Pharmacological Inhibition of Endocytic Pathways: Is It Specific Enough to Be Useful?

ARTICLE *in* METHODS IN MOLECULAR BIOLOGY · FEBRUARY 2008

Impact Factor: 1.29 · DOI: 10.1007/978-1-59745-178-9_2 · Source: PubMed

1 AUTHOR:

Andrei I Ivanov
Virginia Commonwealth University
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Pharmacological Inhibition of Endocytic Pathways: Is It Specific Enough to Be Useful?

Andrei I. Ivanov

Summary Eukaryotic cells constantly form and internalize plasma membrane vesicles in a process known as endocytosis. Endocytosis serves a variety of housekeeping and specialized cellular functions, and it can be mediated by distinct molecular pathways. Among them, internalization via clathrin-coated pits, lipid raft/caveolae-mediated endocytosis and macropinocytosis/phagocytosis are the most extensively characterized. The major endocytic pathways are usually distinguished on the basis of their differential sensitivity to pharmacological/chemical inhibitors, although the possibility of nonspecific effects of such inhibitors is frequently overlooked. This review provides a critical evaluation of the selectivity of the most widely used pharmacological inhibitors of clathrin-mediated, lipid raft/caveolae-mediated endocytosis and macropinocytosis/phagocytosis. The mechanisms of actions of these agents are described with special emphasis on their reported side effects on the alternative internalization modes and the actin cytoskeleton. The most and the least-selective inhibitors of each major endocytic pathway are highlighted.

Keywords Caveolae; clathrin; lipid rafts; macropinocytosis; phagocytosis; selectivity.

1 Introduction

The plasma membrane of eukaryotic cells represents a crucial interface that mediates communication between the cell interior and its environment. One of the central communication processes involves bidirectional fluxes of membrane-coated

From: Methods in Molecular Biology, Vol.440, Exocytosis and Endocytosis
Edited by A. Ivanov © Humana Press, Inc., Totowa, NJ

vesicles in and out of the cell surface. Formation and inward cytosolic movement of plasma membrane vesicles is termed *endocytosis*. Endocytosis is a fundamental feature of living cells that serves a variety of housekeeping and specialized functions. The former includes nutrient uptake, termination of receptor signaling, and regulation of cell shape and volume, and the latter encompasses neuronal synaptic transmission, transcellular transport, regulation of cell migration, and immune defense functions.

Three major endocytic pathways have been extensively investigated *(1)*. The first pathway involves the assembly of a specific coat protein, clathrin, on the intracellular face of the plasma membrane, resulting in the formation of a clathrin-coated pit. This pathway is characteristic of receptor-mediated endocytosis; in addition to clathrin, it requires a number of adaptor and accessory molecules for controlling different steps in the assembly and maturation of the coated pits. [Au1] Among these proteins, a so-called AP2 adaptor complex is critical for the initial linkages between cargo molecules and clathrin, whereas amphiphysin and dynamin guanosine triphosphatase (GTPase) regulate later conversion of membrane invagination into a vesicle *(1,2)*.

The second pathway involves invagination of cholesterol-enriched microdomains within the plasma membrane that may contain a coat protein known as caveolin. These structures are referred to as *lipid rafts* or *caveolae* ***(3)***. Lipid raft/caveolae-mediated endocytosis participates in the internalization of glycosylphosphatidylinositol-anchored proteins, cholera toxin entry, and intracellular cholesterol trafficking ***(1,3)***. The molecular mechanisms of this pathway involve dynamin activity and tyrosine kinase signaling.

The third major internalization pathway involves the formation of large F-actin-coated vacuoles that serve to uptake either solid particles (phagosomes) or liquid (macropinosomes) from the extracellular space *(4–6)*. Phagocytosis is a characteristic of specialized cells such as leukocytes, whereas macropinocytosis can be induced in many cell types on stimulation with mitogens and growth factors. Phagocytosis and macropinocytosis are initiated by the changes in the dynamics of cortical actin and are regulated by intracellular protein machinery that controls actin polymerization *(4–6)*.

Studies of different internalization pathways attract attention not only from classical cell biologists but also from researchers working in different fields of neurobiology, immunology, and pathophysiology. For such a diverse community, it is critical to have simple, reliable, and affordable tools that can be used to analyze endocytosis in vitro and in vivo. Direct probing of endocytosis in living cells is commonly achieved using pharmacological (chemical) inhibitors. Several advances of these inhibitors over more sophisticated molecular biological tools, targeting particular endocytic proteins, can be envisioned.

First, pharmacological inhibitors equally affect all cells in a population, and these effects can be easily titrated and quantified. Second, cells are usually exposed to inhibitors over a short period of time, which precludes the development of delayed side effects or compensatory mechanisms. Third, pharmacological

inhibitors still represent the tools of choice for in vivo studies. Last but not least, approaches that use chemical inhibitors remain the most time and labor efficient as well as the most affordable.

One major problem that may undermine the use of pharmacological inhibitors of endocytosis is their potential for poor specificity. However, this problem is frequently overlooked because a particular inhibitor might demonstrate high pathway specificity in one experimental condition but cause side effects in different experimental setups. Since pharmacological inhibitors of endocytosis remain extensively used, it is important to have an unbiased view of their specificity and possible side effects.

This review examines the specificity of pharmacological/chemical inhibitors most frequently used to probe different endocytic pathways. An inhibitor's specificity is evaluated based on two criteria. The first criterion implies that the particular agent affects only an endocytic pathway of interest without interfering with alternative internalization modes. The second criterion requires that the inhibitor does not affect actin cytoskeleton. It is particularly important because reorganization of cortical actin filaments may induce nonselective endocytosis-dependent and -independent changes in distribution, biochemical properties, and functions of various plasma membrane proteins. Using these simple criteria, I examine the literature on inhibition of three major endocytic pathways to determine which pharmacological tools can be considered the most selective and cause the fewest side effects on the living cells.

2 Inhibitors of Clathrin-Mediated Endocytosis

The history of chemical inhibition of clathrin-dependent internalization began in the early 1980s when monodansylcadaverine (MDC) *(7)*, potassium depletion *(8)*, and hypertonic sucrose *(9)* were introduced to block receptor-mediated endocytosis. Now, these early tools along with several other drugs and chemical maneuvers are widely used to probe internalization via clathrin-coated pits.

2.1 Hypertonic Sucrose

Hypertonic (0.4–0.5 *M*) sucrose represents probably the most popular chemical inhibitor of clathrin-mediated endocytosis. The underlying mechanism of such inhibition reportedly involves the dispersion of clathrin lattices on the plasma membrane *(10)*. Although blockage of protein internalization by hypertonic sucrose is considered strong evidence for clathrin-mediated endocytosis *(11–13)*, this treatment may also affect non-clathrin-mediated internalization pathways. Indeed, an early ultrastructural study by Carpentier et al. *(14)* observed that hypertonic sucrose not only reduced the number of clathrin-coated pits on

fibroblast plasma membrane but also caused the disappearance of noncoated invaginations. Furthermore, this treatment inhibited internalization of a classical lipid raft ligand, cholera toxin, along with a fluid-phase (macropinocytosis) marker, horseradish peroxidase. It is noteworthy that the decreased uptake of fluid-phase markers in cells incubated with hypertonic sucrose was confirmed by several subsequent reports *(15,16)*. Furthermore, a study in cardiac myocytes showed abnormal swelling of caveolae and disappearance of their resident proteins after an exposure to even moderately hypertonic (150 m*M*) sucrose *(17)*. Overall, these data suggest that hypertonic sucrose may interfere with all three major internalization pathways.

Moreover, it is well known that extracellular hypertonicity induces cell shrinkage, which in its turn causes compensatory activation of a number of plasma membrane ion transporters, pumps, and channels *(18)*. Stimulation of the ion transport activates a variety of intracellular kinases that may lead to the remodeling of the cortical actin cytoskeleton. Indeed, hypertonic medium was shown to trigger the assembly of polygonal F-actin network in aortic endothelial cells *(18)* and to cause redistribution of F-actin from the perijunctional belt into disorganized bundles in kidney epithelial cells *(19)*. Such a dramatic effect on the F-actin architecture adds yet another level of nonspecificity to the intracellular actions of hypertonic sucrose.

2.2 Potassium Depletion

Potassium depletion represents another classical *(8)*, but still useful *(20,21)*, chemical procedure to block internalization via clathrin-coated pits. Technically, this procedure consists of two steps, an initial hypotonic shock followed by an incubation in isotonic potassium-free medium *(8)*. Similar to hypertonic sucrose, potassium depletion is thought to block clathrin-mediated endocytosis by removing plasma membrane-associated clathrin lattices *(10)*. However, potassium depletion appears to be a more selective inhibitor of clathrin-dependent internalization when compared to hypertonic media. To the best of my knowledge, no inhibitory effects of potassium depletion on lipid raft/caveolae-mediated endocytosis have been reported. Although a decreased uptake of fluid-phase markers in potassium-depleted cells has been observed *(22,23)*, this effect may be caused by an increase in the regurgitation of the markers rather than the inhibition of macropinocytosis per se *(22)*.

Nevertheless, two studies have reported that potassium depletion affects the actin cytoskeleton. One study observed that potassium depletion blocked reorganization of F-actin filaments from circumferential bundles into linear stress fibers in fibroblasts, which resulted in the inhibition of cell spreading *(24)*. Another study showed that this treatment blocked the activation of Rho GTPase and attenuated the formation of basal stress fibers in renal epithelial cells acquiring the apico-basal cell polarity *(25)*. These data suggest that potassium depletion might cause some

side effects because of its interference with reorganization of the cortical actin cytoskeleton.

2.3 Cytosolic Acidification

Internalization through clathrin-coated pits can be blocked by decreasing the cytosolic pH of the cell *(13,26–28)*. Two major procedures for cytosolic acidification have been described. The first procedure involves preincubation of cells with 10–30 m*M* NH_4Cl to load them with NH_4^+ ion, followed by transferring cells into an ammonium-free medium *(28)*. Intracellular NH_4^+ dissociates into membrane-permeable NH_3 and a proton, which cannot cross the plasma membrane. Therefore, after the removal of NH_4Cl, NH_3 rapidly diffuses out of the cell, whereas the protons temporarily remain inside and lower the cytosolic pH. The second procedure involves incubating the cells with millimolar concentrations of weak acids, such as acetic or succinic acids *(27,28)*. In the protonated form, these acids rapidly cross the plasma membrane and enter the cells. Once inside the cell, the acids dissociate and lower the cytosolic pH.

In contrast to hypertonic sucrose and potassium depletion, cytosolic acidification does not prevent the formation of clathrin lattices but rather inhibits the budding off of clathrin-coated pits from the membrane *(10)*. Despite these different mechanisms, cytosolic acidification is associated with the same side effects as the two other inhibitors of clathrin-dependent internalization. Examples of these side effects include inhibited uptake of macropinocytosis markers in fibroblasts *(26)* and attenuation of apical endocytosis of nonclathrin ligand ricin in polarized epithelial cells *(29)*. Furthermore, cytosolic acidification has noticeable effects on the actin cytoskeleton, which include triggering global depolymerization of F-actin in neutrophils *(30)* and inducing translocation of actin microfilaments from the lateral plasma membrane to cytoplasmic aggregates in polarized epithelial cells *(31)*. Overall, these data reveal pleiotropic effects of cytosolic acidification on intracellular trafficking and the cytoskeleton that may complicate the interpretation of the actions of this treatment.

2.4 Chlorpromazine

Chlorpromazine is a cationic amphipathic drug that, when used in a micromolar range (50–100 μ*M*) inhibits clathrin-mediated endocytosis of various plasma membrane proteins *(11–13,20)*. Such inhibition likely involves a loss of clathrin and the AP2 adaptor complex from the cell surface and their artificial assembly on endosomal membranes *(13,32)*. There is no evidence in the literature that chlorpromazine affects lipid raft/caveolae-mediated endocytosis. Furthermore, several studies have demonstrated that receptor-mediated endocytosis, which can be inhibited

by chlorpromazine, is insensitive to the agents that block internalization via lipid rafts and vice versa *(11,12)*. However, chlorpromazine was shown to block phagocytosis in immune cells such as neutrophils and macrophages *(33,34)* and inhibit neutrophil degranulation *(33)*.

These data suggest that, in addition to affecting clathrin-coated pits, chlorpromazine may interfere with the biogenesis of large intracellular vesicles such as phagosomes, macropinosomes, and granules. Two possible mechanisms may underline this interference. First, since chlorpromazine is an amphipathic molecule, it can easily incorporate into the lipid bilayers and can increase lipid fluidity within the plasma membrane *(35)*. This alteration in the physical state of the plasma membrane lipids may block the formation of large membrane invaginations similar to the known inhibitory actions of other membrane fluidizers on macropinocytosis *(36)*. The second possible mechanism may involve a reported inhibitory effect of chlorpromazine on phospholipase C (PLC) *(37)*, which is an important regulator of actin dynamics *(38)* and macropinocytosis *(39,40)*. Such multiple interactions of chlorpromazine with intracellular lipids and cytoskeletal regulators should be considered as potential sources for the side effects of this inhibitor.

2.5 Monodansylcadaverine

Since 1980, monodansylcadaverine (MDC) has been extensively used to block clathrin-mediated endocytosis in different mammalian cells *(7,15,41,42)*. The inhibitory activity of MDC is attributed to the stabilization of clathrin-coated pits by the drug *(42)*. However, the evidence for this mechanism has been obtained only in cell-free systems using purified clathrin and very high (0.7–10 m*M*) concentrations of MDC *(43)*. It remains to be investigated if similar stabilization of clathrin-coated pits can be achieved at lower (100–300 μ*M*) concentrations of MDC that attenuate endocytosis in living cells.

Monodansylcadaverine appears to be a relatively specific blocker of clathrin-mediated internalization. No studies have reported inhibitory actions of this drug on the lipid raft/caveolae-dependent pathway. Furthermore, its effects on macropinocytosis and phagocytosis remain controversial, with some studies demonstrating inhibition *(44,45)* and others reporting no observable blockage *(15,46)* of these pathways.

Some possible side effects of MDC treatment may originate from its known inhibitory activity toward the enzymes of the transglutaminase family *(47)*. These enzymes are involved in posttranscriptional transamidation of various proteins. Particularly, transglutaminases have been shown to be important for the activation of Rho GTPases *(48)*, which are the key regulators of actin assembly and dynamics. Furthermore, transglutaminase activity is required for the self-association of the major F-actin motor, myosin II *(49)*. Therefore, inhibition of transglutaminase by MDC may result in global changes in the organization and dynamics of the actin cytoskeleton *(48,49)*.

2.6 Phenylarsine Oxide

Phenylarsine oxide (PAO) is another chemical compound that, at low micromolar (1–20 μM) concentrations, blocks clathrin-dependent endocytosis *(23,27,50,51)*. The exact mechanism of such inhibition remains unknown since no ultrastructural studies examining the effects of PAO on the formation of clathrin-coated pits have been reported. Furthermore, PAO is not selective to the clathrin pathways and has also been shown to inhibit macropinocytosis in adipocytes *(52)* and phagocytosis in mast cells *(53)*.

Phenylarsine oxide is a trivalent arsenite that is known to crosslink vicinal sulfhydryl groups *(54)*. Therefore, it is not surprising that it can inhibit multiple intracellular targets, including major cytoskeletal regulators such as protein tyrosine phosphatases *(55)* and Rho GTPase *(56)*. Such effects together with the reported PAO-dependent depletion of intracellular adenosine triphosphate (ATP) *(50)* may explain the dramatic disorganization of actin cytoskeleton observed in PAO-treated cells *(55,56)*. Overall, these serious side effects outweigh the usefulness of PAO as a selective inhibitor of clathrin-mediated endocytosis.

2.7 Summary of Inhibitors of the Clathrin-Mediated Pathway

Despite the common belief that the above pharmacological agents or chemical maneuvers selectively inhibit internalization via clathrin-coated pits, none of the inhibitors possesses absolute selectivity. In particular, all these inhibitors have been shown to block uptake of fluid-phase markers and therefore cannot be used to distinguish between clathrin-mediated endocytosis and macropinocytosis. In addition, all pharmacological blockers of the clathrin pathway may cause the reorganization of the cortical actin cytoskeleton and therefore grossly affect biochemical and functional properties of plasma membrane proteins via endocytosis-independent mechanisms. However, some of these inhibitors, such as potassium depletion, chlorpromazine, and MDC, can still be used for the initial discrimination between clathrin-mediated internalization and other endocytic pathways. On the other hand, hypertonic sucrose and PAO appear to be a poor choice to probe clathrin-mediated endocytosis because of their abundant side effects.

3 Inhibitors of Lipid Raft/Caveolae-Mediated Endocytosis

A vast majority of pharmacological inhibitors of caveolae/lipid-raft mediated endocytosis target cholesterol, a critical lipid constituent of membrane rafts and caveolae *(3,57)*. Four different strategies have been used to modify the content or chemical properties of membrane cholesterol *(57)*. The first strategy

involves general inhibition of cholesterol biosynthesis by statins. The second approach utilizes the extraction of cholesterol from the plasma membrane using cyclodextrins. The third strategy involves cholesterol sequestration within the membrane by polyene antibiotics such as filipin and nystatin. The fourth commonly used method utilizes the enzymatic modification of plasma membrane cholesterol by cholesterol oxidase. Detailed protocols for alterations of the plasma membrane cholesterol by these approaches have been described *(57)*.

3.1 Statins

One of the most effective ways to deplete intracellular cholesterol involves inhibition of cholesterol synthesis by a group of low molecular weight substances termed statins. Statins such as lovastatin, simvastatin, pravastatin, and the like are reversible inhibitors of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is a rate-limiting enzyme in cholesterol biosynthesis *(58,59)*. Incubation of cells with 10–100 μ*M* of statins for 3–4 h has been shown to result in almost 100% blockage of cholesterol synthesis *(60)*. However, such a dramatic cholesterol depletion appears to have a general and nonspecific inhibitory effect on endocytosis. Indeed, treatment with statins reportedly blocked clathrin-dependent receptor-mediated internalization in lymphocytes and epithelial cells *(60,61)*, inhibited internalization of the fluid-phase marker in epitheliocytes (6o), and blocked phagocytosis in macrophages *(62)*.

The inhibitory effects of statins on different internalization pathways have a simple mechanistic explanation. Statins block the synthesis of L-mevalonic acid, which is a common precursor not only for cholesterol but also for other isoprenoids such as farnesylpyrophosphate and geranylgeranylpyrophosphate *(58)*. These last two intermediates serve as important lipid moieties for post-translational modification (isoprenylation) of various intracellular proteins, including members of Ras, Rho, and Rab families of small GTPases *(58,63)*. A lack of the isoprenylation has been shown to prevent targeting of small GTPases to the cell membranes where they subsequently become activated. Hence, the incubation with statins causes the accumulation of inactive cytosolic forms of Rho and Rab *(58,63,64)*, thus leading to a profound and nonspecific disruption of intracellular vesicle trafficking and the actin cytoskeleton.

3.2 Methyl-β-cyclodextrin

Methyl-β-cyclodextrin (MβCD) is a widely used inhibitor that alters the structure of cholesterol-rich domains in the cell membranes *(57)*. MβCD is a cyclic

heptasaccharide containing a hydrophobic core that has a high affinity for cholesterol *(65)*. When added to cells at 5–10 m*M* concentrations, MβCD rapidly (within 1 h) forms soluble inclusion complexes with cholesterol, thereby depleting this lipid from the cell membranes *(66)*. This treatment was shown to cause the flattening of caveolae and mislocalization of caveolin-1 in mouse fibroblasts *(67)*. Furthermore, MβCD reportedly inhibits internalization of several marker ligands of lipid rafts and caveolae, including cholera toxin *(68)*, glucose transporter *(69)*, and nitric oxide synthase *(70)*.

In addition to its effects on lipid raft/caveolae-mediated endocytosis, acute cholesterol depletion by MβCD was shown to block internalization of a classical ligand of clathrin-mediated pathway, transferrin *(69,71)*. This side effect can be explained by the blockage of invagination of clathrin-coated pits in the MβCD-treated cells *(71)*. In addition, MβCD was shown to block fluid-phase endocytosis in epithelial and endothelial cells *(72,73)*. Such a nonspecific inhibition of several internalization pathways by acute cholesterol depletion is not surprising when considering the dramatic effects of MβCD treatment on cellular cytoskeleton and signaling. These effects include the dispersion of a cortical F-actin in adipocytes *(74)*, inhibition of phosphatidylinositol turnover in A431 carcinoma cells *(75)*, and activation of Ras GTPase in Cos-7 epithelial cells *(76)*.

3.3 Filipin and Nystatin

Polyene antibiotics such as filipin and nystatin readily interact with cholesterol in model and biological membranes and are able to change properties of cholesterol-rich membrane domains. Both antibiotics create large aggregates in the membrane that are visible by freeze-fracture electron microscopy *(77)*. The aggregates accumulate cholesterol, thereby sequestering this lipid from the membrane structures. As a result, filipin (~1 μ*M*) and nystatin (20–50 μ*M*) induce a profound distortion of the structure and functions of the cholesterol-rich membrane domain, including aberrations in the caveolar shape *(78)*, dispersion of GPI-anchored proteins from these structures *(79)*, as well as the inhibition of lipid raft ligands internalization *(78,80,81)*.

[Au2]

Polyene antibiotics appear to be quite selective inhibitors of the lipid raft/caveolae pathway. In contrast to MβCD, nystatin and filipin do not inhibit the internalization of transferrin *(57,78)*, which suggests that these drugs have little effect on the clathrin-mediated endocytosis. Furthermore, one recent study demonstrated that although MβCD blocked macropinocytosis in rat fibroblasts, filipin treatment did not affect this pathway *(82)*. However, two possible side effects of filipin treatment should be considered. The first involves permeabilization of the plasma membrane that occurs because of filipin's interactions not only with cholesterol but also with membrane phospholipids *(83)*. Fortunately, membrane permeabilization occurs at relatively high (above 10 μ*M*) levels of filipin, and this side effect can be minimized by lowering the drug concentration. Another reported side effect of filipin involves

disruptions of the linkage between cortical F-actin and the plasma membrane *(84)*. The mechanisms behind this cytoskeletal effect of filipin and its impact on inhibition of endocytosis remain to be investigated.

3.4 Cholesterol Oxidase

Cholesterol oxidase converts cholesterol into 4-cholesten-3-one *(85)*. The oxysterol remains enriched in caveolae and dramatically changes their properties *(86)*. These changes include translocation of caveolin from the plasma membrane to the Golgi complex in fibroblasts *(86)* and a decrease in the number of caveolae in smooth muscle cells *(87)*. Only a few studies used cholesterol oxidase to block caveolae-mediated endocytosis, but they showed that this treatment attenuates internalization of caveolae ligands as efficiently as the more popular procedures of cholesterol depletion *(27,69)*. In comparison to the related techniques, the treatment with cholesterol oxidase appears to be a more selective method of cholesterol depletion. This superior selectivity is based on the high substrate specificity of the enzyme as well as on its non-permeability thorough the plasma membrane. In addition, this treatment appears to be well tolerated by cells and does not induce significant leakage of the plasma membrane *(88)*.

Nevertheless, two possible problems of using cholesterol oxidase as an inhibitor of lipid raft/caveolae-mediated endocytosis should be considered. The first is inefficient oxidation of cholesterol in living cells. Indeed, the action of cholesterol oxidase depends on a variety of factors, including phospholipid composition, surface pressure of the plasma membrane, presence of divalent cations, and ionic strength of the media *(89)*. The second possible problem of using cholesterol oxidase arises from its enigmatic ability to switch protein internalization from caveolae to clathrin-coated pits *(90)*. Both problems may lead to a false-negative conclusion on the involvement of lipid raft/caveolae-mediated endocytosis.

3.5 Summary of Inhibitors of Lipid Raft/ Caveolae-Mediated Endocytosis

A body of literature suggests that lipid raft/caveolae-mediated endocytosis can be selectively inhibited by an acute depletion of the plasma membrane cholesterol. Among different protocols of cholesterol depletion, the incubation with filipin and nystatin and cholesterol oxidase treatment produce the fewest side effects. However, appropriate controls should be included while performing both procedures, such as a control for plasma membrane integrity during filipin exposure and a control for the efficiency of cholesterol oxidase treatment. It should be stressed that chronic inhibition of cholesterol synthesis by statins or acute cholesterol depletion by MβCD nonspecifically disrupts intracellular vesicle trafficking and the actin

cytoskeleton and should not be used to probe lipid raft/caveolae-mediated endocytosis.

4 Inhibitors of Macropinocytosis and Phagocytosis

Despite the fact that macropinocytosis and phagocytosis are the oldest known types of endocytosis, only a few pharmacological tools have been developed to study these internalization pathways in living cells. This has two explanations. First, unlike clathrin-coated pits and caveolae that possess a specific protein or lipid coat, no unique protein or lipid targets for pharmacological inhibition have been identified in macropinosomes or phagosomes. Furthermore, it is generally believed that macropinosomes and phagosomes can be easily distinguished from other types of plasma membrane-derived vesicles because of their large size (above 0.2 mm diameter; *6*). However, this criterion does not always work since recent studies have demonstrated the clathrin-dependent formation of large intercellular vesicles during internalization of bacteria and epithelial junctions *(91,92)*.

Three major types of pharmacological inhibitors have been used to disrupt macropinocytosis and phagocytosis. They include inhibitors of sodium–proton exchange, F-actin-depolymerizing agents, and drugs that target phosphoinositide metabolism.

4.1 Inhibitors of Sodium–Proton Exchange

An early serendipitous observation that epidermal growth factor simultaneously stimulates pinocytosis and amiloride-sensitive sodium–proton exchange in A431 carcinoma cells prompted researchers to use amiloride for the inhibition of macropinocytosis *(93)*. Subsequently, amiloride and its derivatives 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) and dimethyl amiloride (DMA) were shown to block constitutive and stimulated macropinocytosis and phagocytosis in a variety of mammalian cells *(40,94–96)*. Amiloride blocks macropinocytosis when used at millimolar concentrations *(93)*, whereas EIPA and DMA are effective in the range of 50–100 μ*M* *(94,95,97)*. These differences in potency suggest that the blockage of the activity of the Na^+/H^+ exchanger isoform 1 underlies the inhibitory actions of amiloride and its derivatives.

Although the original study found no effect of amiloride on internalization of the classical clathrin pathway ligand transferrin *(93)*, subsequent publications demonstrated that this group of inhibitors may also block clathrin-mediated endocytosis *(27,98)*. In addition, amiloride was shown to attenuate internalization through lipid rafts *(99)*, whereas EIPA reportedly altered morphology and the intracellular distribution of early and late endosomes *(97)*. Furthermore, EIPA was shown to induce reorganization of the F-actin in epithelial cells, which included the disassembly of

stress fibers, pseudopod retraction, and loss of cell–matrix adhesions *(100)*. These data clearly show multiple side effects of amiloride and its derivatives on intracellular vesicle trafficking and the cytoskeleton.

4.2 F-Actin-Depolymerizing Drugs

Since macropinosomes and phagosomes represent large F-actin-coated vesicles *(4–6)*, disruption of their F-actin coat should prevent the formation of these structures. Submicromolar concentrations of two pharmacological agents, cytochalasin D and latrunculins, have been commonly used to disassemble the actin cytoskeleton in living cells *(101)*. Cytochalasin D blocks actin polymerization by occupying a faster-growing "barbed" end of actin filaments, whereas latrunculins bind to monomeric actin, thus preventing its incorporation into filaments *(101)*. Both types of F-actin-depolymerizing drugs have reportedly blocked membrane ruffling and inhibited macropinocytosis and phagocytosis under various experimental conditions *(102–104)*.

It is noteworthy that recent studies have implicated the actin cytoskeleton in the regulation of different endocytic pathways (*see*, for review, *Refs. 105* and *106*). Indeed, confocal microscopy imaging of living cells revealed that the formation and internalization of both clathrin-coated pits and caveolae are accompanied by the recruitment of actin to these structures. Furthermore, pharmacological inhibition of actin polymerization has been shown to block endocytosis via clathrin-coated pits and caveolae *(105,106)*. These data strongly suggest that cytochalasin D and latrunculins should be considered as global and nonselective inhibitors of all internalization pathways rather than specific blockers of macropinocytosis and phagocytosis.

4.3 Inhibitors of Phosphoinositide Metabolism

Macropinocytosis and phagocytosis have also been targeted by inhibiting either phosphoinositide 3-kinase (PI3K) or phosphoinositide-specific PLC, which are the key enzymes involved in phosphoinositide metabolism *(4,39)*. PI3K is known to phosphorylate the hydroxyl group at the third carbon in the inositide ring of phosphoinositides, generating the so-called D3 products *(107)*, whereas PLC hydrolyzes one of the D3 phosphoinositides to produce a diacylglycerol and inositol 1,4,5-triphosphate *(38)*. Two PI3K inhibitors, wortmannin (100–200 n*M*) and LY290042 (~20 μ*M*) were used to block constitutive and stimulated macropinocytosis and phagocytosis in macrophages, fibroblasts, and epithelial cells *(102–104,108)*. Likewise, these internalization pathways appear to be sensitive to PLC inhibitors nitrocarboxyphenyl-*N, N*-diphenylcarbamate (50–100 μ*M*) and U73122 (1 μ*M*)*(39,40,103)*. The relationship between PI3K and PLC signaling during macropinocytosis remains poorly understood, although one recent study suggested their sequential activation and placed PLC downstream of PI3K in the signaling cascade *(39)*.

Two major mechanisms are likely to underlie the role of phosphatidylinositide-metabolizing enzymes in endocytosis. The first involves the assembly of multiprotein complexes critical for the formation and fusion of intracellular vesicles *(38,107,109)*. This mechanism is determined by the ability of phosphatidylinositides to bind scaffolding and signaling proteins and link them to phospholipid membranes. Proteins possessing phosphatidylinositide-binding domains include several clathrin adaptors, dynamin GTPase, as well as an early endosomal protein EEA1 *(107,109)*. The second mechanism involves reorganization of the actin cytoskeleton since PI3K- and PLC-generated lipid mediators are known to regulate several important steps (nucleation, elongation, and bundling) in the assembly of actin filaments *(38,107)*. The ability of phosphoinositides to modulate different steps in intracellular vesicle trafficking and cytoskeletal reorganizations is inconsistent with their selective involvement in macropinocytosis and phagocytosis. Indeed, PI3K and PLC inhibitors have pleiotropic effects on endocytosis as they are able to block the internalization of known ligands of the clathrin- and caveolae-mediated pathways *(110)*.

4.4 Summary of Inhibitors of Macropinocytosis and Phagocytosis

A wealth of published data casts doubts on the specificity of all pharmacological tools used to block macropinocytosis and phagocytosis. It is obvious that F-actin-depolymerizing drugs and inhibitors of phosphoinositide metabolisms may interrupt different internalization pathways and affect the architecture and dynamics of a cortical F-actin. As a result, their inhibitory effects on endocytosis are difficult to interpret unambiguously. Of all drugs discussed in this section, selective inhibitors of the Na^+/H^+ exchanger such as EIPA and DMA may have the fewest side effects and should be considered as a first choice for the pharmacological probing of macropinocytosis and phagocytosis. However, possible endocytosis-unrelated effects of these drugs on ion transport and cytoskeleton should be considered.

5 Conclusion and Perspectives

One simple conclusion of this review is that none of the popular inhibitors of different endocytic pathways possesses an absolute specificity to the pathway of interest. This lack of absolute specificity does not mean that pharmacological inhibitors should be excluded from probing different endocytic pathways. Some of them remain useful tools, especially when used in combination with modern molecular and cell biology approaches. Furthermore, a renewal of interest in using small molecular inhibitors to probe intracellular vesicle trafficking can be predicted based on the rising popularity of the chemical compound library screenings and on the

increased numbers of successful structural studies of the critical proteins involved in different endocytic pathways. Relevant examples of fruitful chemical library screens include two different inhibitors of dynamin GTPase *(111,112)*, whereas structural studies have already led to the development of a peptide that selectively inhibits clathrin-mediated endocytosis by disrupting the interaction between the clathrin adaptor, amphiphysin, and dynamin *(2)*. We can expect therefore that future work will discover more highly selective pharmacological inhibitors of endocytosis that can be used for experimental and clinical applications.

Acknowledgments I thank Moshe Bachar for his excellent editorial assistance and Drs. Alex Mongin and Andrew Kowalczyk for their valuable comments on the manuscript. This work was supported by a Career Development Award from the Crohn's and Colitis Foundation of America.

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Author Queries:

[Au1]: Define this and all abbreviations/initialisms/acronyms on first mention.
[Au2]: Spell out GPI.