure. The phytosterol-lipid raft interaction can also affect biochemical signaling taking place downstream of the lipid rafts. Other plant sterols, like β-sitosterol, decrease the infectivity of the hepatitis B virus and HIV. Thus a phytosterol-dependent therapeutic approach could decrease the attachment of viruses to host cells (22).

Flavonoids

Flavonoids are natural biomolecules with antiviral properties extracted from plants (23). Flavonoid structure includes a 15-carbon phenyl-benzopyrone backbone consisting of two benzene rings connected through a heterocyclic pyrene ring. Flavonoids can be divided into flavone, flavan, flavanone, flavonol, anthocyanidin, biflavanoid and isoflavanoid classes. There are also flavonoid derivatives, glycosides and aglycones, and methylated derivatives (23,24).

Extensive studies have analyzed the effects of flavonoids on a large number of DNA and RNA viruses. The mechanism of action of flavonoids includes blocking viral attachment and entry into host cells, obstruction of viral replication at various stages, and inhibition of translation or processing of the polyprotein necessary for viral release to infect more cells. Various mechanisms of action are likewise involved in viral inhibition by different flavonoids (25).

Flavonoids with antiviral activity can be further divided into: a) flavonoids binding particular extracellular viral regions like viral capsid proteins, b) flavonoids preventing viral attachment and entry into host cells, c) inhibitors of early stage replication, d) inhibitors of transcription and translation, e) inhibition of maturation, assembly, packaging and release (25).

Flavonoids like kaempferol and its derivatives can noncompetitively inhibit the enzyme neuraminidase, although the exact mechanism of action is not under-

stood (26). Further *in vitro* studies have reported that kaempferol inhibits coronaviruses by blocking the 3a viral channels involved in the assembly and release of viral progeny (27).

Other studies report that luteolin interferes with the entry process of SARS-CoV and influenza A virus through its interaction with S2 protein and hemagglutinin, respectively (28). Furthermore, epigallocatechin, a flavonoid extracted from tea catechins, inhibits influenza A and B viruses by *in vitro* lysosomal acidification and/or acidification of the endosomal micro-environment by clathrin-mediated endocytosis. Similarly, epigallocatechin gallate displays antiviral activity against influenza virus, herpes simplex virus and Zika virus by damaging their lipid envelope. Epigallocatechin gallate also stimulates lysosomal acidification, creating an environment unfavorable for viral replication (29).

Another study found that formononetin inhibits the entry of enterovirus A71 into Vero cells, while quercetin reduces rhinovirus endocytosis (25). Likewise, Yu et al. reported that myricetin acts as a chemical inhibitor of SARS-CoV due to its effects on viral helicase ATPase activity (30).

Animal studies with rat models revealed that flavonols conjugated with phospholipids have higher bioavailability when administered orally (31). The use of mouth washes and nasal spray containing a combination of β-cyclodextrins and bioflavonoids (also called Citrox) decreases the viral load of SARS-CoV-2 in the nasopharyngeal microbiota. The microbiota coats the droplets and aerosol particles produced during sneezing or coughing. More clinical trials are needed to assess the protective effects of these mouth washes in decreasing viral load and slowing the spread of infection (32).

Influence of flavonoids on SARS-CoV-2 main protease

The SARS-CoV-2 main protease (M^{pro}), required for viral proteolytic maturation, is considered a potential target to inhibit replication of the virus (33). The flavonoids rutin and hesperidin have high M^{pro}-binding affinity. Hesperidin is found in sweet oranges, grapefruits and lemons, whereas rutin is mostly found in apples and tea (34). More studies with *in vivo* and *in vitro* models are required to validate the use of these flavonoids and their derivatives (7).

Influence of flavonoids on cell membranes and lipid rafts

Flavonoids can influence the phase transition and lateral lipid segregation required for lipid raft formation. The influence of flavonoids on lipid bilayer physical properties may control the membrane protein arrangement and functional complex formation responsible for signal transduction and metabolic regulation (17,35).

Flavonoids in the hydrophobic region of the lipid bilayer have the ability to initiate raft-like domain formation, called the raft-making effect, whereas flavonoids located at the polar interface of the lipid bilayer tend to fluidize the membrane, called the raft-breaking effect, or initiate the formation of micellar or interdigitated structures. Flavonoids in cell membranes are therefore expected to affect the formation of lipid rafts or the raft-like domains of cell membranes, consequently affecting the lateral diffusion of lipid molecules (17,36).

Prevention of membrane fusion is one way in which flavonoids protect an organism against viral infection. For instance, the two diastereoisomeric flavonolignans, silybin A and B, prevent hepatitis C virus infection of the liver by targeting several steps in the viral life cycle, preventing viral particles from fusing with cell membranes (35).

Dietary flavonoids are membrane-active antioxidant agents that take part in a wide range of cell signaling events. They target specific receptors on the cell membrane or intercalate in the membrane lipid bilayer. Some flavonoids increase lipid viscosity while decreasing hydrocarbon chain melting cooperativity; others significantly decrease lipid melting temperature, providing extra freedom for lipid diffusion (17,37).

Several studies have analyzed the ability of flavonoids to interact with cell membrane lipid rafts and caveolae. Epigallocatechin gallate from green tea has been found to affect cell signaling and is involved in the attenuation of endothelium cell inflammatory

processes by reducing cyclooxygenase COX-2 and caveolin-1 expression and inhibiting Akt and ERK 1/2 kinases of the mitogen-activated protein kinase (MAPK) signaling pathway (38).

Hydroxytyrosol as an antiviral agent

Hydroxytyrosol is a phenolic compound with antioxidant properties found in the leaves and fruits of the olive (*Olea europaea*). It is a metabolite of oleuropein, the major polyphenolic constituent of olive derivatives. Hydroxytyrosol is already known for its antiviral activity against various subtypes of influenza A virus, such as H1N1, H3N2, H5N1 and H9N2. A viral envelope is required for the antiviral activity of hydroxytyrosol. Oleuropein and hydroxytyrosol inhibit the fusion of viruses with cell membranes (39). Since SARS-CoV-2 is an enveloped virus with spike glycoproteins, hydroxytyrosol may inhibit its endocytosis. Additional research is required into the antiviral activities of hydroxytyrosol towards this coronavirus.

Conclusion

The recent outbreak of COVID-19 caused a public emergency of international concern, for which no drugs or vaccines are currently available. This review focuses on finding possible molecular targets for inhibiting the spread and breaking the circle of SARS-CoV-2 transmission. Endocytosis takes place in the early phases of coronavirus infection and is directly involved in viral infectivity. Various molecular targets involved in this process, like ACE2 receptors, lipid rafts and proteases, are explored and the action of inhibitors such as cyclodextrin and phytosterols, and natural inhibitors such as flavonoids, citrus, galangal, curcuma and hydroxytyrosol, are discussed. In particular, flavonoids seem very promising for developing COVID-19 therapeutic strategies.

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

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