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Endocytosis Inhibitors Prevent Poly(amidoamine) Dendrimer Internalization and Permeability across Caco-2 Cells

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Abstract: Previous studies from our group demonstrated visual evidence that endocytosis mechanism(s) contribute to the internalization and intracellular trafficking of cationic and anionic poly(amidoamine) (PAMAM) dendrimers across Caco-2 cells. These dendrimers colocalized with established endocytosis markers, which suggested PAMAM dendrimers may be internalized by a clathrin-dependent endocytosis mechanism and are rapidly trafficked to endosomal and lysosomal compartments. In the present study, generation 4 PAMAM-NH_2 ($G4NH_2$) dendrimer was labeled with tritium to measure the rate of uptake and permeability in Caco-2 cells. The effect of endocytosis inhibitors brefeldin A, colchicine, filipin, and sucrose on $G4NH_2$ absorption and transport was examined to give further insight into the endocytosis mechanisms that transport PAMAM dendrimers across Caco-2 cell monolayers. $G4NH_2$ showed linear uptake at lower concentrations, and rapidly increased as a function of concentration. The rate of $G4NH_2$ uptake significantly declined at high concentrations in the presence of the endocytosis inhibitors, and the apparent permeability similarly reduced in the presence of these inhibitors. A significant reduction in $G4NH_2$ permeability was observed in the presence of brefeldin A and colchicine, which generally disrupt vesicular trafficking and formation during the endocytosis process. Coincubation with filipin and sucrose reduced $G4NH_2$ permeability to a lesser extent, which suggests $G4NH_2$ could be nonspecifically internalized in coated vesicles at the plasma membrane. The observations from this study further confirm that $G4NH_2$ internalization and transport involves an endocytosis pathway.

Keywords: poly(amidoamine) dendrimers; endocytosis; Caco-2 cells; riboflavin; absorption; transport

Introduction

Dendrimers are a class of polymers that have been widely used for the delivery of therapeutic and diagnostic agents.[1] Their potential as drug carriers arises from the large number of surface groups to immobilize drugs, enzymes, targeting moieties, or other bioactive and imaging agents. For instance, cationic dendrimers poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) with surface amine groups have been used as gene transfection agents,[2] since they are capable of condensing nucleic acids through interactions with anionic phosphate groups and serve as an endosomal pH buffer, which results in enhanced cellular delivery of nucleic acids.[3] Such principles have led to the development of Superfect and PolyFect transfection reagents by Qiagen, Inc. Similarly, the surface amines of cationic dendrimers can be conjugated to gadolinium(III) chelates to enhance magnetic resonance imaging through prolonged blood circulation, greater extravasation, and improved excretion compared to other macromolecular contrast agents.[4] These macromolecules are capable of enhancing drug solubility, increasing drug circulation due to their nanoscopic size, and targeted delivery with the incorporation of specific ligands to the surface groups. Clearly, dendrimers offer many advantages in the delivery of bioactive and diagnostic agents. However, little information is available on the mechanisms of dendrimer uptake and intracellular trafficking.

Oral drug administration offers several advantages over parenteral drug administration, such as the elimination of pain and discomfort associated with injections, the ease of administration, and lower costs to produce oral formulations. Thus, as increased new therapeutic agents continue to be

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produced, substantial research has taken place to develop oral drug delivery systems that enhance their intestinal absorption. The unique architecture of PAMAM dendrimers consists of a branching mechanism that increases exponentially with an increase of amidoamine groups, and each consecutive series of branching is termed a generation (G).[5] The branching multiplicity of dendrimers allows for therapeutic agents to be either encapsulated within the dendritic cavity or conjugated/complexed to their surface groups.[5] Recent studies from our laboratory and others have demonstrated the transepithelial transport of PAMAM dendrimers across the intestinal epithelial barrier,[6–10] and initial mechanistic studies suggested that cationic PAMAM dendrimers are transported via a combination of the paracellular pathway and adsorptive endocytosis.[11–13] Subsequent research in our laboratory demonstrated that cationic and anionic PAMAM dendrimers colocalize with endosomal and lysosomal markers in Caco-2 cells and the intracellular trafficking from primary endosomes to secondary endosomes and lysosomes is dependent on time and surface charge.[14] Overall, these studies offered visual and semiquantitative confirmation that endocytosis mechanisms contribute to the internalization and intracellular trafficking of PAMAM dendrimers.

The aim of this study was to explore the rate of absorption and transport of PAMAM dendrimers in Caco-2 cells to provide further insight into the mechanisms by which PAMAM dendrimers are internalized and transported. Cationic generation 4 dendrimers ($G4NH_2$) were labeled with tritium to measure their cellular internalization and transport in Caco-2 cell monolayers. Furthermore, the effect of endocytosis inhibitors brefeldin A, colchicine, filipin, and sucrose on $G4NH_2$ absorption and permeability was evaluated. The present study quantifies the extent by which $G4NH_2$ dendrimers are transported across the intestinal epithelial barrier.

Experimental Section

Materials. PAMAM dendrimer generation 4 solution ($G4NH_2$), Triton X-100, Hank's balanced salt solution (HBSS), and *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) hemisodium salt (HEPES) buffer were purchased from Sigma-Aldrich Co. (St. Louis, MO). [^{3}H]-Acetic anhydride was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]-Riboflavin was purchased from Moravek Biochemicals, Inc. (Brea, CA). Caco-2 cells were purchased from American type Cell Culture (Rockville, MD). Cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA). WST-1 cell proliferation reagent was purchased from Roche Applied Science (Indianapolis, IN). BCA Protein Assay Kit was purchased from Pierce Biotechnology (Rockford, IL).

Synthesis and Characterization of Radiolabeled $G4NH_2$. $G4NH_2$ was radiolabeled as previously described.[15] Briefly, $G4NH_2$ PAMAM dendrimers were reacted with [^{3}H]-labeled acetic anhydride (5 mCi/mmol of the polymer) in the presence of excess triethylamine to quench acetic acid formed as a side product.[16] $G4NH_2$ (0.100 g, 0.03 mmol) was dissolved in dry methanol (10 mL) followed by addition of [^{3}H]-labeled acetic anhydride (5 mCi/mmol of the polymer) (0.062 g, 0.61 mmol) and triethylamine (0.12 g, 1.2 mmol). The solution was stirred for 12 h at room temperature, and then methanol was evaporated to obtain crude acetylated $G4NH_2$. The crude product was redissolved in water and dialyzed against distilled water using 1000 MWCO dialysis membranes (Spectrum Laboratories, Inc., Rancho Dominguez, CA). Small molecular weight impurities were not detected from size exclusion chromatography using a PD-10 column. The specific activity for radiolabeled $G4NH_2$ was in the range of 2–3 mCi/mmol.

Caco-2 Cell Culture. Caco-2 cells (passages 30–60) were grown at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells were maintained in T-75 flasks using

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Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 10000 units/mL of penicillin, 10000 μg/mL of streptomycin, and 25 μg/mL of amphotericin. Growth medium was changed every 2 days. Cells were passaged at 70–90% confluency using 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) solution. Transport medium consisted of HBSS supplemented with 10 mM HEPES buffer (pH 7.4).

Cytotoxicity of PAMAM Dendrimers in Rapidly (Undifferentiated) Proliferating Cells. Caco-2 cells were seeded at a seeding density of 30,000 cells/well in 96-well cell culture plates (Corning, Inc., Corning, NY) and maintained for 48 h under cell culture conditions described in the previous section. The cells were washed twice with HBSS transport medium and then incubated with 100 μL (1–10 μM) of $G4NH_2$. After 2 h, the cells were washed three times with HBSS to remove $G4NH_2$ and replaced with 100 μL of HBSS. Thereafter, 10 μL of cell proliferation reagent WST-1 was added to each well, followed by an incubation period of 4 h. Absorbance was measured at 440 nm using a SpectraMax 384 Plus plate reader (Molecular Devices, Sunnyvale, CA).

Cellular Uptake of $G4NH_2$ in (Undifferentiated) Caco-2 Cells. Caco-2 cells were seeded at 80000 cells/mL on cell culture-treated 24-well plates (Becton Dickinson). After 4 days, cells were washed twice with HBSS and incubated with 0.5 mL of HBSS for 20 min, followed by 200 μL of [^{3}H]-riboflavin (0–60 nM) or 200 μL of [^{3}H]-$G4NH_2$ (0–8 μM) for 20 min. At the end of the incubation, cells were washed twice with ice-cold HBSS buffer. The cells were then lysed with 250 μL of 1% Triton X-100. The cell-associated radioactivity was measured using liquid scintillation counting (Beckman Coulter, Fullerton, CA). The uptake data was normalized to total protein content determined with the BCA Protein Assay Kit. To study the effect of endocytosis inhibitors on riboflavin and $G4NH_2$ uptake, cells were preincubated with 500 μL of inhibitor solution for 20 min at 37 °C, followed by solutions of 200 μL of [^{3}H]-riboflavin (0–60 nM) or [^{3}H]-$G4NH_2$ (0–8 μM) that also contained the same concentration of inhibitor used for preincubation for 20 min: 5 μM brefeldin A; 10 μM colchicine; 1 μg/mL of filipin; 200 mM sucrose.

$G4NH_2$ Permeability Across (Differentiated) Caco-2 Cell Monolayers. Caco-2 cells were seeded at 80000 cells/cm^2 onto polycarbonate 12-well Transwell filters of 3.0 μm mean pore size, 1.0 cm^2 surface area (Corning Inc., Corning, NY). Caco-2 cells were maintained under incubation conditions described above and used for transport experiments 21–28 days postseeding. The transport of radiolabeled PAMAM dendrimers was investigated in triplicate in the apical-to-basolateral (AB) direction at a donor concentration of 1.0 μM. Permeability experiments were conducted in a humidified atmosphere of 37 °C while maintaining sink conditions. Samples were collected from the receiver chamber at 30, 60, 90, and 120 min to obtain the permeability rate. Samples were analyzed by liquid scintillation counting

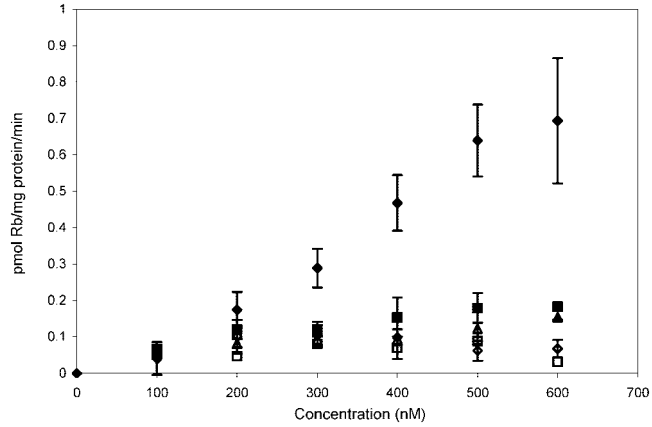

Figure 1. Uptake of [^{3}H]-riboflavin in Caco-2 cells is concentration-dependent. Cells were seeded at 80000 cells/ml in 24-well plates and incubated for 20 min with various concentrations of [^{3}H]-riboflavin. Uptake of [^{3}H]-riboflavin was measured in the absence (♦) and presence of various endocytosis inhibitors: (◊) 5 μM brefeldin A; (■) 10 μM colchicine; (□) 1 μg/mL of filipin; (Δ) 200 mM sucrose. Results are reported as mean ± SD (n = 3).

(Beckman Coulter, Fullerton, CA). The apparent permeability (P_{app}) coefficients were calculated as follows:

$$P_{app} = \frac{\partial Q}{AC_0 \partial t}$$

where $\partial Q/\partial t$ is the permeability rate, A is the surface area of the membrane filter, and C_0 is the initial concentration in the donor chamber.[17] To study the effect of endocytosis inhibitors on riboflavin and $G4NH_2$ permeability, cells were preincubated with 500 μL of inhibitor solution for 20 min at 37 °C, followed by solutions of 500 μL of [^{3}H]-riboflavin and [^{3}H]-$G4NH_2$ that also contained the same concentration of inhibitor used for preincubation for 30, 60, 90, and 120 min.

Statistical Analysis. Student's t-test was used to statistically compare two sets of data, e.g. mean percent cell viability of Caco-2 cells (treated vs untreated), cellular uptake of dendrimers (in presence vs absence of endocytosis inhibitors) and apparent permeability of dendrimers (in presence vs absence of endocytosis inhibitors) (SPSS for Windows).

Results

Determination of $G4NH_2$ Cytotoxicity in Caco-2 Cells. The influence of $G4NH_2$ on Caco-2 cell viability was investigated using the WST-1 cytotoxicity assay (Figure 1). Triton X-100 significantly ($p < 0.00001$) reduced Caco-2 cell viability (8.5%), whereas $G4NH_2$ showed more than 86% cell viability at all the concentrations tested after 2 h. The

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uptake of [^{3}H]-G4NH$_2$ was measured at donor concentrations of 1–8 μM, and [^{3}H]-G4NH$_2$ permeability was measured at 1 μM.

Rate and Mechanism of G4NH$_2$ Uptake. Previous studies in our group have conclusively established the involvement of a riboflavin specific receptor-mediated endocytic process regulating this vitamin's internalization and intracellular trafficking.[18–20] Thus, [^{3}H]-riboflavin was chosen as a positive control ligand for endocytosis in the present studies. The cellular uptake of [^{3}H]-riboflavin increased linearly, and slightly tapered off at higher concentrations with uptake rates of 0.64 pmol/mg protein/min and 0.69 pmol/mg protein/min for 500 and 600 nM, respectively (Figure 1). The application of brefeldin A resulted in significant reduction of [^{3}H]-riboflavin absorption at concentrations $\geq$300 nM ($p < 0.05$), and the rate of [^{3}H]-riboflavin absorption declined 10-fold (0.068 pmol/mg protein/min) in Caco-2 cells at the maximum [^{3}H]-riboflavin concentration tested (Figure 1). Similarly, coincubation with colchicine caused significant reduction in [^{3}H]-riboflavin absorption at concentrations $\geq$400 nM ($p < 0.05$) to 0.18 pmol/mg protein/min. Filipin significantly reduced the rate of [^{3}H]-riboflavin internalization to 0.032 pmol/mg protein/min ($p < 0.05$), while coincubation with sucrose reduced [^{3}H]-riboflavin uptake to 0.15 pmol/mg protein/min ($p < 0.05$).

[^{3}H]-G4NH$_2$ uptake increased linearly at lower concentrations (1–4 μM), and rapidly increased at concentrations of 5−8 μM to a maximum rate of 51.9 pmol/mg protein/min (Figure 2). The application of endocytosis inhibitors reduced the rate of [^{3}H]-G4NH$_2$ uptake, which was also observed for [^{3}H]-riboflavin uptake. Brefeldin A reduced [^{3}H]-G4NH$_2$ uptake 2-fold to 22.1 pmol/mg protein/min at 8 μM ($p < 0.05$). Likewise, [^{3}H]-G4NH$_2$ absorption was reduced almost 3-fold to 18.5 pmol/mg protein/min ($p < 0.05$) in the presence of colchicine. The coincubation with filipin resulted in reduced [^{3}H]-G4NH$_2$ uptake 3-fold to 16.5 pmol/mg protein/min ($p < 0.05$), and sucrose reduced the rate of [^{3}H]-G4NH$_2$ uptake almost 3-fold to 20.1 pmol/mg protein/min ($p < 0.05$) (Figure 2).

Effect of Endocytosis Inhibitors on G4NH$_2$ Permeability. The apparent permeability of [^{3}H]-riboflavin (500 nM) and [^{3}H]-G4NH$_2$ (1 μM) was measured in the absence and presence of endocytosis inhibitors. [^{3}H]-Riboflavin had an apparent permeability of 9.3×10^{-6} cm/s after 2 h incubation time, and the apparent permeability of [^{3}H]-G4NH$_2$ was 11.6 $\times 10^{-5}$ cm/s. Brefeldin A significantly reduced [^{3}H]-riboflavin and [^{3}H]-G4NH$_2$ permeability to 5.3×10^{-6} cm/s ($p < 0.01$) and 5.7×10^{-6} cm/s ($p < 0.001$), respectively (Figure 3). The application of colchicine caused [^{3}H]-riboflavin and [^{3}H]-G4NH$_2$ permeability reduction to 5.8×10^{-6} cm/s ($p < 0.01$) and 3.4×10^{-6} cm/s ($p < 0.001$), respectively. Filipin reduced [^{3}H]-riboflavin and [^{3}H]-G4NH$_2$ permeability to 5.3×10^{-6} cm/s ($p < 0.01$) and 7.3×10^{-6} cm/s ($p < 0.001$), respectively.

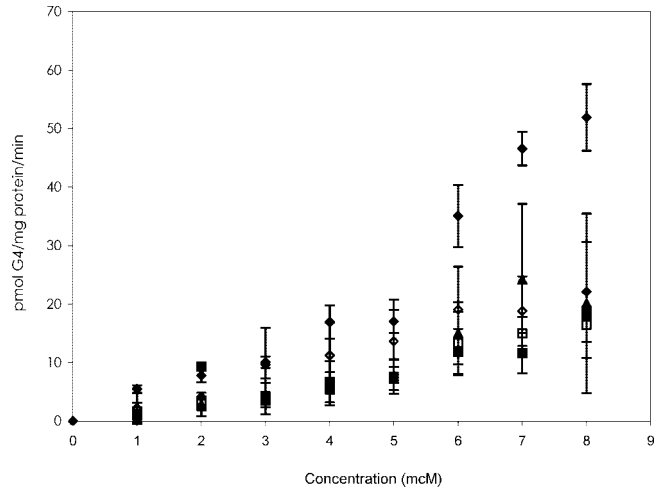

Figure 2. Uptake of [^{3}H]-G4NH$_2$ in Caco-2 cells is concentration-dependent. Cells were seeded at 80000 cells/mL in 24-well plates and incubated for 20 min with various concentrations of [^{3}H]-G4NH$_2$. Uptake of [^{3}H]-G4NH$_2$ was measured in the absence (♦) and presence of various endocytosis inhibitors: (◊) 5 μM brefeldin A; (■) 10 μM colchicine; (□) 1 μg/mL of filipin; (Δ) 200 mM sucrose. Results are reported as mean ± SD ($n = 3$).

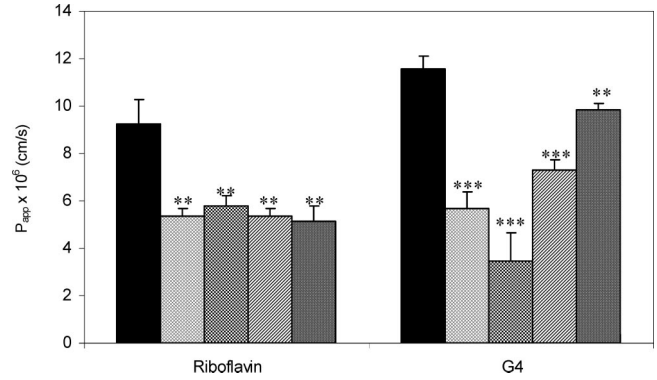

Figure 3. Reduced apparent permeability ($P_{app} \times 10^{-6}$ cm/s) of ^{3}H-riboflavin (500 nM) and ^{3}H-G4NH$_2$ (1 μM) across Caco-2 cell monolayers in the presence of endocytosis inhibitors. Cells were seeded at 80000 cells/cm^2 in 12-well Transwell filters for up to 120 min. [^{3}H]-riboflavin and [^{3}H]-G4NH$_2$ permeability was measured in the absence (solid) and presence of various endocytosis inhibitors: (diamond pattern) 5 μM brefeldin A; (square pattern)10 μM colchicine; (diagonal pattern) 1 μg/mL of filipin; (gray pattern) 200 mM sucrose. Results are reported as mean ± SD ($n = 3$). **, $p < 0.01$: ***, $p < 0.001$.

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Finally, coincubation of [^{3}H]-riboflavin and [^{3}H]-G4NH$_2$ with sucrose caused a decline in permeability to 5.1×10^{-6} cm/s ($p < 0.01$) and 9.7×10^{-6} cm/s ($p < 0.01$), respectively.

Discussion

Cationic dendrimers such as PPI and PAMAM are commonly reported to undergo cellular internalization by endocytosis mechanisms.[11–13,21] This is due to the macromolecular nature of dendrimers, and the strong electrostatic interaction between cationic amine groups and the negatively charged cell membrane that facilitates the nonspecific, adsorptive endocytosis process of such macromolecules. Studies in our laboratory revealed high levels of colocalization between PAMAM dendrimers and classical endosomal/lysosomal markers in Caco-2 cells,[14] which along with temperature-dependent and colchicine studies,[6,12] confirm that both cationic and anionic PAMAM dendrimers are internalized in Caco-2 cell monolayers via adsorptive endocytosis, and these dendrimers undergo intracellular trafficking that is associated with the endocytosis process. In addition, it is well-established that receptor-mediated endocytosis contributes to the uptake and transport of riboflavin in human placental trophoblasts as well as Caco-2 cells.[18,20,22] As a tool to investigate the transport mechanisms of G4NH$_2$ dendrimers in the intestinal epithelium, the effect of various inhibitors on riboflavin and G4NH$_2$ absorption and permeability was investigated in Caco-2 cell monolayers with riboflavin as a positive control for receptor-mediated endocytosis. Caco-2 cells are known to undergo enterocytic differentiation and have been well-established as a transport model system that mimics the physiology of the small intestinal epithelium.[23–25]

Brefeldin A induces tubulation of secondary endosomes and lysosomes,[26] which compromises microtubule trafficking of transport vesicles within the cell. This antibiotic is suggested to interfere with GTPase activation, which promotes transport vesicle formation and therefore inhibits the transport of proteins that would otherwise shuttle to late endosomes/lysosomes from the endoplasmic reticulum to the Golgi apparatus.[27] Similarly, colchicines interfere with microtubule trafficking through binding to tubulin subunits.[28] Filipin is known to inhibit caveolae-mediated endocytosis by binding to cholesterol, a major component of glycolipid microdomains and caveolae. This interaction disrupts caveolae structure and function.[29,30] Hyperosmolarity conditions generated by increased sucrose concentration primarily blocks membrane internalization and clathrin recycling via the coated-pit pathway that involves the formation of coated pits.[31] This occurs through reversible inhibition of low density lipoprotein (LDL) internalization, which results in random dispersion of LDL receptors on the cell membrane and consequently reduces the number of available coated pits for clathrin assembly.[31]

In the present study, we observed significant reduction in the uptake and permeability of riboflavin and G4NH$_2$ in the presence of all inhibitors tested. Since the microtubule network contains α-tubulin and β-tubulin subunits that drive vesicle movement with the motor proteins kinesin and dynein during the endocytosis process,[28] a reduction in transport is anticipated for substrates that use specific or nonspecific endocytosis transport mechanisms. Our results show that the cellular uptake of riboflavin and G4NH$_2$ was hindered at high concentrations by 10 μM colchicine as well as 5 μM brefeldin A (Figure 1). Riboflavin has been detected in clathrin-coated vesicles in BeWo cells, which suggests riboflavin internalization occurs, in part, via a clathrin-mediated endocytosis process.[20] Concurrent with these findings, riboflavin uptake significantly reduced by the formation in hypertonic conditions, as well as with caveolae structure disruption by 1 μg/ml filipin (Figure 2). It has been demonstrated that reduced cholesterol levels do disrupt the formation of clathrin-coated compartments,[32] as well as reduced levels of caveolin. And the application of filipin with chlorpromazine, which reduces the number of coated-pit receptors at the cell membrane,

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almost completely reduced the internalization of cholera toxin in Caco-2 cells.[29] These findings demonstrate that these endocytosis inhibitors reduced riboflavin permeability to the same extent after treatment for 2 h.

Previous studies from our laboratory demonstrated a high extent of colocalization between cationic $G2NH_2$ and clathrin in Caco-2 cells,[14] suggesting cationic dendrimers are internalized via clathrin-mediated endocytosis. Similarly, $G4NH_2$ internalization was demonstrated to be cholesterol-dependent in B16f10 melanoma cells, indicated by 44% inhibition in the presence of methyl-β-cyclodextrin,[11] which blocks cholesterol-dependent pathways.[32] These findings are in agreement with the present findings that $G4NH_2$ uptake (Figure 2) and permeability (Figure 3) were significantly reduced in the presence of sucrose and filipin, respectively. Thus, it is unlikely that PAMAM dendrimers are transported by receptor-mediated endocytosis, especially since PAMAM dendrimers have not been reported as ligands for cell-surface receptors in Caco-2 cells. Rather, our studies suggest $G4NH_2$ is nonspecifically internalized partly by clathrin vesicles, which has also been demonstrated for nanoparticle uptake in human aortic vascular smooth muscle cells.[33]

In conclusion, our studies provide further confirmation that $G4NH_2$ dendrimers are transported by an adsorptive endocytosis process which is supported by their cationic and macromolecular nature, coupled with the rapid, linear rate of uptake in Caco-2 cell monolayers. The endocytosis inhibitors reduced riboflavin and $G4NH_2$ uptake in undifferentiated cells and similarly reduced their permeability in differentiated cells. These findings support our previous results that cationic dendrimers are transported by an energy-dependent process and dendrimer internalization and intracellular trafficking occurs by a classical endocytosis pathway. Knowledge of the transport mechanisms utilized by these potential drug carriers paves the way for future oral drug delivery applications of PAMAM dendrimers to enhance the subcellular or transcellular delivery of therapeutic agents.

Table 1. Cytotoxicity of $G4NH_2$ in Caco-2 Cell Monolayers Based on the WST-1 Assay

$G4NH_2$ concentration (μM)	% cell viability
HBSS	100.0
Triton X-100	***8.5 ± 3.3***
1	87.8 ± 20.4
2	86.9 ± 13.9
4	89.6 ± 13.1
6	93.7 ± 16.5
8	96.4 ± 26.1
10	92.6 ± 17.6

Cells were seeded at 30000 cells/well in 96-well plates and treated with $G4NH_2$ for 2 h. Results are reported as mean percentage ± standard deviation (SD) of the negative control, HBSS ($n = 5$). Italicized values indicate a significant reduction in cell viability compared to the negative control ($p < 0.00001$).

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