REVIEW

Insight into the role of clathrin-mediated endocytosis inhibitors in SARS-CoV-2 infection

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Abstract

Emergence of SARS-CoV-2 variants warrants sustainable efforts to upgrade both the diagnostic and therapeutic protocols. Understanding the details of cellular and molecular basis of the virus–host cell interaction is essential for developing variant-independent therapeutic options. The internalization of SARS-CoV-2, into lung epithelial cells, is mediated by endocytosis, especially clathrin-mediated endocytosis (CME). Although vaccination is the gold standard strategy against viral infection, selective inhibition of endocytic proteins, complexes, and associated adaptor proteins may present a variant-independent therapeutic strategy. Although clathrin and/or dynamins are the most important proteins involved in CME, other endocytic mechanisms are clathrin and/or dynamin independent and rely on other proteins. Moreover, endocytosis implicates some subcellular structures, like plasma membrane, actin and lysosomes. Also, physiological conditions, such as pH and ion concentrations, represent an additional factor that mediates these events. Accordingly, endocytosis related proteins are potential targets for small molecules that inhibit endocytosis-mediated viral entry. This review summarizes the potential of using small molecules, targeting key proteins, participating in clathrin-dependent and -independent endocytosis, as variant-independent antiviral drugs against SARS-CoV-2 infection. The review takes two approaches. The first outlines the potential role of endocytic inhibitors in preventing endocytosis-mediated viral entry and its mechanism of action, whereas in the second computational analysis was implemented to investigate the selectivity of common inhibitors against endocytic proteins in SARS-CoV-2 endocytosis. The analysis revealed that remdesivir, methyl-β-cyclodextrin, rottlerin, and Bis-T can effectively inhibit clathrin, HMG-CoA reductase, actin, and dynamin I GTPase and are more potent in inhibiting SARS-CoV-2 than chloroquine. CME inhibitors for SARS-CoV-2 infection remain understudied.

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry into the host cell is crucial for viral infectivity. It is well-established that the SARS-CoV-2 spike (S) glycoprotein interacts with the host cell surface receptors. Many previous studies have used purified spike (S) glycoprotein and lentivirus pseudo-typed with spike glycoprotein and found that SARS-CoV-2 undergoes rapid clathrin-mediated endocytosis (CME) to gain entry into cells.[1–4] This scenario is quite similar to the paradigms of receptor-mediated endocytosis (RME), in which cells internalize their own liganded surface receptors for signal management or to uptake nutrients, such as iron, and cholesterol, from their microenvironment. Although CME is regarded as the most common pathway for virus entry, some reports suggested that different viruses may adopt other routes to infect host cells, such as the transmembrane serine protease 2 (TMPRSS2) pathways and caveolae-mediated endocytosis (CvME).[5] Organs, especially the lung and intestines, for example, are highly susceptible to viral infection owing to the large amounts of TMPRSS2 found in their cell membranes.[6,7] Moreover, the virus may utilize an endocytic pathway when TMPRSS2 is absent.[7] These mechanisms provide efficient routes that enable the virus to evade host immune surveillance, gain sustainable intracellular replication, and develop the associated pathogenesis. The recent SARS-CoV-2 pandemic, with its associated high mortality rate, elicited tremendous research efforts to explore the biology, viral–host cell interactions, and screening methods for the virus and to suggest therapeutic approaches via either vaccination or the use of antiviral drugs. The rapid appearance and transmission of new SARS-CoV-2 variants as well as the associated high mortality rate have imposed more challenges on research communities to modify existing detection methods and therapeutic protocols.[8–11] Although vaccination strategies have become the gold standard for preventing SARS-CoV-2 infections, challenges have emerged because of mutational variants gaining increased infectivity potential and becoming less responsive to vaccines.[12,13] Recent studies compared the titer of neutralizing antibodies of natural SARS-CoV-2 infection and Sinovac vaccination to evaluate the efficacy of vaccination. People vaccinated with Sinovac were found to have a lower titer of neutralizing antibodies than the naturally infected patients,[14] indicating that live SARS-CoV-2 virus may induce a stronger immune response than the inactivated virus. Heterogeneous vaccination may be required to induce a strong immune response against the virus. Several vaccines that are effective against SARS-CoV-2 variants have been developed, such as molnupiravir. Molnupiravir can inhibit the replication of SARS-CoV-2 by inducing lethal mutagenesis.[15] Its bioavailability and tolerability have been confirmed in clinical trials. Although molnupiravir has been shown to be effective against SARS- CoV-2, its genotoxicity is concerning; thus, longer-term clinical trials are needed to complete the safety evaluation.[15] This increases the importance of developing variant-independent therapeutic strategies. Because of the close resemblance between ligand–receptor CME and the proposed SARS-CoV-2 entry route, endocytic proteins serve as potential anti-SARS-CoV-2 targets. Even before the emergence of SARS-CoV-2, this infection route was determined to be the preferred route for other viruses such as human hepatitis C virus (HCV),[16] enterovirus 71,[17] Ebola virus, Dengue-3 virus,[18] and many others. The clathrin-independent endocytosis (CIE) has also been proposed as an additional route of SARS-CoV-2 cell entry.[19] The SARS-CoV-2 outbreak accelerated the use of endocytic proteins involved in CME, making endocytic proteins and other cellular entry pathways antiviral targets for potentially blocking SARS-CoV-2 entry into host cells.[20] CME inhibitors have been shown to inhibit spike (S) protein endocytosis and pseudo-virus infectability in recent studies. This is accomplished by preventing the formation and disassembly of clathrin lattices on cytoplasmic surfaces and endosomes.[5] In the same context, destruction of clathrin heavy chains decreases the internalization of viral spike protein.[20] Additionally, inhibitors targeting dynamins I and II and dynamin-related protein-1 (Drp-1) disrupted the endocytic pathway and prevented viral entry through CME pathways.[21] Other studies revealed that inhibition of β-arrestin blocked viral host cell entry.[20,22] Accordingly, this review summarized endocytic protein inhibitors that potentially block SARS-CoV-2 entry through endocytic pathways, with a focus on the most common CME-related proteins. Additionally, molecular docking analysis of these inhibitors was conducted to provide a predictive model of their relative potential as anti-SARS-CoV-2 therapeutic drugs.

2 | ENDOCYTIC PATHWAYS AND TRAFFICKING

2.1 | Phagocytosis and pinocytosis

Phagocytosis is a mechanical processes that allows the ingestion of particles larger than 0.5 μm, such as apoptotic cells, foreign substances, and microorganisms.[23,24] Phagocytosis occurs via a series of well-studied steps, which include phagocyte detection, recognition of foreign molecules, and movement toward the target cargo for elimination. After physically binding to the targets, pseudopodia formation is initiated, and the pseudopodial membrane fuses to form a vesicle with the targeted material inside, thus forming a phagosome.[24] Finally, the phagosome is merged with a lysosome, where its components are enzymatically hydrolyzed.

Conversely, pinocytosis is used to uptake small particles (less than 0.1 μm) and to ingest fluid by modulating the cellular plasma membrane without pseudopodia formation.[25] Pinocytosis occurs

through the formation of vesicles, followed by endocytosis into the cell and fusion with lysosomes to achieve complete digestion Figure 1. Based on particle size, pinocytosis is classified into micropinocytosis and macropinocytosis. The former occurs via the formation of a vesicle bud from the cell to engulf a small particle with a diameter of ≤0.1 μm, whereas the latter occurs indirectly without the formation of vesicles and is commonly observed in white blood cells that engulf larger particles (0.5–5 μm).

2.2 | CME involves a machinery of proteins, adaptors, and modifiers

In response to extracellular stimuli, such as hormones and growth factors, and to changes in the cellular microenvironment containing nutrients and oxygen, the relative abundance of cell surface receptors is accommodated to perform a range of functions, such as nutrient uptake and signaling. The relative abundance of these receptors is mainly controlled by their endogenous expression, trafficking, and internalization via CME.[26,27] The latter mechanisms which include endogenous expression, trafficking, and internalization regulates receptor internalization, recycling, and degradation. These processes require several endocytic cellular proteins that sequentially function to engulf liganded receptors into a fully formed endosome containing ligand–receptor complexes.

CME occurs through two approaches, clathrin-coated pits (CCPs) and clathrin-coated vesicles (CCVs).[20,28] CME is primarily a clathrin- and dynamin-dependent mechanism and includes several other proteins, such as β-arrestin, and adaptor proteins, such as adaptor protein-2 (AP2). The process involves a series of steps, including initiation of CCPs, followed by their stabilization and maturation, and finally, membrane fission. The AP2 heterotetramer, comprised of α, β2, μ2, and σ2 subunits, plays a fundamental role in the initiation of CCPs.[29] Thereafter, scaffolding molecules, including FCHo and eps15, are recruited to participate in either nucleating or stabilizing nascent CCP. This process aids the newly formed CCP to develop into a CCV.[28] The second stage of CCP is maturation and fission and is dependent on regulatory proteins such as amphiphysin, endophilin, Wiskott–Aldrich Syndrome Protein (N-WASP), cortactin, myosin 1E, sorting nexin 9 (SNX9), synaptojanin, phosphatidylinositol 3-kinase C2α (PI3KC2α), and phosphatidylinositol phosphate (PIP5K).[30,31] Dynamin, a large GTPase, is activated at low levels in nascent CCPs and regulates CCP initiation and maturation.[32] It then forms short helical rings around the necks of deeply invaginated CCPs to catalyze their membrane fission. In CCVs, dynamin is used by proteins with Bin–Amphiphysin–Rvs (BAR) lipid specificity domains, including amphiphysin, endophilin, and SNX9.[33] Subsequently, GTP hydrolysis occurs to drive membrane fusion. After the detachment of the vesicle from the plasma membrane, both heat shock cognate 70 (HSC70) and its cofactor auxilin disassemble the clathrin coat.[28] This allows the uncoated vesicle to progress to lysosomal fusion or be recycled to the cell membrane. For lysosomal fusion, multiple homotypic fusion events occur. This step involves cyclin G-associated kinase (GAK), HSC70, and oculocerebrorenal Lowe syndrome protein (OCRL).[34] Lysosomal fusion transports the CCV cargo to early endosomes. The internalization of low-density lipoprotein (LDL) receptors and iron-
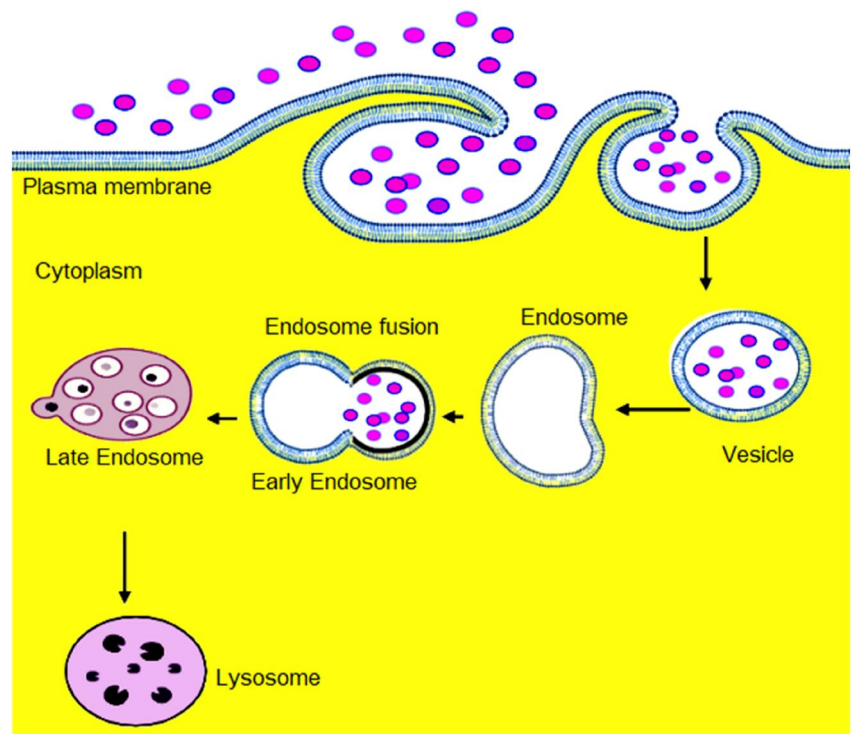

FIGURE 1 Pinocytosis pathway in which the cell absorbs fluid by invagination and formation of a separate tiny vacuole around each droplet called a vesicle
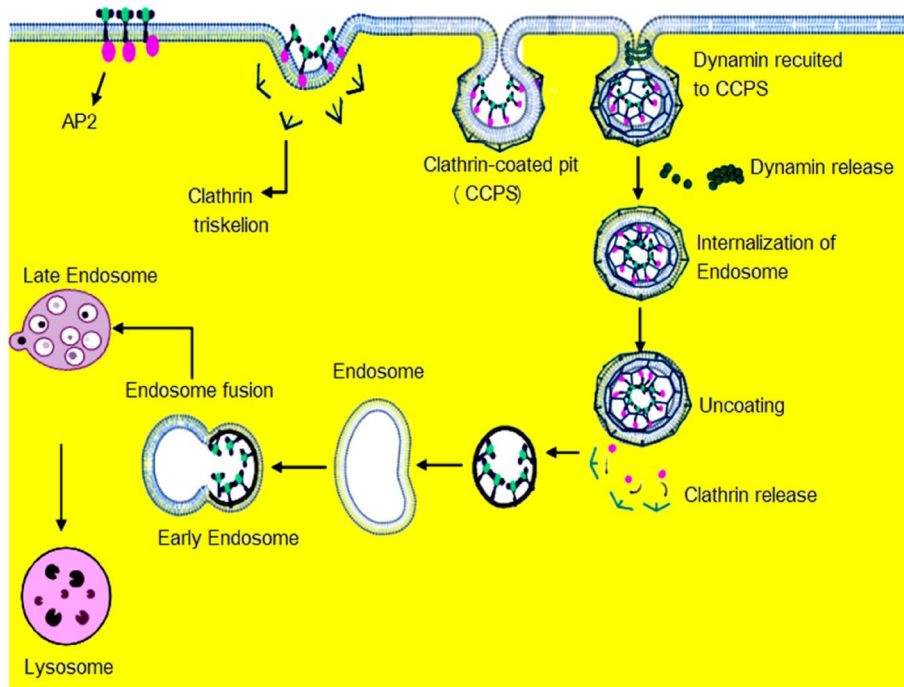
FIGURE 2 Clathrin-dependent endocytosis pathways in which the metabolites, proteins and viruses transported into the cell through binding to cell surface receptor. The molecule-receptor complex enters the cell by in-folding of the plasma membrane, which eventually gets pinched off into a vesicle. During receptor-mediated endocytosis (RME), endocytic proteins including, AP2, and clathrin triskelion coats the budding vesicle give spherical form called clathrin-coated pits (CCPs) and the dynamin recruited to the CCPS. Then the dynamin releases from CCPS forming endosome and un-coating the clathrin release and bind to the lysosome.

binding protein are the best examples of CME, which enable the cellular uptake of LDL cholesterol and iron from the microenvironment Figure 2.

2.3 | Caveolae-mediated endocytosis (CvME)

Caveolae/glycolipid raft-mediated endocytosis is the best-characterized clathrin-independent but dynamin-dependent endocytic pathway.[35] Caveolae are flask-shaped plasma membrane invaginations with a diameter of 50–80 nm. Caveolins, considered the main structural proteins, are members of the caveolin gene family and comprise caveolins 1, 2, and 3. Caveolar membranes are enriched with sphingolipid, cholesterol, signaling proteins, and clustered glycosylphosphatidylinositol-anchored proteins (GPI-APs).[36] Caveolar-mediated endocytosis occurs when foreign particles, such as viruses, adhere to the membrane and are trapped in the caveolae. This event initiates a signal transduction cascade in which certain proteins are phosphorylated, resulting in the depolymerization of the cortical actin cytoskeleton of the caveolae cargo, which is followed by the recruitment of actin monomers, forming an actin patch. Concurrently, dynamin is recruited to the caveolae cargo, causing it to be detached from the cell membrane and translocated to the cytosol.[37] According to some reports, endocytic cargo can be internalized by various mechanisms in different cell types or it can switch

pathways in a single cell type under certain conditions. In human skin fibroblasts and endothelial cells, for example, albumin is internalized by caveolae, whereas in Chinese hamster ovary cells, it is internalized via a RhoA-dependent mechanism.[35,38]

2.4 | CLIC/GEEC endocytic pathway

The involvement of crescent-shaped tubular clathrin-independent carriers (CLICs) that evolve into glycosylphosphatidylinositol (GPI)-anchored protein-enriched early endocytic compartments (GEECs) is referred to as the CLIC/GEEC pathway, which is CIE. This pathway is regulated by some proteins including small GTPases, such as Arf1 and CDC42,[39,40] GTPase activating factor GRAF1,[41] actin nucleation factor ARP2/3,[42] and the BAR domain protein IRSp53. These regulators are involved in the uptake of cargo such as hyaluronic acid receptor/CD44, GPI-anchored proteins (CD59 and Thy-1), and cholera toxin.[43] Notably, CLIC/GEEC endocytosis is dynamin-independent for endogenous cargo and not strictly dynamin-dependent for exogenous cargo such as toxins.[44,45] The CLIC/GEEC pathway, unlike micropinocytosis, is a high-capacity pathway in which the internalized fluid phase is delivered to the cell. Furthermore, in several cell types, the CLIC/GEEC pathway is responsible for the rapid recycling of cell membranes. For instance, the entire cell membrane of mouse embryonic fibroblasts is recycled in less than

15 min via the CLIC/GEEC pathway. In addition to nutrient and toxin uptake, the CLIC/GEEC pathway is recognized as a portal for viral infection.[46]

2.5 | Flotillin-dependent endocytosis

Flotillin proteins are ubiquitous in cells and play several roles, including cell adhesion, signal transduction through tyrosine kinases receptors, and cellular trafficking via pathways involving flotillin-1/reggie-2 and flotillin-2/reggie-1.[47] Flotillins are acylated at the N-terminal stomatin/prohibitin/flotillin/HflK/C (SPFH) domain and oligomerized at the C-terminal (flotillin domain). Therefore, they constitutively associate with cholesterol-enriched lipid microdomains, which participate in endocytosis and other cellular trafficking processes. Flotillin microdomains are dynamic, and upon stimulation, specifically cluster cargo molecules, such as Alzheimer's amyloid precursor protein, dopamine transporter, and epidermal growth factor receptor, at the plasma membrane into flotillin rafts for pre-endocytic cluster formation before endocytosis. Notably, following their clustering, cargo molecules are endocytosed without any involvement of flotillin. Flotillins have been shown to be functionally dependent on each other, where deletion of one perturbs the stability of the other. Flotillin-1 seems to be more dependent on flotillin-2.[48] For instance, in neuroblastoma cells and primary hippocampal neurons, depletion of flotillin-2 impairs amyloid precursor protein endocytosis,[49] whereas flotollin-1 remains intact.

2.6 | Post-endocytic events and factors controlling endocytosis

Typically, after macromolecules are internalized by one of the above-described pathways, they are sorted by endosomes,[50] where they may either be recycled to the plasma membrane, degraded by lysosomes, or sent across the epithelium, endothelium, and blood–brain barrier through endocytosis.[50,51] Moreover, the endocytic process is involved in cell signaling from surface receptors such as G protein-coupled receptors (GPCRs), neurotransmission, and viral infections. Endocytic pathways are multistep mechanisms and are controlled at several stages including scission mechanisms, coat-associated protein assembly, and in the integration of these steps.[52] In such ordered events, the cell membrane and the soluble endocytic machinery are involved in endocytosis and endosome maturation and trafficking. Endocytic trafficking relies on membrane structural heterogeneity, including the presence of specific lipid and derived lipid components as well as protein domains. This structural pattern plays a pivotal role in defining a region of the membrane to initiate the endocytic event.[52] Endocytic pathways participate in the propagation of membrane heterogeneity, which switches phosphoinositide species throughout the endocytic trafficking process. These metabolic changes are important for the regulation of each step of the intracellular sorting process following endocytosis.[53]

3 | TYPES OF ENDOCYTIC PROTEIN INHIBITORS

Endocytic inhibitors include various low-molecular-weight compounds and peptides. A standardized classification system is yet to be established; however, these inhibitors may be subgrouped according to their chemical nature, therapeutic use, sources, or mode of action as follows: chemical, pharmacological, natural, and genetic inhibitors.[54,55] This primitive categorization system is based on the available literature in which various endocytic pathways were targeted. Other classification approaches may consider their targeted endocytic pathway or their selective cellular proteins. This may include inhibitors known to interfere with CME, lipid rafts, CIE, macropinocytosis, phagocytosis, and β-arrestin.[54] Additional suggested classification approaches may differentiate between clathrin- and/or dynamin-dependent pathways, in which case other intervening factors should be considered, such as evidence-based selectivity (specific vs. non-specific), sustainable effects (reversibility), and the affected subcellular organelles.

3.1 | Chemical inhibitors

Chemical inhibitors include various molecules and peptides known to inhibit CME, CvME, CIE, CLIC/GEEC, phagocytosis, and micropinocytosis Table 1. Chemical inhibitors may alter the cellular physiology (physiological modifiers) or directly interact with integral endocytic proteins. Physiological modifiers non-specifically inhibit the endocytic pathway by altering the normal physiological conditions of cells and/or subcellular organelles. For example, clathrin lattices on the plasma membrane may be inhibited by chemical inhibitors such as hypertonic sucrose and 2-deoxy-d-glucose/sodium azide, which trap clathrin in microcages.[56] Endocytosis may also be inhibited by chemical inhibitors that reduce ATP and NADH levels by inhibiting glycolysis.[57] Another chemical inhibitor, monensin, inhibits the endocytic pathway through disruption of the proton gradient.[58] Other chemical inhibitors directly and specifically interact with specific endocytic proteins, including arrestin and dynamins. Barbadin, for example, inhibits the molecular interaction between β-arrestin and AP2.[59,60] Additionally, the GTPase activity of dynamins can be targeted by several compounds including Rhodadyn-D10, Naphthaladyn 29, Dynole, Dyngo, and Dynasor. These compounds act as competitive inhibitors in the GTP domain and inhibit the GTPase activity of Dyn 1.[61] Similarly, bisphosphonates can inhibit the effect of Dyn II by inhibiting GTPase activity.[62] Disrupting cell membrane structural integrity may be another mechanism for inhibiting endocytosis via a different route, considering the critical role of bisphosphonates in the plasma membrane in the endocytic pathway. Other chemical inhibitors, such as methyl-β-cyclodextrin and nystatin, remove cholesterol from the plasma membrane and interfere with the fluid phase, thereby inhibiting CvME.[63] Additionally, the chemical inhibitors filipin, LG186, and 7-keto-cholesterol interact with membrane cholesterol and prevent the close packing of acyl chains, resulting in inhibition of the CLIC/GEEC pathway.

TABLE 1 Summary of chemical inhibitors with their mode of actions

No	Endocytosis of inhibitors	Mode of action	Target for inhibition	Summary	References
	Chemical inhibitor				
1	Hypertonic sucrose	Inhibition takes place by the dispersion of clathrin lattices on the plasma membrane. It also traps clathrin in microcages.	CME	Nonspecific inhibitor. affects non-clathrin-mediated internalization pathway and interferes with fluid phase macropinocytosis.	57,160–162
2	Cytosol acidification	Inhibits the scission of the clathrin pits from the membrane.	CME	Nonspecific inhibitor interferes with macropinocytosis and the actin cytoskeleton.	99,163,164
3	Potassium depletion	Activates the aggregation of clathrin.	CME	Nonspecific inhibitor and affects the actin cytoskeleton.	164,165
3	2-Deoxy-d-glucose/ Sodium azide	Decreases ATP and NADH by inhibiting glycolysis.	CME	-	57,166
4	Barbadin	Inhibits β-arrestin/AP2.	CME	Blocks agonist-promoted endocytosis of the prototypical β2-adrenergic (β2AR), V2-vasopressin (V2R) and angiotensin-II type-1 (AT1R) receptors.	60,61,168
5	Monodansylcadaverin	Stabilizes clathrin-coated vesicles.	CME	Causes changes in actin dynamics.	168-170
6	Phenylarsine oxide	Inhibits O_2 consumption and decreases cellular ATP content overlap with those used to inhibit protein internalization.	CME	Inhibits macropinocytosis and phagocytosis.	172–174
7	Bolinaquinone	Affects the function of clathrin-coated vesicles.	CME	–	175,176
8	Peptide inhibitors Wbox 2	Wbox2 is non-specific and inhibits clathrin-independent endocytosis; proposed to be due to either upregulation of clathrin-independent endocytosis (CIE) during clathrin terminal domain overexpression or that SNX9 is required for CIE, and inhibition by Wbox2 sequesters it from CIE.	CME	–	176
9	Bis-Ts	Most potent of the dimeric tyrphostins (tyrosine phosphorylation inhibitors and inhibits dynamin).	CME	–	178–180
10	Iminodyns	Interacts with and inhibits dynamin.	CME	–	181,182
11	Rhodadyn D10	Inhibits dynamin 1 by blocking GTPase activity.	CME	–	183,184
12	Pthaladyn 23	Inhibits dynamin 1 by blocking GTPase activity.	CME	–	185,186
13	Naphthaladyn 29	Competitive inhibitor in GTP domain and blocks GTPase activity of dynamin 1.	CME	–	61
14	Pyrimidyn 6–7	Most potent dynamin 1 inhibitors.	CME	–	186
15	Bisphosphonates	Blocks dynamin 2.	CME	Inhibits the uptake of transferrin as well as adenovirus and simian virus 40.	63
16	Monensin	Affects proton gradient.	CME	Inhibits SARS-COV-2 infection.	59,188
17	ES9-17	It is a potent and selective CME that binds to N-terminal domain of clathrin heavy chain.	CME	Acts as an inhibitor of the CHC function; cytosol acidification	33

TABLE 1 (Continued)

No	Endocytosis of inhibitors	Mode of action	Target for inhibition	Summary	References
18	Methyl-β-cyclodextrin	Removes cholesterol from plasma membrane.	Caveolae-mediated endocytosis/lipid rafts	Interferes with fluid-phase endocytosis and clathrin-mediated endocytosis.	63,188,189
19	Filipin	Binds to cholesterol in the membrane.	CIE	Toxic at higher concentrations. Inhibits clathrin-mediated endocytosis.	190-192
20	Nystatin	Binds to cholesterol.	Lipid rafts/cholesterol-enriched microdomains/caveolae	Interferes with other uptake mechanisms because of changes in membrane fluidity.	194,195
21	7-keto-cholesterol	Prevents the close packing of acyl chains.	CLIC/GEEC	–	141
22	LG186	Reversible inhibitor of Arf-GEF function.	CLIC/GEEC	–	140,196
23	Cytochalasin D, latrunculin	Depolymerizes F-actin.	Phagocytosis macropinocytosis	Affects most endocytic pathways.	197–199
24	Amiloride derivatives (EIPA and HOE-694)	Inhibits Na^+/H^+ exchange	Macropinocytosis	May affect actin.	65,66,200
25	Dynasore	Inhibits dynamin by blocking GTPase activity.	CME	Interferes with actin.	27,62,138,201
26	Dynole, Dyngo	Inhibits dynamin by blocking the GTPase activity of dynamin I.	CME	Interferes with actin.	202,203
27	Pitstop 2	Interferes with binding of proteins to the N-terminal domain of clathrin.	CME	Affects most forms of clathrin-independent endocytosis and causes decreasing in PM mobility.	204,205

In another context, although the principles governing the activation and organization of the actin cytoskeleton during CME are not fully understood, there is a spatiotemporal relationship between the generation of actin platform and the formation of CCVs. Accordingly, a few studies investigated the inhibition of CME by disrupting actin formation, where amiloride derivatives (EIPA and HOE-694), cytochalasin D, and latrunculin were found to inhibit Na^+/H^+ exchange and disrupt actin formation.[65,66]

3.2 | Pharmacological inhibitors

Pharmacological inhibitors include U.S. Food and Drug Administration (FDA)-approved medications used for therapeutic purposes that simultaneously affect endocytic pathways as shown in Table 2. Chlorpromazine (CPZ), for example, is used to treat psychotic disorders, such as schizophrenia or manic-depression, in adults.[66] Some studies reported that phenothiazine derivatives, including CPZ, may affect the formation of CCVs and then inhibit CME.[67,68] Similarly, during viral infection, chloroquine inhibits the formation and functioning of CCVs and its analogs inhibit the acidification of endosomes.[69,70] Other pharmacological inhibitors, including itraconazole, vinblastine, imipramine, auranofin, terfenadine, and flubendazole,

inhibit plasma membrane ruffle formation, which is an early step in macropinocytosis.[27,65] Furthermore, the PI3K inhibitors wortmannin and LY294002 inhibit the activity of protein kinase C delta, thereby inhibiting phagocytosis and macropinocytosis.[71] Lipid rafts are rich in glycosphingolipids, cholesterol, and signaling proteins, all of which play a vital role in the internalization and disruption of parasitic, bacterial, and viral infectious particles.[72] Statins, which are commonly used to treat hyperlipidemia, block cholesterol synthesis by inhibiting the activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which is used for the therapeutic reduction of cholesterol-containing plasma lipoproteins, and then affect the CvME pathway.[73] Another pharmacological inhibitor, genistein, interferes with dynamin and may affect endocytic pathways such as CME.[74]

3.3 | Natural products as anti-endocytic factors

Some natural inhibitors can interact with lipid raft components, reducing cholesterol and glycosphingolipids in the cell membrane and thus inhibiting viral endocytosis.[73,76,77] Phytosterols, for example, interact with the components of lipid rafts, thereby reducing cholesterol content in the cell membrane[76,78] Table 3. Flavonoids also play a role in endocytic inhibition as they can fluidize the cell

TABLE 2 Summary of pharmacological inhibitors with mode of action

No	Endocytosis inhibitors	Mode of action	Target of inhibition	Summary	References
Pharmacological inhibitors					
1	Chlorpromazine	Translocates clathrin and AP2 adaptor protein from the cell surface to intracellular endosomes.	CME	Inhibits clathrin-independent endocytosis in some cells.	64,189,206
2	Phenothiazines	Affects formation of clathrin-coated vesicles.	Phagocytosis CME	Inhibits dynamin II (dynII) at similar concentrations.	68,69,207
3	Chloroquine	Affects the function of clathrin-coated vesicles.	CME	Chloroquine and its analogs inhibit SARS-COV-2 acidification of endosomes during the events of viral replication and infection.	70,71,208
3	Itraconazole	Inhibits plasma membrane ruffle formation, a critical early step in macropinocytosis.	Macropinocytosis	–	66,209
4	Vinblastine		Macropinocytosis	–	66,153,209
5	Imipramine		Macropinocytosis	Inhibits phagocytosis.	66,209,210
6	Auranofin		Macropinocytosis	–	65,210
7	Terfenadine		Macropinocytosis	–	66,212
8	Flubendazole		Macropinocytosis	–	66,212
9	Genistein	Interferes with dynamin and may affect other endocytic processes.	CME	Inhibits several tyrosine kinases. Genistein interferes with caveolae/raft-mediated endocytosis by blocking the Src kinase-dependent phosphorylation of caveolin-1 and preventing vesicle fusion.	64,75,193,213
10	Statins	Affects most endocytic mechanisms.	Caveolae-mediated endocytosis/lipid rafts	Blocks cholesterol synthesis. Inhibitors of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase used for the therapeutic reduction of cholesterol-containing plasma lipoproteins.	213-215
11	Wortmannin and LY294002	Affects most endocytic mechanisms	Phagocytosis, macropinocytosis	Inhibits the activity of phosphatidylinositol 3-kinase.	72,217,218
12	Rottlerin			Inhibits the activity of protein kinase C delta.	218,219

membrane (raft-breaking effect), which leads to inhibition of both membrane fusion and CvME Table 3. Other reports demonstrated that oleuropein and hydroxytyrosol, which are phenolic compounds with antioxidant properties found in the leaves and fruits of olive plants, can inhibit the fusion of viruses with host cell membranes[76,79] Table 3. Furthermore, ikarugamycin, isolated from *Streptomyces phaeochromogenes*, affects CME,[80] whereas brefeldin A, TENin1, and auxin inhibit the formation of clathrin coats pits and ion transport.[81–83]

3.4 | Genetic inhibitors

Although chemical inhibitors are the most commonly identified inhibitors, the absolute selectivity of their actions remain a major concern. Selective CME inhibitors that target specific molecular

targets may either directly or indirectly interfere with other molecules Table 4. Barbadin, for example, is a selective inhibitor of β-arrestin/AP2 interaction,[83] ERK1/2, and cAMP accumulation in human embryonic kidney cells.[59] Barbadin also affects cellular viability and induces both apoptosis and autophagy in breast cancer cells.[84-88] This may give genetic inhibitors more leverage to avoid the anticipated adverse effects of chemical inhibitors. Genetic inhibitors are used to inhibit CME by suppressing the expression of specific endocytic proteins.[75] The expression of mutant forms of critical proteins involved in endocytosis and siRNA-mediated depletion of these proteins are the most commonly used methods.[89] The mutant dynamin, K44A, is an example of a genetic inhibitor of CME.[90] Dyn2K44A was also used to inhibit CME under conditions of increased clathrin-independent fluid endocytosis.[91] Clathrin adaptor proteins including AP180 and Epidermal growth factor receptor substrate 15 (Eps15) target regulatory proteins of the CME pathway

TABLE 3   Summary of natural products with mode of action

No	Endocytosis inhibitors	Mode of action	Target of inhibition	Summary	References
	Natural products				
1	Phytosterols	Interact with lipid raft components of the cell membrane resulting in lowering of cell membrane cholesterol or the destabilization of lipid raft structure.	Caveolae-mediated endocytosis/lipid rafts	The phytosterol-lipid raft interaction can affect biochemical signaling taking place downstream of the lipid rafts.	76,78
2	Flavonoids	It can 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.	Caveolae-mediated endocytosis/lipid rafts	Induce prevention of membrane fusion is one way in which flavonoids protect an organism against viral infection.	76,221,222
3	Hydroxytyrosol	Phenolic compound with antioxidant properties found in the leaves and fruits of the olive (*Olea europaea*).	Caveolae-mediated endocytosis/lipid rafts	Oleuropein and hydroxytyrosol inhibit the fusion of viruses with cell membranes.	76,79
4	Ikarugamycin	Affects clathrin-mediated endocytosis.	CME	Isolated from *Streptomyces phaeochromogenes* and has antiprotozoal activity.	80,146
5	Brefeldin A	Inhibits the formation of clathrin coats and ion transport.	CME	Inhibitor of vesicle trafficking, on internodal cells of *Chara australis*.	82,83
6	TENin1	Inhibits endocytosis, causes endomembrane protein accumulation at the pre-vacuolar compartment, and impairs gravitropic response in *Arabidopsis thaliana*.	CME	–	223,224
7	Auxin	Promotes efflux from cells by a vesicle-trafficking-dependent mechanism.	CME	–	81,225

and thus inhibit endocytosis. The C-terminal clathrin-binding domain of AP180 or the truncated form of Eps15 inhibits the initial step (formation of CCPs) of CME.[92,93] Furthermore, the C-terminal (Hub) region of clathrin expression effectively inhibits CME.[94] Endophilin A2 (ENDOA2) depletion prevents the reshaping of the membrane before scission of the vesicle and inhibits fast endophilin-mediated endocytosis (FEME).[95] The same approach was used to inhibit other endocytic pathways. Caveolin-1 (CAV1) and caveolae-associated protein 1 (CAVIN1), for example, prevented caveola formation.[96,97] Similarly, CtBP1 and Rabankyrin-5 (ANKFY1) inhibited macropinosome fission from the plasma membrane and blocked macropinocytosis.[98,99]

4 | ENDOCYTOSIS AND TRANSCYTOSIS OF SARS-CoV-2

Viral entry into host cells occurs through two distinct pathways: viruses deliver their nucleic acid material into the cytosol after their envelopes fuse with the plasma membrane (pH-independent entry),[101] or they use the host cell's endocytic machinery (pH-dependent entry), such as CME and CvME, in addition to poorly characterized routes such as clathrin- and caveolae-independent endocytic pathways Figure 3. A previous study reported that SARS-CoV-2 is similar to SARS-CoV, which can fuse with host cell

membranes and internalize into the host cell. Following endocytosis-mediated entry at an acidic pH, viral and endosomal membranes fuse, and subsequently, the viral genome is released into the cytosol.[102] Initially, the viral spike (S) protein binds to the host cell receptor angiotensin-converting enzyme 2 (ACE2).[19] This binding triggers endocytic infection similar to the GPCR internalization process. During the entry process, CCPs are formed by AP2 complexes,[2] following which virus-containing CCVs are released. During recycling, structural proteins, including the viral protein capsid, are moved to late endosomes containing lysosomes for degradation; however, viral nucleic acid RNA initiates the formation of a new virus that egresses from lysosomes.[2]

In contrast to CME-mediated entry, macropinocytosis-mediated entry follows a non-specific internalization pathway that does not require a receptor.[102,103] Although macropinocytosis is not the key entry pathway of SARS-CoV-2,[103,104] the virus may activate the signaling pathways that trigger macropinocytosis and enter the cell by promoting actin-mediated membrane formation and lamellipodia formation at membrane perturbation sites to close them and form large, irregular vesicles.[105,106] Other pathways, such as CIE, could be used by SARS-CoV-2 to infect host cells. This pathway is regarded as a cholesterol-sensitive mechanism. CIE is divided into dynamin-dependent CvME and dynamin-dependent or -independent non-clathrin non-CvME.[105,107,108] Wang et al. clarified that the coronavirus can infect host cells through various mechanisms, including

TABLE 4 Summary of genetic approaches with mode of action

No	Endocytosis inhibitors	Mode of action	Target of inhibition	Summary	References
	Genetic approachs				
1	Dynamin mutant, Dyn K44A	Causes defects in GTP hydrolysis.	CME	Enhances fluid-phase uptake.	90,91,226
2	AP180C	Causes clathrin sequestration.	CME	May cause changes in gene expression.	94,226
3	Eps15 mutant	Inhibits clathrin pit assembly.	CME	–	228–230
4	Clathrin Hub mutant	Acts as a dominant-negative mutant of clathrin.	CME	–	231,232
5	siRNA of clathrin	Blocks the formation of clathrin pits.	CME	–	233,234
6	siRNA of AP2	Blocks formation of AP2-dependent clathrin pits.	CME	–	232,234
7	Clathrin (CLTC)	Clathrin depletion prevents the formation of clathrin-coated pits.	CME	Changes in gene expression are observed due to overexpression and knocking down.	236,237
8	Endophilin A2 (ENDOA2)	Endophilin A2 depletion prevents the reshaping of the membrane before scission of the vesicle.	(FEME)	Changes in gene expression are observed due to overexpression and knocking down.	45,95
9	Caveolin-1 (CAV1)	Caveolin-1 depletion prevents caveola formation.	CAV	Changes in gene expression are observed due to overexpression and knocking down.	97,98
10	Caveolae associated protein 1 (CAVIN1)	Cavin-1 depletion prevents caveola formation.	CAV	Changes in gene expression are observed due to overexpression and knocking down.	238,239
11	IRSp53	IRSp53 depletion interferes with actin dynamics.	CLIC/GEEC	Changes in gene expression are observed due to overexpression and knocking down.	240,241
12	PICK1	PICK1 depletion interferes with actin dynamics, specifically the Arp2/3 complex.	CLIC/GEEC	Changes in gene expression are observed due to overexpression and knocking down.	242,243
13	CtBP1 (CTBP1)	CtBP1 depletion inhibits macropinosome fission from the cell surface.	Macropinocytosis	Changes in gene expression are observed due to overexpression and knocking down.	100,244
14	Rabankyrin-5 (ANKFY1)	Depletion of rabankyrin-5 inhibits the formation of macropinosomes.	Macropinocytosis	Changes in gene expression are observed due to overexpression and knocking down.	245,246

RME, CIE, caveolae-independent endocytosis, and lipid raft formation.[19,105] The plasma membrane contains sphingolipids and cholesterol (lipid rafts), which are domains enriched with signaling and transport proteins.[105] Entry of SARS-CoV-2 by endocytic pathways involving lipid rafts provides new insight into the pathogenesis of acute respiratory distress syndrome and the elevated levels of proinflammatory cytokines associated with it.[105,106] Wang et al. demonstrated that coronaviruses, including SARS-CoV-2, can infect host cells deficient in CME.[19]

On the other hand, Cholesterol-mediated, dynamin-dependent, and receptor-mediated pathways include CvME. CvME pathway forms a specific type of flask-shaped structure called caveola existing on the cell membrane enriched in cholesterol and sphingolipid as shown in Figure 3. Bastiani, Parton, Cheng, and Nichols clarified that caveolae are microdomains of membrane rafts that can bud into cells

as caveosomes, which fuse to form early endosomes for delivery.[109,110] The caveolar coat consists of an inner layer containing caveolins and an outer membrane containing cavins, which help to support the caveolin scaffold, promote membrane curvature, and caveolae budding.[111,112] SARS-CoV-2 is internalized via the ACE-2 receptor into the host cell by CvME, forming caveosomes and undergoing degradation after accumulation of overexpressed Cav1.[113] The endocytic vesicles, which are formed in lipid rafts, can be stabilized by flotillin (flotillin-dependent endocytosis).[105] Many studies do not support viral entry by the flotillin-dependent endocytic pathway because the mechanisms remain unclear.[114,115] Glebov showed that SARS-CoV-2 can infect host cells via the flotillin-dependent endocytic pathway, which should be supported with an in vivo clinical study.[106] Furthermore, viruses such as adenoviruses can enter via non-clathrin non-CvME in lipid rafts. These viruses can
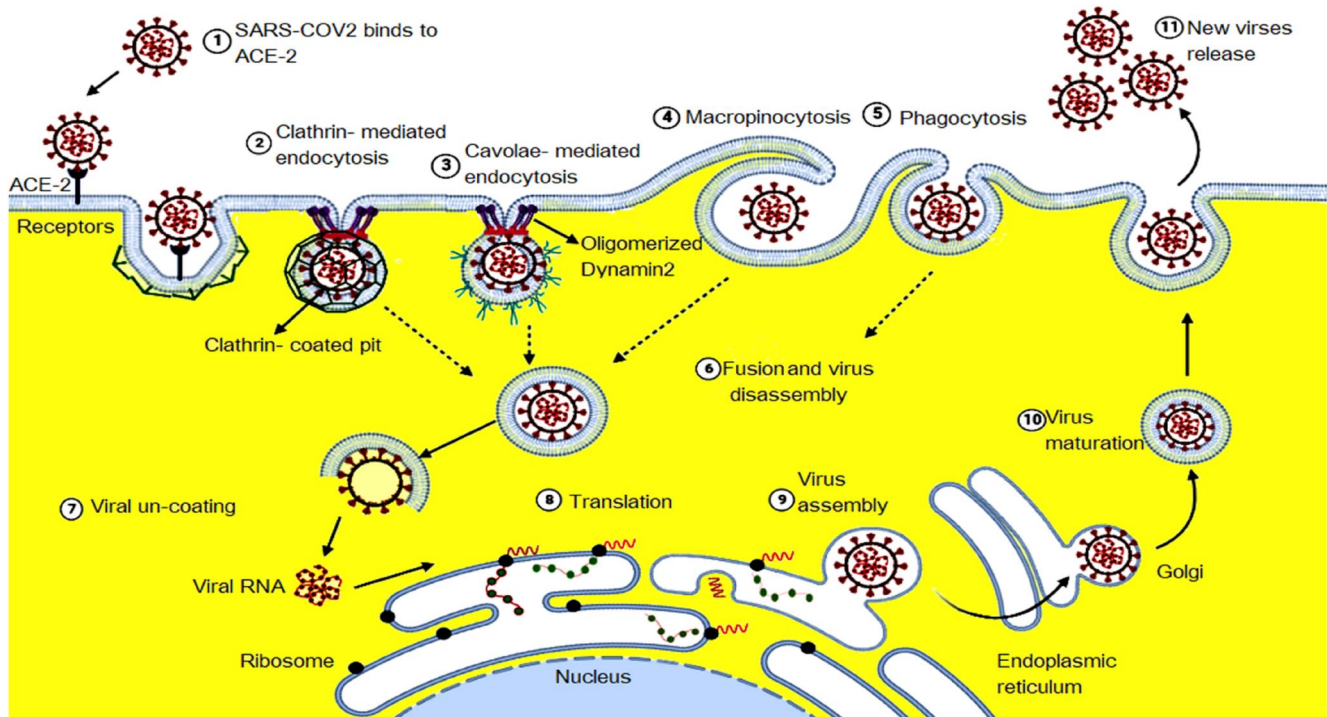
FIGURE 3 Mechanisms of SARS-CoV-2 entrance through binding to the surface receptor (1) and enter to the cell by clathrin-mediated endocytosis (CME) (2), or caveolae-mediated endocytosis (3), macropinocytosis (4), and phagocytosis (5) depending on the types of endocytic proteins forming endosome (6) and then viral uncoating (7) releasing viral RNA and then viral translation (8) and eventually virus assembly (9) and maturation (10) then viral release (11).

infect host cells through GRAF1-dependent endocytosis, which is also named as the CLIC/GEEC pathway.[116] The CLIC/GEEC pathway is sensitive to cholesterol depletion and may decrease viral entry when it is cholesterol-dependent but raft-independent.[105,117] Glebov showed that SARS-CoV-2 can infect host cells via the CLIC/GEEC pathway by forming GEECs, which are free of clathrin, caveolae, dynamin, and Rac1.[106] SARS-CoV-2 is internalized with ACE-2 into host cells by forming GEECs from the fusion of smaller CLICs.[105] CLICs and GEECs are formed after actin polymerization, which is regulated by Cdc42, Arf1, and GRAF1.

Transcytosis is the transport of macromolecules, supramolecular complexes, and microorganisms through the cell via membrane-bound carriers. It is mediated by two major transportation mechanisms. The first mechanism employs CCVs to internalize transported molecules, which enter cell compartments and are then released into the cell via exocytosis. The second mechanism employs caveolae, without their entry into cell compartments.[118,119] Notably, the former mechanism is used in intestinal cells and the latter in endothelial cells. SARS-CoV-2 enters the intestinal epithelial and M cells by binding to ACE-2. Thereafter, ACE-2 is split by cellular proteases such as TMPRSS2.[120] Following entry into the cell with common recycling endosomes, SARS-CoV-2 is translocated into the multivesicular bodies/late endosomes. In these endosomes, the viral envelope fuses with endolysosomal membranes, which is triggered by cathepsins, and ultimately releases the genetic material in the cell.[121]

5 | ENDOCYTIC PROTEINS AS ANTIVIRAL TARGETS AGAINST SARS-CoV-2 ENTRY

Since the emergence of SARS-CoV-2, various efforts have been made to present new medications for inhibiting its infection with minimal adverse effects.[122,123] To achieve this goal, two strategies were adopted. The first was to target the biology of the virus, such as inhibiting its replication in host cells, whereas the second involved targeting the biology of host cells.[106] In both strategies, complete elimination or minimizing the associated undesirable effects remains the primary challenge. Although both approaches have some limitations, drugs targeting the host cell might be advantageous owing to the relative structural and physiological stability of the host cells compared with drugs targeting the virus, which is constantly mutating, resulting in new variants and escalating drug resistance.[124,125] A study showed that disruption of the Na^+/K^+-ATPase of the host cell's plasma membrane by cationic steroid inhibitors, including ouabain and bufalin, led to the inhibition of the CME pathway and inhibited coronavirus entry.[126] Similarly, ouabain and bufalin inhibited SARS-CoV-2's entry by the same mechanism.[127,128] Other studies targeted endosomes to inhibit coronavirus-2 infection.[129] It has also been discovered that endosomal acidification factors, such as bafilomycin A1, inhibit endosomal SARS-CoV-2 via the CLIC/GEEC endocytic pathway, which is pH dependent[3] Figure 5. Additionally, Prabhakara et al. showed that niclosamide is considered as a probable SARS-CoV-2 entry inhibitor.[93]

6 | SARS-CoV-2 ENTRY INHIBITION

SARS-CoV-2 uses endocytic pathways to deliver its virion components into the cytoplasm. The virus infects lung cells through CME in addition to clathrin-independent processes.[19,104] To achieve this, the viral spike protein binds to human ACE2 through its receptor-binding domain. This binding is activated by human proteases. Previous studies have shown that other receptors and cellular proteases, such as CD147, neuropilin-1, dipeptidyl peptidase 4, alanyl amino peptidase,[129] and transferrin receptor,[130] also facilitate viral entry and transmission into host cells. During tRNA-derived RNA fragment (tRF)-mediated infection, the viral membrane fuses with the lumen of the endosomal membrane, allowing viral RNA to enter the cytosol to infect the host cell.[2,100] The viral components undergo the tRF recycling pathway, which exposes SARS-CoV-2 capsid proteins to degradation by lysosomes in late endosomes. During this process, SARS-CoV-2 RNA drives the formation of a new virus that egresses from lysosomes.[2] These scenarios predict the direct involvement of endocytic machinery in SARS-CoV-2 infection. Accordingly, because of their potential as promising broad-spectrum antiviral targets, endocytic pathway inhibitors have been proposed as a potential treatment Figure 4 and 5. Although the endocytic machinery includes more than 50 proteins and adaptor proteins, some of these proteins serve as key factors; clathrin, for example, is an integral member of the CME and other clathrin-dependent endocytic pathways. This makes clathrin useful as a primary target. Additionally, clathrin is involved in cell signaling and other activities that may affect viral replication before the stage of nucleocapsid assembly. Chlorpromazine, 2-deoxy-d-glucose/sodium azide, bolinaquinone, and Pitstop 2 inhibit 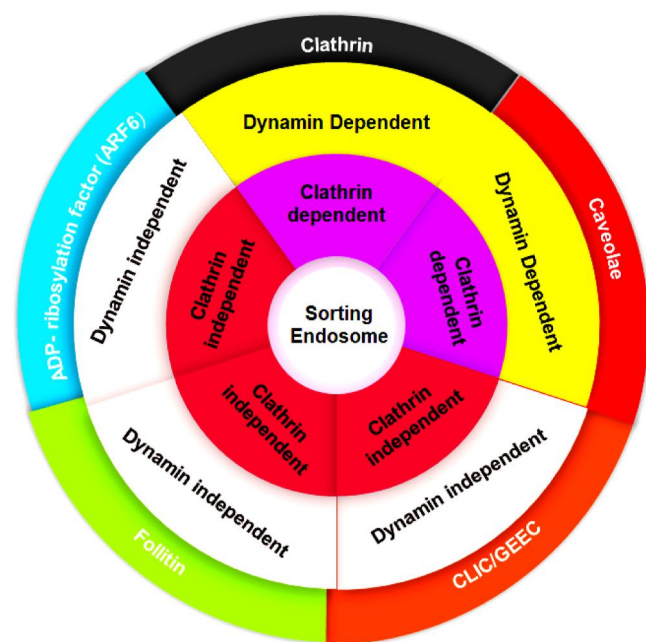the scission of CCVs from the plasma membrane and affect their subsequent functioning[21,67,131,132] Figure 5 as well as block entry of SARS-CoV-2 via the CME pathway.[133] Unfortunately, clinical trials have shown that these drugs have harmful adverse effects, including retinal damage when used in the long term, photosensitivity, liver damage, seizures, headaches, stomach pain, and muscle or nerve damage.[134,135] Inhibitor-mediated complete inhibition of CME is not fully effective, where many studies demonstrated a partial decrease in the number of endosomes Figure 5. Nevertheless, according to clinical trials, even partial virus blockade is of significance, as viral load is related to severity of disease progression.[105] This increases the importance of using multiple endocytic protein inhibitors, such as combinations of dynasore and Pitstop 2, which can result in a substantial reduction in virus titers without affecting early to late viral gene expression[136] Figure 5.

Membrane rafts, which are enriched with cholesterol and sphingolipids, are considered portals for viral entry.[104] Cholesterol chelating agents, including methyl-β-cyclodextrin, phytosterols, and flavonoids, can block the entry of SARS-CoV-2 through CvME/lipid rafts.[63,74] They reduce the cholesterol content of the host cell membrane and destabilize the lipid raft structure by interacting with lipid raft components. Additionally, many agents, such as dynasore, Dynoles, and dyngoes, can inhibit the CME pathway and CvME/lipid rafts by inhibiting dynamin GTPase activity.[137] Furthermore, there are two types of macropinocytosis inhibitors. The first type, which includes flubendazole, terfenadine, itraconazole, vinblastine, and imipraminecan, inhibits plasma membrane ruffle formation, a crucial early step in macropinocytosis, thereby inhibiting viral entry into host cells.[65] The other type, which includes amiloride and its derivatives (EIPA and HOE-694), inhibits Na^+/H^+ exchange, thus affecting macropinocytotic pathways.[65] SARS-CoV-2 can use FEME for entry and infection.[138] ENDOA2 has been identified as a FEME inhibitor whose depletion prevents the remodeling and reshaping of the plasma membrane before vesicle scission, thereby preventing viral entry.[138] Furthermore, viral entry can occur via the CLIC/GEEC pathway, which can be inhibited by LG186 and 7-keto-cholesterol, thereby preventing the close packing of acyl chains and affecting the structure of the cell membrane.[139,140] Some pharmacological inhibitors including chlorpromazine, nystatin, amiloride, methyl-β-cyclodextrin, vinblastine, and itraconazole approved by FDA for human use, although others are still being evaluated.[105] In addition to improving the efficacy of endosomal inhibitors, scientists are investigating natural products for use as potential endosomal inhibitors in an effort to reduce the adverse effects caused by chemical and pharmaceutical endosomal inhibitors.

FIGURE 4 Classification of endocytic pathways according to dynamin and clathrin involvement

7 | COMPUTATIONAL AND PRECLINICAL STUDIES OF ENDOCYTIC INHIBITORS

We conducted a molecular docking analysis of some of the endocytic protein inhibitors discussed in this review to provide more insight into their potential inhibitory effect against SARS-CoV-2. These computational analysis-based investigations were performed to
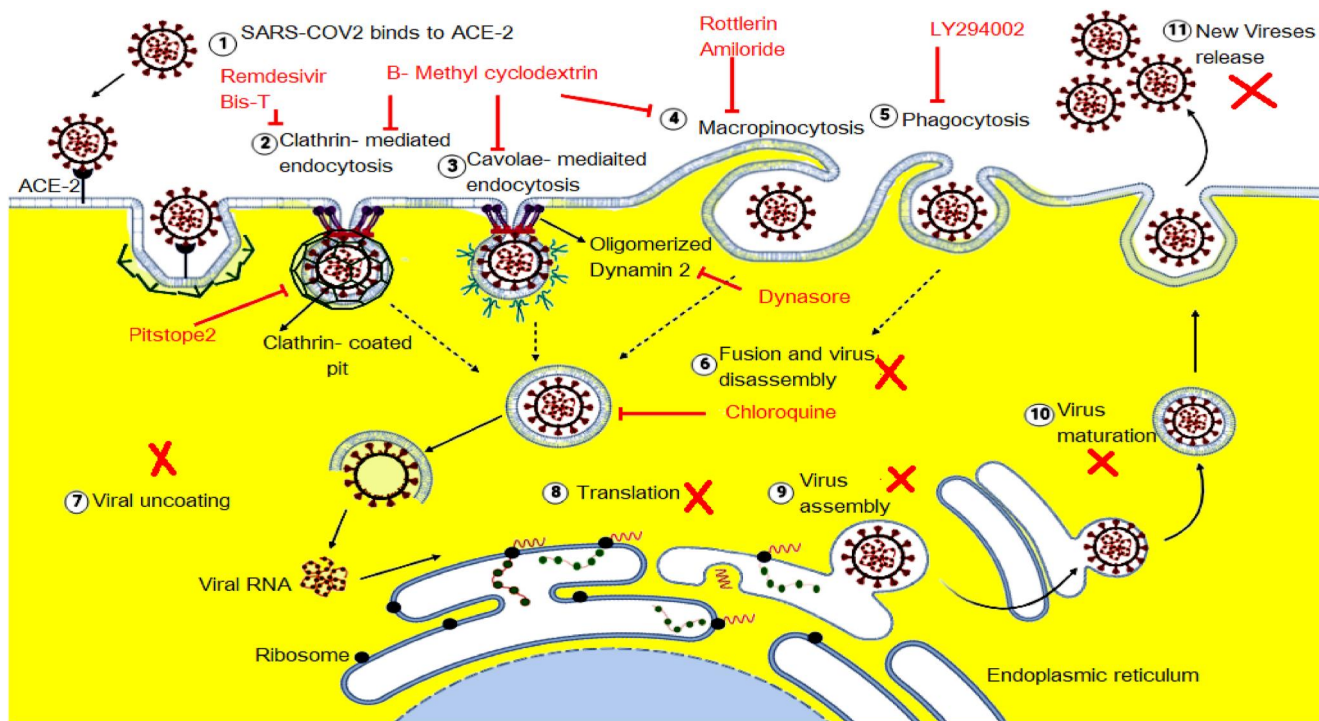
FIGURE 5 Inhibition of SARS-CoV-2 entrance by the large scale of endocytic proteins inhibitors including Remdesivir, Bis-T inhibits clathrin-mediated endocytosis (CME), β-methyl cyclodextrin inhibits caveolae-mediated endocytosis, CME, and macropinocytosis. Rottlerin and Amilorides inhibit the macropinocytosis pathway. LY294002 inhibits the phagocytosis process, and dynasore inhibits oligomerized dynamin2. And chloroquine inhibits the entrance of viral endosomes.
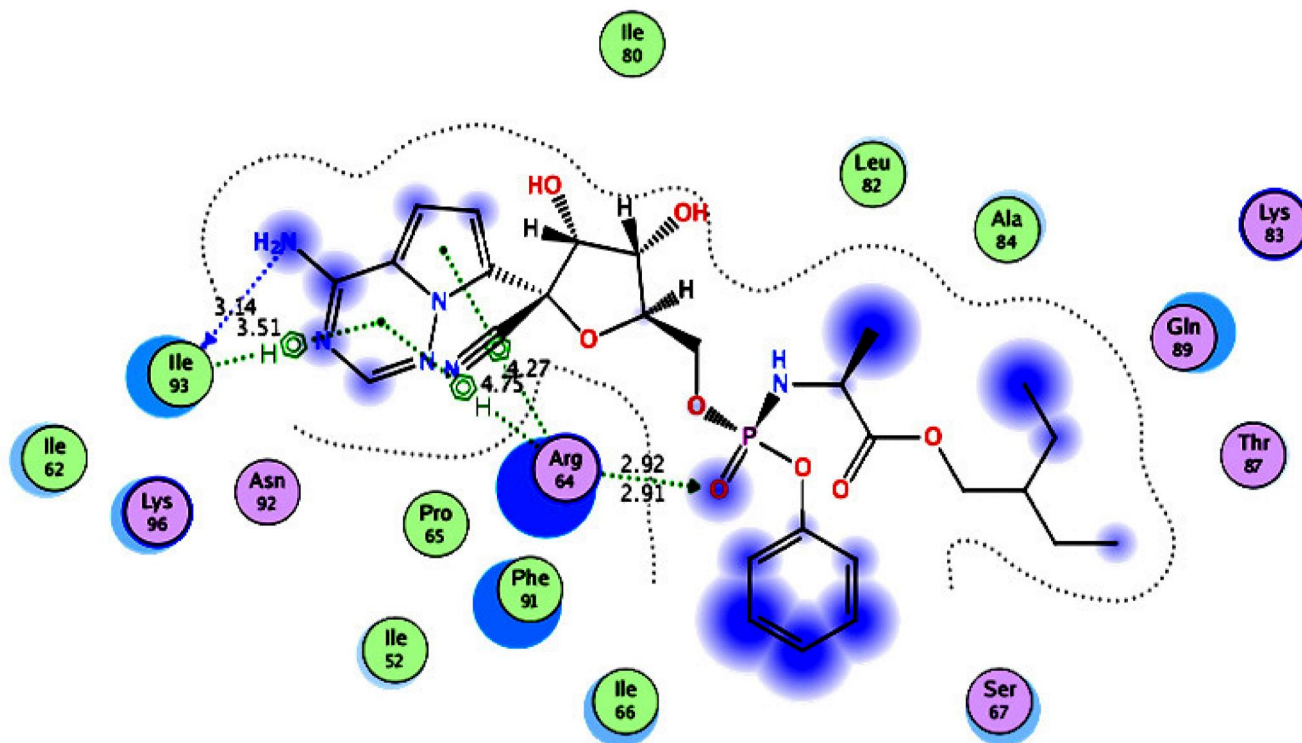

FIGURE 6 2D diagram (Distances in Å) of remdesivir (Group 1 inhibitor) showing its interaction with the Clathrin domain active site

Compound	Docking score (kcal/mol)	Amino acids involved in binding
Pitstop 1	−11.23	Phe91, Arg64, Gln89, and Lys96
Chloroquine	−11.09	Phe91
Sucrose	−11.75	Phe91, Arg64, and Ile93
Chlorpromazine	−10.73	Arg64 and Ile93
Lopinavir	−14.42	Arg64 and Lys96
Remdesivir	−14.46	Arg64 and Ile93
Monodansylcadaverine	−12.11	Arg64, Ile93, and Lys96
Phenylarsine oxide	−6.45	Phe91
Bolinaquinone	−11.61	Phe91 and Arg64
ES9-17	−9.47	Phe91, Arg64, Ile93, and Ile62
Pitstop 2	−12.48	Phe91, Ile93, and Ile62
Ikarugamycin	−11.29	Lys98
Brefeldin A	−9.62	Ile93 and Lys96
TENin1	−11.17	Arg64 and Ile93
Clathrin A	−11.03	Ser67
Clathrin B	−10.42	Ser67
Clathrin C	−10.06	Phe91

TABLE 5 Docking energy scores and amino acids involved in binding for pitstop 1, chloroquine, and Group 1 inhibitors docked with clathrin terminal domain

compare the inhibitory activity of the tested substances to that of the standard drug chloroquine by determining the plausible modes of binding between these substances and their target binding sites. The interactions of the substances with the protein hot spots (key amino acids) were also recorded. Docking studies were performed using the Molecular Operating Environment (MOE 2010) software[141] according to a previously reported procedure[142] and are described in the Supporting Information. X-ray crystallographic structures of target proteins were obtained from the Protein Data Bank (PDB).[143]

For clathrin inhibitors (Group 1), the clathrin terminal domain co-crystallized with Pitstop 1 (IC$_{50}$ = 1.80 × 10^4 nM)[145] as an inhibitor (PDB ID: 2XZG)[146] was used for the docking studies. Remdesivir (docking score; S = −14.46 kcal/mol) was found to have the highest inhibitory activity within the group (Figure 6, Supporting Information Figure S1, and Table 5), which was also higher than those of the co-crystalized ligand Pitstop 1 (S = −11.23 kcal/mol) and the reference drug chloroquine (S = −11.09 kcal/mol). Remdesivir interacted with the clathrin domain active site via three hydrogen bonding interactions: two with the key amino acid Arg64 backbone using its phosphoryl group's oxygen atom and one with the key amino acid Ile93 through its amino group. It also formed three arene–H bonds with these two key amino acids. Remdesivir interacted with the active site through hydrophobic interactions with the hydrophobic side chains of Arg64, Phe91, and Ile66.

For 3-hydroxy-3-methylglutaryl CoA inhibitors (Group 2), the catalytic portion of human HMG-CoA reductase co-crystallized with simvastatin (IC$_{50}$ = 4.3 nM)[147] as an inhibitor (PDB ID: 1HW9)[148] was used for the docking studies. The results (Figure 7, Supporting Information Figure S2, and Table 6) revealed that methyl-β-cyclodextrin

(S = −13.07 kcal/mol) had the highest inhibitory activity within the group, which was also higher than those of simvastatin (S = −10.55 kcal/mol) and chloroquine (S = −8.38 kcal/mol). Methyl-β-cyclodextrin interacted with the catalytic portion of the human HMG-CoA reductase active site via two hydrogen bonding interactions with the key amino acid Glu559 backbone using two hydroxyl groups. It also interacted with the active site through hydrophobic interactions with the hydrophobic side chains of Gly860, Ala856, Leu857, and Leu853.

For actin protein inhibitors (Group 3), a monomer of actin cytoskeleton from the dimer complexed with swinholide A (IC$_{50}$ = 0.04 μg/ml)[149] (PDB ID: 1YXQ chain A)[150] was used for the docking studies. The results (Figure 8, Supporting Information Figure S3, and Table 7) revealed that rottlerin (S = −13.89 kcal/mol) was the most potent inhibitor within the group and had a higher inhibitory activity than those of swinholide A (S = −12.35 kcal/mol) and chloroquine (S = −10.79 kcal/mol). Rottlerin interacted with the actin cytoskeleton active site via one arene–H bond with the key amino acid Tyr133 backbone using the phenyl ring. It also interacted with the active site through hydrophobic interactions with the hydrophobic side chains of Met355, Thr148, Tyr169, and Tyr143.

For dynamin I GTPase inhibitors (Group 4), molecular docking was performed using the crystal structure of dynamin I GTPase domain complexed with phosphomethylphosphonic acid guanylate ester (GMPPCP) ligand (PDB ID: 3ZYC).[151] The results (Figure 9, Supporting Information Figure S4, and Table 8) showed that Bis-T (S = −25.73 kcal/mol) had the highest inhibitory activity within the group, which was also higher than those of GMPPCP (S = −23.73 kcal/mol) and chloroquine (S = −14.12 kcal/mol). Bis-T interacted with the dynamin I GTPase active site through hydrogen bonding with Asp208,
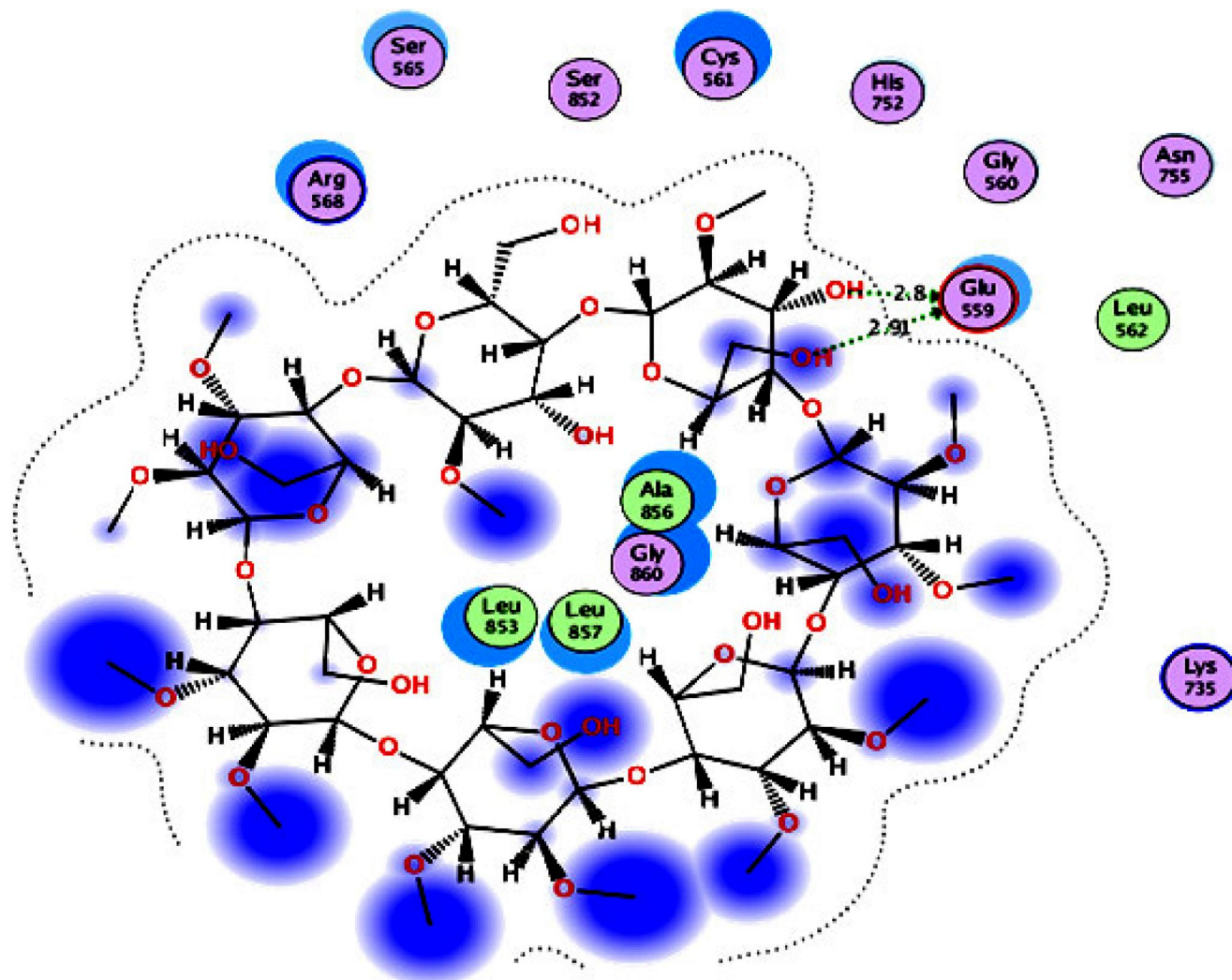
FIGURE 7 2D diagram (Distances in Å) of methyl-β-cyclodextrin (Group 2 inhibitor) showing its interaction with the catalytic portion of the human HMG-CoA reductase active site

TABLE 6 Docking energy scores and amino acids involved in binding for simvastatin, chloroquine, and Group 2 inhibitors docked with the catalytic portion of human HMG-CoA reductase

Compound	Docking score (kcal/mol)	Amino acids involved in binding
Simvastatin	−10.55	Glu559, Lys735, and Asn755
Chloroquine	−8.38	Cys561
Methyl-β-cyclodextrin	−13.07	Glu559
Filipin	−11.23	Glu559 and Asn755
Nystatin	−12.72	Glu559
2-Deoxy-d-glucose	−7.91	Glu559 and Ser565
Chlorpromazine	−7.40	His752
Betulinic acid	−7.63	Lys735, Asn750, and Asn755
Kaempferol	−9.42	Cys561
Hydroxytyrosol	−7.90	Gly560

Ser46, Arg59, Leu137, and Gly62 amino acids in addition to that with the Mg^{2+} ion. It also formed hydrophobic interactions with the allosteric hydrophobic back pocket of the hydrophobic side chains of Gln239, Ser41, Lys206, Leu209, Ser238, and Asn236, thereby maximizing the binding interaction with the active site.

Overall, it was concluded from the docking simulations that remdesivir, methyl-β-cyclodextrin, rottlerin, and Bis-T can effectively inhibit clathrin, HMG-CoA reductase, actin, and dynamin I GTPase, respectively, and therefore, considered potent inhibitors of SARS-CoV-2.

8 | CYTOTOXIC EFFECT OF ENDOCYTIC PROTEINS INHIBITORS

Not all drugs have been proven to have an antiviral effect against SARS-CoV-2, but some can block various endocytic pathways in cell culture and potentially inhibit SARS-CoV-2 endocytosis.[151] However,
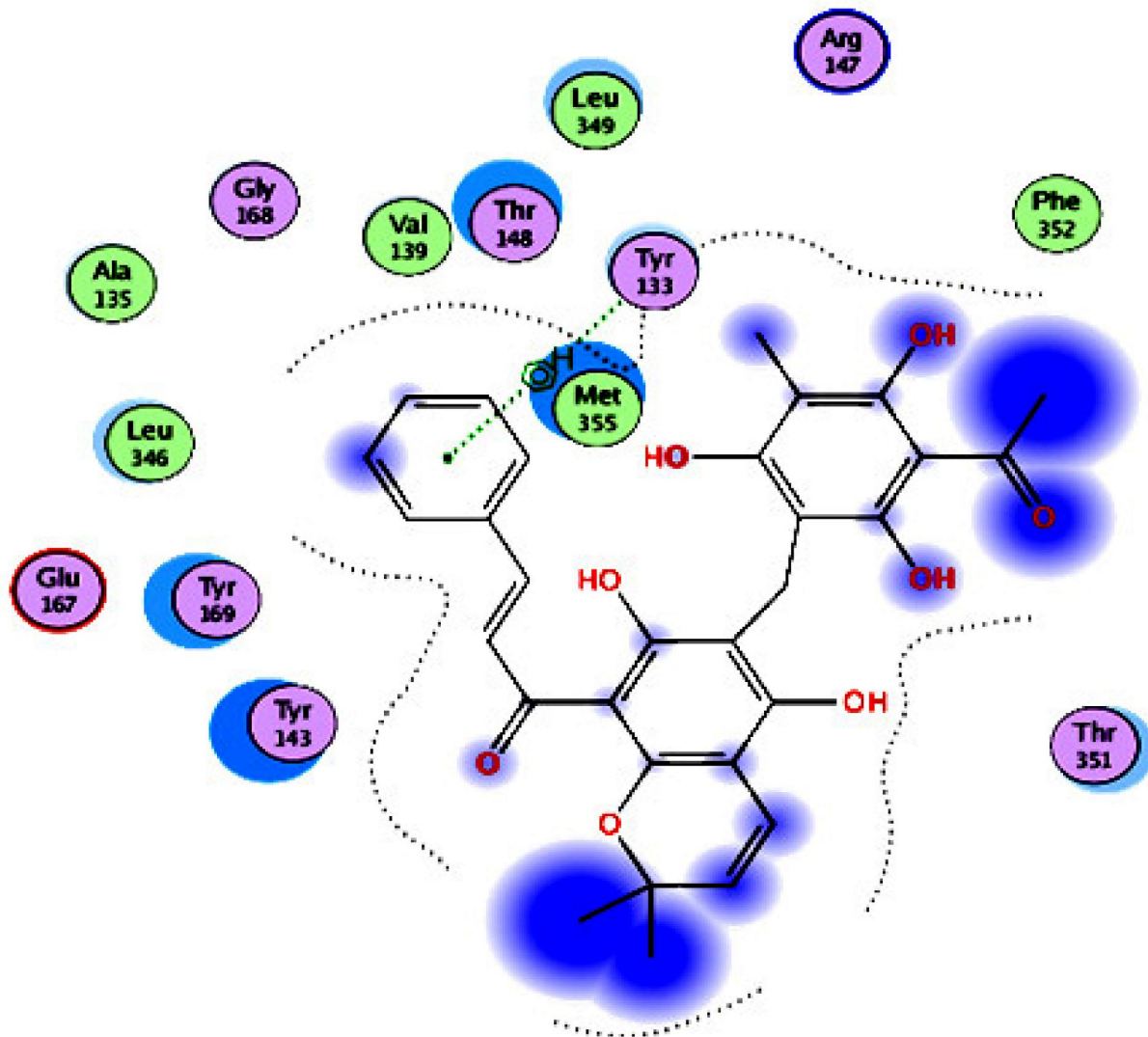
FIGURE 8 2D diagram of rottlerin (Group 3 inhibitor) showing its interaction with the actin cytoskeleton active site

there are many issues associated with the use of these drugs/inhibitors, such as non-specificity, affecting normal cellular processes, and cytotoxicity.[152] Although various FDA-approved drugs, such as chlorpromazine, amiloride, nystatin vinblastine, and itraconazole, are currently available, there use is restricted. Chlorpromazine may have adverse effects in patients, such as allergic reactions, agranulocytosis, severe hypotension, and blockade action of the receptor of chlorpromazine (D2 receptor), which may reduce the efficacy of other medications such as levodopa or cabergoline. Patients with cerebrovascular and cardiovascular diseases should take chlorpromazine with caution.[153] Nystatin also has adverse effects, such as those affecting the gastrointestinal tract including nausea, vomiting, diarrhea, stomach upset and stomach pain, as well as rare adverse effects such as mouth irritation, skin redness, and breathing difficulties. Amiloride has adverse effects such as nausea, vomiting, diarrhea, headache, increased reabsorption of uric acid in the proximal tubule, and potassium retention, which can be critical in patients with compromised renal function. Moreover, amiloride affects the central nervous

system, gastrointestinal tract, endocrine system, musculoskeletal system, dermatological system, and hematological systems.[154-156] Vinblastine, a chemotherapeutic agent, can cause hematological adverse effects, such as leukopenia, anemia, and thrombocytopenia, which can lead to intestinal bleeding; gastrointestinal symptoms, such as nausea, vomiting, and abdominal pain; and neurological adverse effects, such as peripheral neuritis, mental depression, headache, and convulsions. Itraconazole is used to treat dermatomycosis and onychomycosis using continuous therapy and may have serious adverse effects, including hepatotoxicity, liver failure and death, granulocytopenia, leukopenia, cardiac failure, congestive heart failure, neutropenia, thrombocytopenia, and immune system disorders.[157] Itraconazole may also cause vertigo, gastrointestinal symptoms, vomiting, pyrosis, constipation, fatigue, fever, allergic reaction, and skin rash.

Vercauteren et al. found that chlorpromazine and methyl-β-cyclodextrin, which are supposed to be SARS-CoV-2 endocytic inhibitors, exhibited cytotoxic effects on different cell types.[64]

TABLE 7 Docking energy scores and amino acids involved in binding for swinholide A, chloroquine, and Group 3 inhibitors docked with actin cytoskeleton

| Compound	Docking score (kcal/mol)	Amino acids involved in binding
Swinholide A	−12.35	Ser145, Arg147, and Ala331
Chloroquine	−10.79	Tyr133
2-Deoxy-d-glucose	−9.76	Tyr133, Ile136, and Gly168
Cytochalasin D	−12.38	Gly168
Latrunculin A	−9.83	Met355
Latrunculin B	−12.14	Tyr169
Flubendazole	−10.00	Ile136
Terfenadine	−11.13	Met355 and Ile136
Itraconazole	−12.25	Ile136
Vinblastine	−8.73	Met355
Wortmannin	−10.31	Thr351
LY294002	−10.59	Tyr133 and Tyr143
Rottlerin	−13.89	Tyr133
Imipramine	−9.81	Tyr133
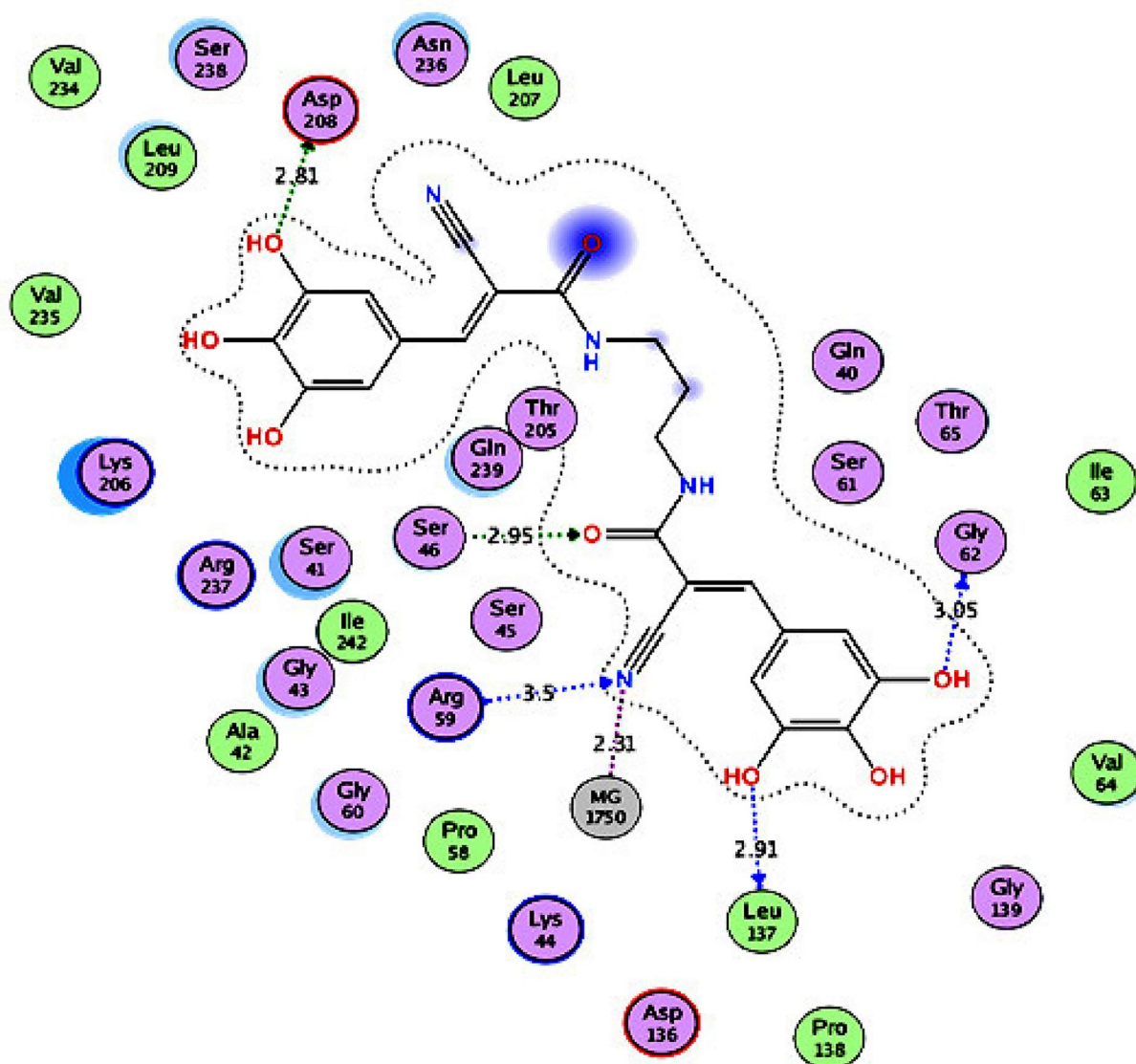
FIGURE 9 2D diagram (Distances in Å) of Bis-T (Group 4 inhibitor) showing its interaction with the dynI GTPase domain active site

TABLE 8 Docking energy scores and amino acids involved in binding for GMPPCP, chloroquine, and Group 4 inhibitors docked with dynI GTPase domain

Compound	Docking score (kcal/mol)	Amino acids involved in binding
GMPPCP	−23.73	Gly60, Ser41, Arg237, Lys206, Asn236, and Asp208
Chloroquine	−14.12	Lys206
Dynole 25	−14.67	Lys206
Dyngo 4a	−18.16	Gln239
Dyngo 6a	−18.70	Gly60, Ser46, Lys206, and Lys44
Dynasore	−18.52	Gly60
Iminodyn 21	−22.23	Lys206 and Asp208
Iminodyn 22	−24.16	Gly60, Arg237, Lys206, Leu137, Asn236, and Asp208
Iminodyn 23	−21.93	Thr65, Gly139, Asn236, and Lys206
Rhodadyn D10	−17.42	Gly139, Asn236, and Lys206
Bis-T	−25.73	Gly62, Leu137, Arg59, Gln239, Ser46, and Asp208
Pyrimidyn 6	−16.88	Gly60, Gly139, and Thr65
Pyrimidyn 7	−14.18	Ser41, Val64, and Thr65
Chlorpromazine	−12.3	Ser46 and Lys206
Genistein	−16.95	Gly60 and Lys206
7-Keto-cholesterol	−17.2	Ser45 and Ser46
LG186	−18.68	Gly60, Gln239, and Lys206

Therefore, continuous efforts are being made to develop endocytic protein inhibitors with less cytotoxicity that are safer for human use. FDA approved chlorpromazine, nystatin, amiloride, vinblastine, and itraconazole as safe drugs for human use worldwide, whereas others are under evaluation.[106] Table 1 summarizes different types of inhibitors and their cellular targets, with the underlying mechanisms. Understanding the membrane-trafficking mechanisms of the cell can aid in the development of new promising drugs against SARS-CoV-2 and other viruses.[159]

9 | SUMMARY AND FUTURE PERSPECTIVES

The coronavirus disease 2019 pandemic is still a global health threat. Despite the rapid spread of the virus and the viral-related morbidity decreased, research community still struggling in the detection and treatment of SARS-CoVs variants. The endocytic pathways are possible mechanisms involved in the entry of many viruses like SARS-CoVs, MERS-CoVs, and possibly SARS-CoV-2 into the host cell. In this review, we summarized the efficacy of different endocytic inhibitors, such as Dyngo 4a, Dyngo 6a, dynasore, Iminodyn 21, Iminodyn 22, Rhodadyn D10, remdesivir, and rottlerin, in combating SARS-CoV-2 by inhibiting the CME pathway and CME/lipid rafts. This could be achieved by inhibiting the GTPase activity of dynamin such as dynasore, dynoles, and dyngoes. The virus can also invade the cells via the CLIC/GEEC pathway, which can be blocked by LG186 and 7-keto-cholesterol that prevent the close packing of acyl chains and affect the structure of the cell membrane. The molecular docking simulations we carried out, revealed that remdesivir ($S = −14.46$ kcal/mol), methyl-β-cyclodextrin ($S = −13.07$ kcal/mol), rottlerin ($S = −13.89$ kcal/mol), and Bis-T ($S = −25.73$ kcal/mol) are more potent inhibitors of SARS-CoV-2 than chloroquine and act by inhibiting clathrin, HMG-CoA reductase, actin, and dynamin I GTPase, respectively. Although endocytic inhibitors can potentially inhibit endocytosis-mediated viral entry, they still have some limitations, such as the lack of information about their specificity and cytotoxicity on the non-endocytic targets in host cells. Therefore, additional computational analyses along with basic and clinical studies are required to achieve maximum drug-based variant independent antiviral effects associated with less side complications. Finally, there are a huge number of investigations are still running to investigate the potential antiviral activity of small molecules including endocytic inhibitors against COVID-19.

AUTHOR CONTRIBUTIONS

Samar Sami Alkafaas: Conceptualization; supervision; software; data curation; writing – original draft; editing and validation. **Abanoub Mosaad Abdallah**: Molecular docking; data curation; and writing – original draft. **Soumya Ghosh**: Data curation; writing – original draft and editing. All authors read and approved the final manuscript. **Samah A Loutfy**: Data curation; writing – original draft and editing. **Sara Sami ElKaffas**: Data curation; writing – original draft and editing. **Nasra F. Abdel Fattah**: Data curation; writing – original draft and editing. **Mohamed Hessien**: Conceptualization; data curation; and writing – original draft.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

CONSENT FOR PUBLICATION

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