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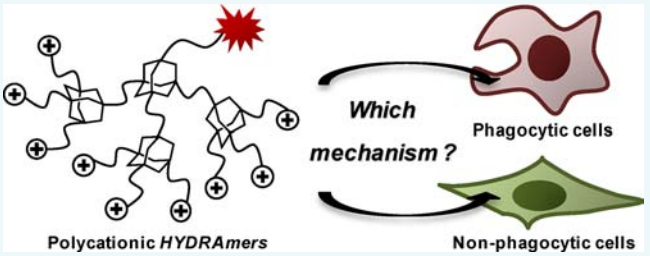
Elucidation of the Cellular Uptake Mechanisms of Polycationic HYDRAmers

Julie Russier, Maxime Grillaud, and Alberto Bianco*

CNRS, Institut de Biologie Moléculaire et Cellulaire, Immunopathologie et Chimie Thérapeutique, 67000 Strasbourg, France

Supporting Information

ABSTRACT: Dendrimers and dendrons appeared to potentially fulfill the requirements for being good and well-defined carriers in drug and gene delivery applications. We recently demonstrated that polycationic adamantane-based dendrons called *HYDRAmers* are easily internalized by both phagocytic and nonphagocytic cells in vitro. The aim of the present study was to investigate which of the different pathways of cellular internalization is involved in the cellular uptake of the first and second generation ammonium and guanidinium *HYDRAmers*. For this purpose, we have evaluated the internalization of fluorescently labeled *HYDRAmers* in both phagocytic murine macrophages and nonphagocytic human cervix epithelioid carcinoma cells in the presence of different well-known active uptake inhibitors. Our data revealed that the first and second generation *HYDRAmers* are internalized via different endocytic pathways based on the cellular type and on the type of functional groups present at the periphery of the dendrons. In particular, it was registered that the first generations were mainly internalized by clathrin-mediated endocytosis and macropinocytosis while the cellular internalization of the second generations was less affected by the inhibitory conditions of the endocytic pathways. These results suggest the possibility of addressing dendrimers toward specific subcellular compartments by tuning their structure properties and, in particular, the functional groups at their periphery.



INTRODUCTION

Among molecular nanostructures with well-defined particle size and shape, dendrons have been investigated as ideal nanoscale carriers for the delivery of bioactive molecules into the cells.[1,2] Multivalency consisting in the possibility of binding multiple surface groups at the periphery of a dendron, could promote higher drug and gene delivery together with enhanced affinity for ligand/receptor interactions making dendrons suitable for biomedical applications in therapy and imaging.[3] We and others have demonstrated that trivalent systems constructed around C_3-symmetric cores play an important role in the area of molecular recognition.[4−6]

In this context, we have decided to synthesize dendrons starting from adamantane cores which combine both multivalency and C_3-symmetry properties.[7,8] A wide range of adamantane derivatives have been developed in the field of medicinal chemistry, and several drugs, already on the market, incorporate the adamantane motif.[9] We have recently reported the synthesis and full characterization of a novel type of dendrons based on an adamantane core that we called *HYDRAmers*.[10] The adamantane core with a well-defined 3D conformation serves as multivalent scaffolds orienting four arms tetrahedrally into the space.[7,8] This tripodal arrangement on a rigid molecule permits the introduction of additional functionality into the fourth bridgehead position without disturbing the geometry of adamantane-based scaffolds.[11] Moreover, these features give to the arborescent structures less steric hindrance between the attached entities, leading to an improvement of multivalent ligand/receptor interactions of this novel type of dendron.[12,13]

Investigations on the impact on cellular viability of *HYDRAmers* revealed that they had good dispersibility and stability in the physiological media and they did not induce cytotoxic effects in vitro.[10] Furthermore, we could demonstrate the advantage of multimerizing a well-known anti-inflammatory agent (i.e., ibuprofen) in comparison to the free form.[14] Indeed, the anti-inflammatory activity of ibuprofen was enhanced by its complexation to *HYDRAmers*. This better anti-inflammatory effect is probably related to the better bioavailability of the drug, likely correlated to its better internalization by cells in its multivalent form. In fact, we have very recently demonstrated that our polycationic *HYDRAmers* are well internalized by both phagocytic and nonphagocytic cells in vitro without inducing cytotoxicity.[15]

Considering the possibility to use *HYDRAmers* as vectors for biomedical applications, cellular internalization represents a critical parameter to assess their efficacy, especially as drug and gene delivery systems. Up to date, not much is known about the mechanisms involved in the cellular internalization of dendrons and dendrimers, despite their wide use to enhance

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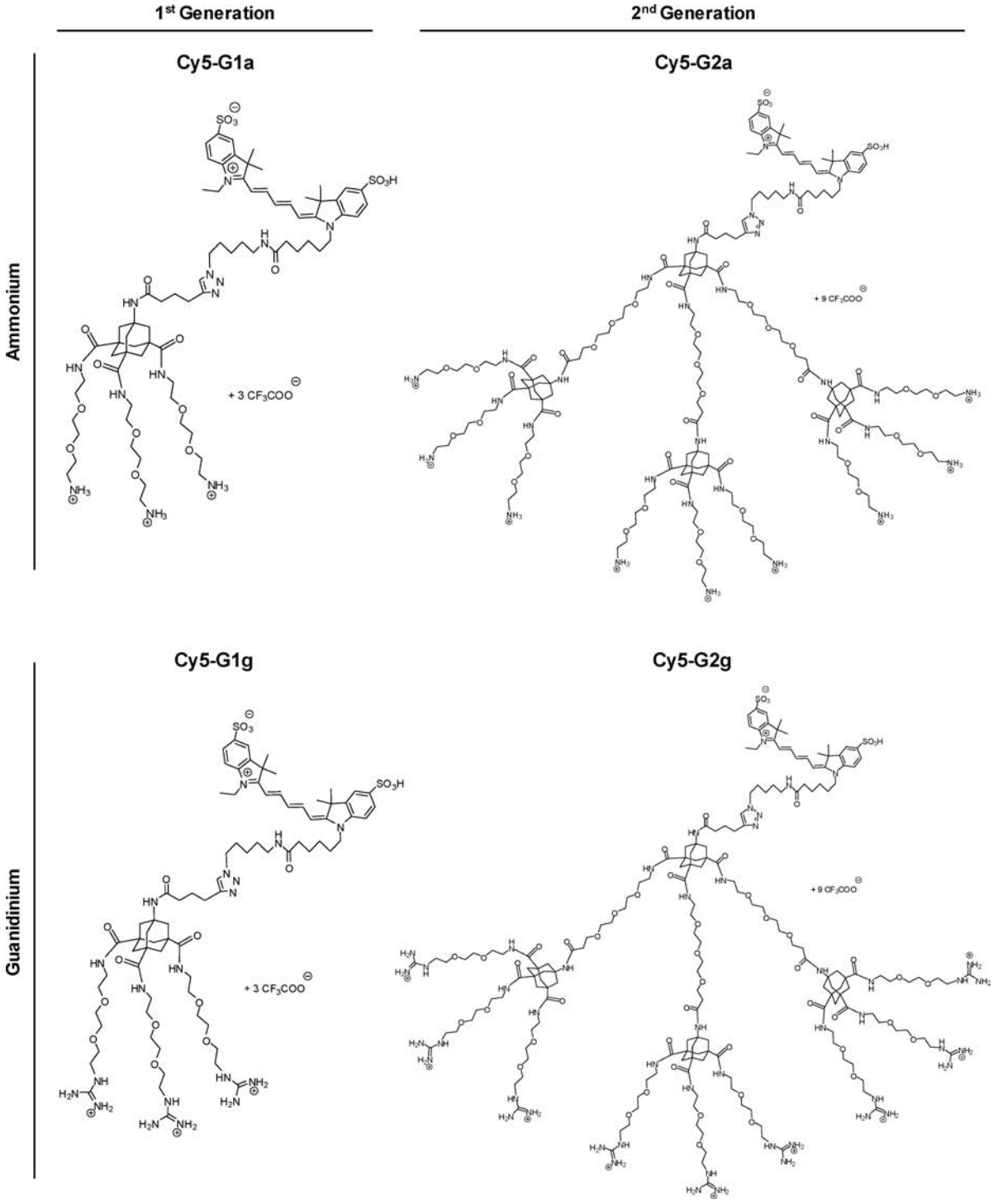

Figure 1. Molecular structures of fluorescent Cyanine 5-labeled ammonium first and second generation *HYDRAmers* (**Cy5-G1a** and **Cy5-G2a**, respectively) and guanidinium first and second generation *HYDRAmers* (**Cy5-G1g** and **Cy5-G2g**, respectively).

targeting and cellular uptake of small drugs and nanoparticles.[16,17] The knowledge of the mechanisms responsible for the cellular internalization of these compounds is of great importance in the development of strategies to build new dendritic structures for dynamic cell studies.[18,19] For this purpose, some dendrimers were fluorescently labeled to evaluate their dynamics of cellular entry such as polyamine-based carriers which present positive charges at the physiological pH.[20,21] The periphery and the surface charge of dendrimers mainly tune their internalization process and their cytotoxicity,[22] while the charge density plays a role in the conformational change of the dendritic structure.[23] Hydrophilic poly(ethylene glycol) (PEG)-based dendrimers displayed low cytotoxicity as well as a reduction in systemic clearance with enhanced permeability and retention (EPR).[24,25] Guanidino groups are highly basic (pK_a = 12.5) and they can be fully protonated under physiological pH. This feature renders polyguanidinium carriers[26−28] highly positively charged, and they can form both ionic and hydrogen-bonding interactions with the negatively charged carboxylates, phosphates, and

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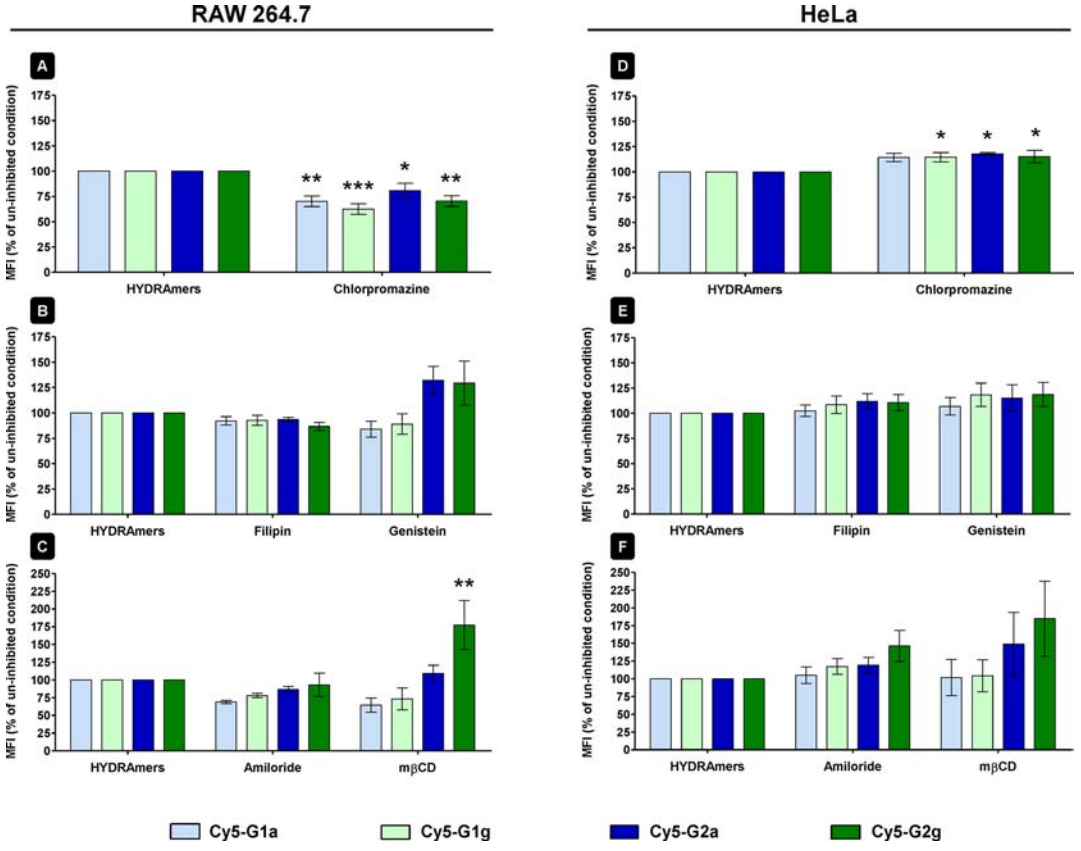

Figure 2. Quantification of the extent of cellular uptake of cyanine 5-labeled first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* (5 μM) in RAW 264.7 and HeLa cells by flow cytometry. Conditions that inhibit different internalization pathways were: clathrin-mediated endocytosis (A and D), caveolae-mediated endocytosis (B and E), and macropinocytosis-dependent internalization (C and F). Mean values ± SEM were obtained from at least five experiments run in triplicate in serum-free conditions. Data are presented relative to the uninhibited conditions which correspond to 100% of the intracellular fluorescence signal. One-way ANOVA, followed by Dunnett's post test, was carried out to determine the statistical significance of the data obtained with the different inhibitors compared to the cyanine 5-labeled first and second generation ammonium and guanidinium *HYDRAmers* alone (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

sulfates present in the lipid cell membrane bilayers. The bidentate hydrogen-bonding network formed by guanidinium groups is strong and can facilitate cellular uptake of the carriers.[29,30]

Beyond the interest in understanding which mechanisms account for the cellular uptake of the *HYDRAmers*, it is important to remember that their mode of internalization can have a direct impact on the fate of the loaded drugs and/or nucleic acids. In particular, if we consider the potential risk of inactivation of a therapeutic agent by acid hydrolases from the endolysosomal compartments, adamantane-based drug/gene delivery systems that can escape the endocytotic/phagocytic pathways would be of great interest. On the other hand, if active uptake is predominant, it will be necessary for the *HYDRAmers*, as suggested for other nanovectors, to evade from the endosomes for their cargoes to exert the therapeutic activity.[27,31]

The aim of the present study was to investigate the different pathways of cellular internalization that could account for the intracellular presence of our first and second generation ammonium and guanidinium *HYDRAmers*. For this purpose, we evaluated the internalization of fluorescently labeled-*HYDRAmers* in both phagocytic murine macrophages (RAW 264.7) and nonphagocytic human cervix epithelioid carcinoma (HeLa) cells in the presence of different well-known active uptake inhibitors to selectively block the different active ways of cellular internalization.

RESULTS

The first and second generation dendrons were synthesized starting from multifunctional adamantane building blocks via a protection/deprotection strategy of the amines and the carboxylic acids. The core of the *HYDRAmers* is constituted of the rigid and lipophilic adamantane motif and tri- and tetraethylene glycol chains are covalently attached to this core via amide bonds to improve flexibility, biocompatibility, and water solubility (Figure 1). Their periphery provides ammonium or guanidinium cationic functional groups at physiological pH. Primary amino groups were easily guanidinylated using 1*H*-pyrazole-1-carboxamidine hydrochloride reagent in one simple step. To study the cell internalization capacity of our dendrons, the fluorescent probe cyanine 5 (Cy5) was initially modified with an alkyl-azide chain and then covalently linked at the focal point of the dendrons bearing an alkyne group via "click" chemistry. We called **Cy5-G1a** and **Cy5-G1g** the first generation dendrons bearing ammonium and guanidinium groups, respectively, and **Cy5-G2a** and **Cy5-G2g** the second generation dendrons (Figure 1).

Besides good biocompatibility, another key characteristic for a promising drug and gene delivery system is its capability to reach the intracellular compartments. Polycationic dendrimers

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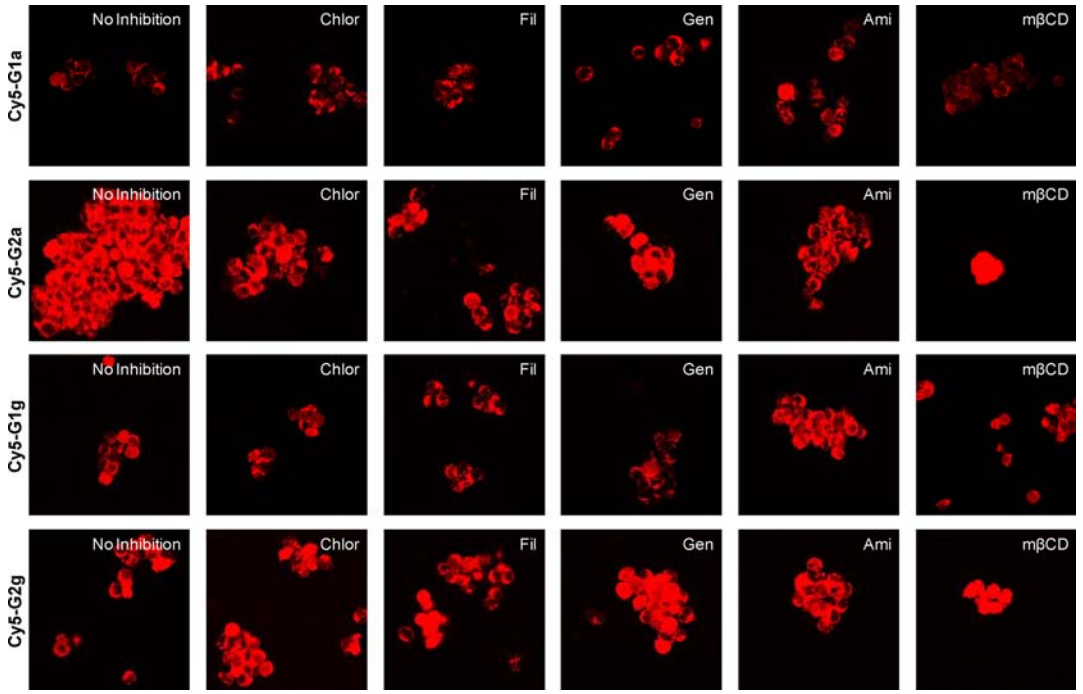

Figure 3. Intracellular localization of the first and second generation ammonium and guanidinium *HYDRAmers* by RAW 264.7 cells using confocal microcopy. Cells were incubated with dendrons alone (No Inhibition) or under conditions that inhibited clathrin-dependent endocytosis using chlorpromazine (Chlor), caveolae-mediated endocytosis using Filipin (Fil) and genistein (Gen), and macropinocytosis using amiloride (Ami) and methyl β cyclodextrin (mβCD). Dendron localization is evidenced in red. The images represent one of at least five experiments with similar results. Images displaying cellular membrane and nuclei staining are also available in SI Figure S5.

have been shown to be differently internalized based on the properties of their peripheral functions and on the type of cells under investigation.[22,32] In order to follow the cellular uptake of our first and second generation ammonium and guanidinium *HYDRAmers*, we used cyanine 5-conjugated dendrons (Figure 1). Following the incubation with the first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) dendrons, the presence of *HYDRAmers* inside cells was assessed. Then, the effect of different selective inhibitors of endocytic pathways on cellular internalization of *HYDRAmers* was evaluated by flow cytometry and confocal laser scanning microscopy.

Starting with flow cytometry analysis in RAW 264.7 macrophages, Figure 2 shows how all inhibitory conditions affected the cellular internalization of the first and second ammonium and guanidinium *HYDRAmers*. Some differences in the effects of the inhibitors were observed based on the dendrimer generation and functionalization. The extent of inhibition also varied from one inhibitor to the other. Even though a modification of cellular uptake of *HYDRAmers* was registered in all the inhibitory conditions, only chlorpromazine and mβCD provoked a significant effect on cellular internalization of our *HYDRAmers* (Figure 2A,C). In more detail, chlorpromazine determined a reduction of the cellular internalization of both first and second generation ammonium and guanidinium *HYDRAmers* (Figure 2A; see SI Table S2). Regarding the first generation, the residual fluorescence signal was indeed 70.2 ± 5.2% of the uninhibited condition for **Cy5-G1a** and 62.6 ± 5.3% of the uninhibited condition for **Cy5-G1g**. Chlorpromazine also reduced the internalization of the second generation *HYDRAmers* by RAW 264.7 macrophages, but to a lesser extent with respect to their corresponding first generation. Indeed, a significant reduction of the **Cy5-G2a** internalization could be registered (80.7 ± 7.4% of uninhibited condition); chlorpromazine only induced a small reduction of the uptake of **Cy5-G2g** (70.4 ± 5.3% of uninhibited condition).

The other condition that significantly affected the internalization of the *HYDRAmers* in RAW 264.7 was the incubation with mβCD (Figure 2C; see SI Table S2). Nevertheless, only the internalization of **Cy5-G1a** and **Cy5-G1g** dendrons was reduced (64.5 ± 10.0% and 73.4 ± 15.4% of the uninhibited condition, respectively). Surprisingly, the cellular uptake of the second generations was not modified but rather increased for **Cy5-G2a** (109.3 ± 11.4% of the uninhibited condition) and for **Cy5-G2g** (177.4 ± 34.5% of the uninhibited condition).

In conditions that inhibited the caveolae-mediated endocytosis pathway, no significant reduction of the internalization of the different *HYDRAmers* was registered, but some modifications were observed (Figure 2B). The incubation with filipin only slightly reduced the cellular internalization of *HYDRAmers* from 10% (**Cy5-G1a**, **Cy5-G1g**, and **Cy5-G2a**) to 15% (**Cy5-G2g**; see SI Table S2).

The incubation with the second caveolae-mediated endocytosis inhibitor, genistein, modified the cellular internalization of the first generation **Cy5-G1a** and **Cy5-G1g** *HYDRAmers* similarly to filipin, determining around 10−15% of inhibition, while an increased uptake of around 30% was registered for the second generation dendrons (see SI Table S1).

Finally, it is important to note that the macropinocytosis inhibitor amiloride also modified the cellular uptake of the *HYDRAmers* (Figure 2C; see SI Table S2). In fact, the remaining fluorescence intensity was 69.0 ± 2.3% for **Cy5-G1a** and 79.0 ± 3.2% for **Cy5-G1g**, while internalization of the second generation *HYDRAmers* was less affected by the inhibitory conditions, as 86.8 ± 3.9% and 93.1 ± 6.6% of the

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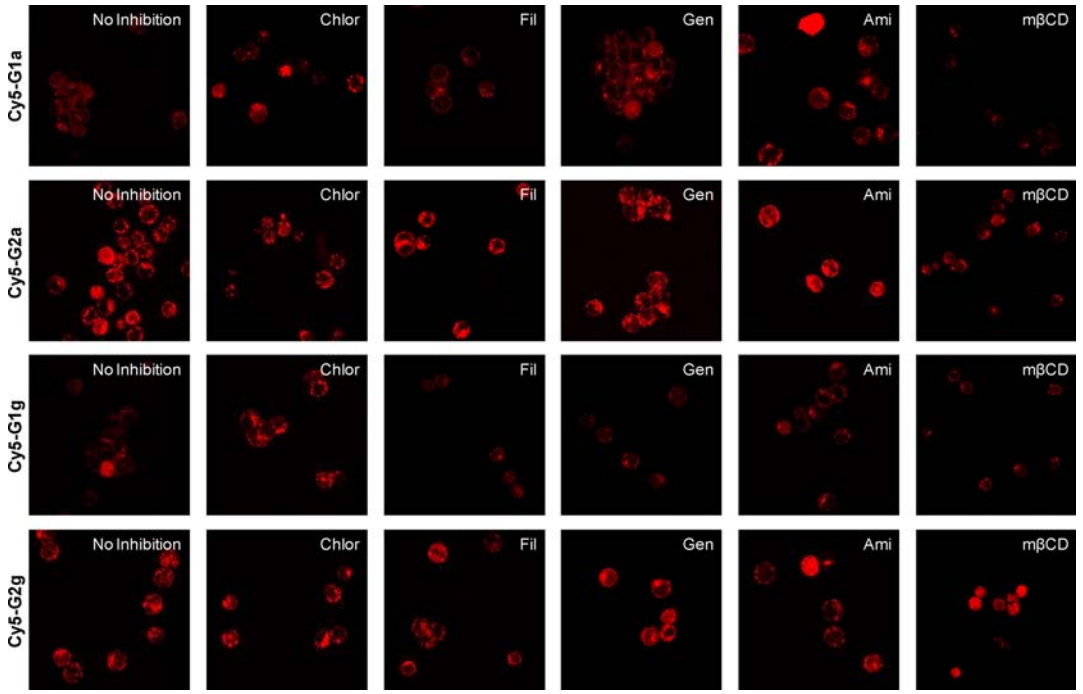

Figure 4. Intracellular localization of the first and second generation ammonium and guanidinium *HYDRAmers* by HeLa cells using confocal microcopy. Cells were incubated with dendrons alone (No Inhibition) or under conditions that inhibited clathrin-dependent endocytosis using chlorpromazine (Chlor), caveolae-mediated endocytosis using Filipin (Fil) and genistein (Gen), and macropinocytosis using amiloride (Ami) and methyl β cyclodextrin (mβCD). Dendron localization is evidenced in red. The images represent one of at least five experiments with similar results. Images displaying cellular membrane and nuclei staining are also available in SI Figure S6.

initial fluorescence intensity were still registered for **Cy5-G2a** and **Cy5-G2g**, respectively.

Regarding HeLa cells, the same inhibitory conditions used for RAW 264.7 were applied, but no significant reduction of the cellular internalization of the different polycationic *HYDRAmers* was measured, consistent with the results registered with the control molecules (i.e., transferrin, dextran, and Bodipy; see SI Table S1 and Figure S1). Nevertheless, a slight increase in the cellular internalization of second generation *HYDRAmers* was registered when macropinocytosis inhibitory conditions were applied (Figure 2F; see SI Table S2). Indeed, amiloride determined an increase of the cellular internalization of **Cy5-G2g** (146.4 ± 21.8), while mβCD determined a higher rate of internalization of either **Cy5-G1g** (149.1 ± 44.7) or **Cy5-G2g** (184.8 ± 52.8). As previously observed with RAW 264.7, the second generation guanidinium dendron **Cy5-G1g** seemed to be less affected by the inhibitory conditions (see SI Table S2).

Interestingly, the same trends were observed in RAW 264.7 and HeLa cells under inhibitory conditions in media containing FBS (10%) even if none of the induced modifications of the *HYDRAmer* cellular internalization reached statistical significance (see SI Table S3 and Figure S2). These results are not due to the modification of the basal internalization of the *HYDRAmers*, as no differences were registered between serum containing and serum free uninhibited conditions (see SI Figure S3).

Confocal microscopy studies were performed to further investigate the cellular internalization of the first and second generation ammonium and guanidinium *HYDRAmers* (Figures 3 and 4). It is important to notice that, in a general manner, the confocal microscopy observations were in good agreement with the flow cytometry analysis under either normal or inhibitory conditions. Confocal microscopy also allowed assessing the subcellular localization of the different *HYDRAmers*. Similarly to the observations by flow cytometry under uninhibited conditions,[15] it was possible to observe that the second generation ammonium and guanidinium *HYDRAmers* (**Cy5-G2a** and **Cy5-G2g**) were more internalized than their relative first generations (**Cy5-G1a** and **Cy5-G1g**, respectively) in both types of cells (see SI Figure S3 and Figure S4). We also observed another trend showing that guanidinium compounds were more internalized with respect to their counter ammonium *HYDRAmers* in both types of cells, and especially in RAW 264.7 macrophages (Figures 3 and 4; see SI Figure S3 and Figure S4). In addition, we could observe that the intracellular fluorescence was diffused into the cytoplasm, suggesting a passive penetration of the *HYDRAmers* across the cellular membrane,[33] or confined into well-defined dotted areas, representative of an active endocytic internalization.[34] In more detail, the incubation of RAW 264.7 macrophages with chlorpromazine and mβCD reduced the cellular internalization of first generation ammonium and guanidinium *HYDRAmers*. Chlorpromazine was also responsible for the reduced cellular internalization of the second generation guanidinium *HYDRAmers*. It is important to highlight that amiloride also determined a slight decrease of cellular uptake of all *HYDRAmers*. On the other hand, incubation with genistein and mβCD provoked an increased intracellular fluorescence for the second generation *HYDRAmers*.

It is noteworthy that, when inhibitory conditions were applied, the cellular uptake of *HYDRAmers* was reduced, but it was still possible to observe a diffused fluorescence inside the cells, confirming the previous flow cytometry results and supporting the contribution of a passive pathway in the internalization of our dendrons.[33] Moreover, it was also possible to detect that well dotted areas of fluorescence were

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present close to the cellular membrane, evidencing that polycationic *HYDRAmers* were blocked at the surface or that the endocytic pathways were somehow frustrated and incomplete. In HeLa cells, confocal observations again confirmed the previous flow cytometry analysis. None of the inhibitors had an effect on the intracellular fluorescence intensity correlated to the internalization of *HYDRAmers*.

The capacity of *HYDRAmers* to passively cross cell membrane under inhibitory conditions was further sustained by adaptive partitioning experiments. Adaptive partitioning experiments of all *HYDRAmers* were performed following a previous procedure.[35] The fluorescent dendrons were added to a bilayer of octanol and water. All different cationic *HYDRAmers* partitioned exclusively (>99%) into the water phase (see SI Figure S7). However, when the fatty acid salt, sodium laurate, was added to the mixture, the dendrons migrate differently into the two phases. The relative partitioning was quantified by separation of the layers and analysis of UV absorbance (at 650 nm) of the organic and the aqueous layers. While polyammonium *HYDRAmers* mainly stayed in the aqueous layer (84% and 85% for **Cy5-G1a** and **Cy5-G2a**, respectively) the polyguanidinium *HYDRAmers* significantly partitioned into the organic layer (72% and 49% for **Cy5-G1g** and **Cy5-G2g**, respectively).

DISCUSSION

Considering the possibility to use the *HYDRAmers* as vectors for biomedical applications, cellular internalization represents a critical parameter in their efficacy as transporters of drugs and genes. The successful delivery of therapeutics to the specific cells and their availability at the intracellular target location are crucial requirements for this type of application. Nevertheless, relatively few studies have systematically evaluated the fundamental properties of dendrimer-based carriers on their cellular internalization mechanism(s) and intracellular trafficking. In particular, there are no studies on the assessment of the cellular internalization mechanism of adamantane-based dendrons.

In order to take up substances from their surrounding environment, cells possess two main mechanisms: (i) endocytosis (active), and (ii) passive diffusion. Both have been reported to participate in the cellular internalization of nanoparticles,[31,36,37] and in particular, endocytosis is the main route of the cellular uptake of dendrimers.[38] Endocytosis is a constitutive cell mechanism for the internalization of the molecules[39] which can be distinguished in phagocytosis and pinocytosis, based on the cellular type. Phagocytosis is part of the immune defense and it is typically restricted to specialized cells such as macrophages, monocytes, and neutrophils which role is the degradation and elimination of foreign elements.[40] These cells can also use pinocytosis. Nonphagocytic cells use pinocytosis to internalize small essential nutrients.[39] Pinocytosis can be further divided into (i) macropinocytosis,[41] a non-specific mechanism by which fluids are taken up;[42] (ii) clathrin-dependent pathway,[43] a receptor-mediated endocytosis; (iii) caveolae-mediated endocytosis[44] in which the formation of vesicles is due to the caveolin protein; and (iv) clathrin- and caveolae-independent pathways.[45]

As a result of these multiple possibilities, differences can be registered in cellular internalization mechanisms operated by phagocytic and nonphagocytic cells.[46] By using phagocytic RAW 264.7 macrophages and nonphagocytic HeLa epithelial cells under the same conditions, we aimed to highlight possible differences in the mechanisms involved in the cellular internalization of the first and second generation ammonium and guanidinium *HYDRAmers*, between the two types of cells and between the different *HYDRAmers* themselves.

The results obtained here with RAW 264.7 macrophages and HeLa cells, demonstrated that the cellular internalization of polycationic *HYDRAmers* is the result of the combination of clathrin-mediated endocytosis, macropinocytosis, phagocytosis, and passive diffusion. Under normal cellular uptake conditions, we could notice that *HYDRAmers* were present in the cytoplasm, mainly as well-defined dotted red areas. This subcellular organization of the internalized *HYDRAmers* supports an active mechanism of internalization leading to a preferential phagosomal/endosomal localization of the different dendrons in both types of cells. Moreover, the fact that well dotted areas of fluorescence were still present under conditions that inhibited clathrin- and caveolae-mediated endocytosis also suggests that other endocytic pathways are involved in the internalization of the *HYDRAmers*.

The results obtained with HeLa cells under inhibitory conditions support the involvement not only of diffusion, but also of clathrin- and caveolae-independent endocytosis in the cellular internalization of the polycationic *HYDRAmers*. Furthermore, the data of RAW 264.7 cells incubated with mβCD reinforced the importance of macropinocytosis in the cellular internalization of the *HYDRAmers*. In particular, the fact that mβCD had a major effect on macropinocytosis respect to amiloride accounts for the important involvement of cholesterol in their cellular uptake. Our data reveal that the first and second generation *HYDRAmers* are internalized via different endocytic pathways based on the cellular type and on the type of functional groups present at the periphery of the dendrons. Our data show that the first generations were mainly internalized by clathrin-mediated endocytosis and macropinocytosis. The second generations were less affected under the inhibitory conditions of the endocytic pathways, especially considering the guanidinium dendrons (i.e., **Cy5-G2g**). These results confirm the previous data that we obtained with active broad range endocytic pathway inhibitors (i.e., 4 °C and NaN_3) showing that the *HYDRAmers* are able to be passively internalized by both phagocytic and nonphagocytic cells.[15] The remaining cellular internalization of our *HYDRAmers* under conditions that inhibit the clathrin- and caveolae-mediated revealed that other receptor-mediated pathways are involved in their cellular internalization. In fact, we could still observe well-defined spots of fluorescence at the cellular membrane under these conditions.

The confined fluorescence found in the periphery of the cells suggests that the *HYDRAmers* have affinity for the cellular membrane probably leading to passive diffusion across the membrane. We previously demonstrated that guanidinium dendrons displayed higher passive diffusion than ammonium dendrons.[15] As guanidinium is more basic than ammonium, its degree of protonation is favored. This property is hypothesized to be responsible for its capacity to form strong and stable bidentate hydrogen bonds with the negatively charged cellular membrane components leading to an enhanced cellular internalization, a phenomenon called adaptive translocation.[35,47] Once anchored to negatively charged cellular membrane components (e.g., phosphates, sulfates, carboxylates), the actual polarity of the dendron changes allowing its penetration in the nonpolar membranes through adaptive noncovalent association with membrane constituents, finally

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sliding into the cytosol under the influence of a membrane potential.[47−49] This capacity to passively cross the cellular membrane was not affected by the presence of FBS (10%) in the cellular culture media under inhibitory conditions. In fact, while the presence of FBS did not modify the cellular internalization of control molecules (i.e., transferrin, dextran and Bodipy) under inhibitory conditions (see SI Figure S1), the presence of serum made these conditions almost ineffective in reducing the cellular uptake of *HYDRAmers* (see SI Figure S2), but still conserving the same trends observed in the serum free conditions. This latter observation suggests that the interactions with the serum proteins did not hamper the cellular internalization of the dendrons.

Adaptive partitioning experiments confirmed the possible occurrence of an adaptive translocation mechanism for polyguanidinium *HYDRAmers*. Indeed, both first and second generation guanidinium dendrons significantly partitioned into the octanol layer, while both first and second generation ammonium dendrons principally stayed in the water phase, after adding fatty acid salts. This difference may be explained in part by more effective bidentate hydrogen bonding for guanidinium groups compared to monodentate hydrogen bonding for the ammonium groups.[47] The cationic guanidinium *HYDRAmers* may adaptively diffuse into the nonpolar membrane by recruiting negatively charged cell surface constituents, producing a less polar ion-pair complex, more effectively than ammonium *HYDRAmers*. The passive internalization of polycationic dendrons is then correlated not only to the nature of the moieties they are bearing on their surface (with guanidinium better than ammonium), but also on the properties of the cellular membrane such as its composition and its potential.[32]

In this context, it is obvious that the composition of the cellular membrane plays a very important role in the interactions between the *HYDRAmers* and the cellular membrane. In particular, the reduction of the cellular internalization of first generation as well as the increased cellular uptake of the second generation dendrons in the presence of mβCD particularly highlights the role played by cholesterol in the cellular internalization of our *HYDRAmers*. mβCD is a water-soluble cyclic oligomer of glucopyranoside, which acts strictly on the cell surface, selectively extracting cholesterol without being incorporated into plasma membrane,[50−54] thus blocking cholesterol-dependent pathways.[55]

Several reports have shown that cholesterol and membrane rafts are involved in membrane trafficking, signaling, protein and lipid sorting, bacterial infection, binding, and internalization of viruses.[56−58] These membrane functional dynamic structures also play a role in cellular uptake and endocytosis of other nonviral vectors, such as liposomes.[59] Cholesterol may not only maintain the structure of the rafts, but may also be involved in a signaling cascade that initiates endocytosis. In particular, it was reported that cholesterol has a crucial role in cellular uptake of dendriplexes.[50]

Finally, the fact that the fluorescence is present inside cells as well-defined areas suggests that the *HYDRAmers* could form clusters once inside the cells. In this hypothesis, the same characteristics of the cellular membrane that determine the affinity of the *HYDRAmers* with the cellular membrane thus leading to their internalization could also account for their subsequent reaggregation once inside cells. Indeed, the internalized *HYDRAmers* are localized preferentially in some areas of the cellular membrane. This determines an enhancement of the local concentration of the *HYDRAmers* leading to their aggregation. To exclude the possibility that the *HYDRAmers* aggregated and formed clusters before the internalization, we performed dynamic light scattering measurements at high concentrations. The *HYDRAmers* were solubilized in water, in culture medium without serum, and in culture medium with FBS (10%), at 5 μM (concentration of cellular uptake experiments) and at 50 μM. The data indicated that none of these samples displayed the presence of aggregates in solution at 20 °C or at 37 °C (data not shown). This result indicated that the well-defined fluorescent areas inside originated from the *HYDRAmers* clustered inside the vesicles rather than from aggregates of dendrons formed before the uptake.

In conclusion, it is important to remember that the mode of internalization of the *HYDRAmers* can have a direct impact on the fate of the loaded drugs and/or nucleic acids. In particular, if we consider the potential risk of inactivation of a therapeutic agent by acid hydrolases from the endolysosomal compartments, adamantane-based drug/gene delivery systems that can escape the endocytotic/phagocytic pathways would be of great interest. Our noncytotoxic *HYDRAmers* are internalized by a combination of active endocytic pathways and passive diffusion. These characteristics highlight the possibility of addressing the *HYDRAmers* toward specific subcellular organelles or districts by modifying their generation and the functional groups at their periphery.

■ MATERIALS AND METHODS

Synthesis of Fluorescently Labeled *HYDRAmers*. The detailed synthesis and characterization data for all compounds are provided in the Supporting Information (SI) of our recently reported work.[15] All intermediates and final compounds were synthesized with good yields and they were fully characterized by NMR and FT-IR spectroscopy and mass spectrometry. Fluorescently labeled *HYDRAmers* were purified by preparative RP-HPLC to afford pure compounds as blue solids used in the cellular investigations.

Dynamic Light Scattering Measurements. Dynamic light scattering analyses were performed on a Nanostar from Wyatt Technology (www.wyatt.com) equipped with a 100 mW laser and using disposable plastic cells for particles with radii in the 1−1000 nm range. Disposable cuvettes used are UVette (Eppendorf, 0030 106.300) certified RNase, DNA, and protein free. All data were collected and analyzed with DYNAMICS Software.

Cell Cultures. Murine macrophage cell line RAW 264.7 and human HeLa cells (cervix epithelioid carcinoma) were obtained from American Type Culture Collection (ATCC). Both cellular types were cultured under controlled atmosphere (37 °C, 5% CO_2) in RPMI 1640 supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and 100 U/mL gentamycin. In addition, for the murine cell line, the culture medium contained 2-β-mercaptoethanol (50 μM) and HEPES (20 mM).

When confluency reached 70−80%, RAW 264.7 and HeLa cells were detached with SE buffer (PBS containing 2 mM EDTA and 2% FBS) or tripsinized, counted, reseeded in 96 well plates at a density of 3−4 × 10^5 cells/well, and allowed to adhere overnight (37 °C, 5% CO_2) prior to *HYDRAmer* exposure.

Cellular Uptake Experiment. Internalization studies included (i) RAW 264.7 and HeLa untreated cells, (ii) RAW 264.7 and HeLa control cells incubated with cyanine 5-labeled

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first and second generation ammonium and guanidinium *HYDRAmers* (5 μM) for 2 h, and (iii) RAW 264.7 and HeLa cells pretreated with cellular uptake inhibitors for 30 min and then incubated with cyanine 5-labeled first and second generation ammonium and guanidinium *HYDRAmers* (5 μM) for 2 h. The inhibitor concentrations were preliminarily tested for cytotoxicity and working concentrations were chosen based on RAW 264.7 response in order to balance cellular viability[31] and inhibitory activity on control molecules (see SI Table S1 and Figure S1). The same conditions were then applied to both RAW 264.7 and HeLa cells which were pretreated in order to selectively inhibit the different cellular internalization pathways. Flow cytometry analysis of the cellular internalization of control molecules and *HYDRAmers* were conducted both in serum free media and in serum containing 10% FBS. Nevertheless, as serum free conditions allowed to highlight the effects of some of the inhibitors, we decided to focus on these results in the main text and to further explore the intracellular localization of the *HYDRAmers* by confocal microscopy under serum free conditions. To inhibit clathrin-dependent endocytosis, cells were pretreated with 2 μg/mL chlorpromazine.[54,60] Caveolae-dependent endocytosis was disrupted by pretreating the cells with 20 μM genistein (2 h at 37 °C) or 0.1 μg/mL filipin.[54,60] Macropinocytosis was inhibited with 40 μM amiloride or 5 mM methyl-β-cyclodextrin (mβCD).[61,62] After incubation with both cellular uptake inhibitors and *HYDRAmers*, cells were washed twice with PBS, detached with SE buffer (RAW 264.7) or by trypsinization (HeLa), analyzed by flow cytometry, or stained for fluorescence microscopy (vide infra).

Flow Cytometry Analysis of the Different Dendron Cellular Uptake. To quantify the effect of the inhibitory treatments on cellular uptake of the different *HYDRAmers*, the mean fluorescence intensity associated with the internalization of our labeled dendrons was compared to the mean fluorescence intensity derived from the dendron uptake in the absence of the inhibitors. The mean fluorescence intensity of 50 000 individual cells was measured using a Gallios flow cytometer (Beckman Coulter-Villepinte, France) and analyzed with FlowJo software.

Immunofluorescence Analysis of the Different Dendron Cellular Uptake. After the treatment, RAW 264.7 macrophages and HeLa cells were stained for fluorescence confocal microscopy studies. Briefly, cells were detached using SE buffer or trypsinization and washed twice with PBS. For cellular membrane staining, RAW 264.7 and HeLa cells were first incubated with an anti-mouse CD11b- or an anti-human CD95- (Fas/APO-1) biotinylated primary antibody (diluted 1:100), respectively (1 h at 4 °C), and then with Streptavidin-Alexa^{488} (diluted 1:500, 30 min at 4 °C). Cells were then fixed with 4% paraformaldehyde (1 h at 4 °C) and nuclei were counterstained with DAPI (0.1 μg/mL, 10 min at room temperature). Finally, cells were mounted on glass slides with Vectashield and images were acquired using a Zeiss LSM 780 laser scanning microscope with a 63× oil immersion objective (Carl Zeiss Inc.) applying identical acquisition parameters to all samples.

Statistical Analysis. The experiments shown are a summary of the data from at least five separate experiments of cellular uptake evaluation, each run in triplicate. All data are presented ± SEM (standard error of the mean). Statistical analyses were performed using Graphpad software. Cellular uptake inhibition effect on intracellular content of labeled dendrons was analyzed using one-way ANOVA, followed by Dunnett's post test, to compare the inhibitory conditions to the uninhibited uptake of the different *HYDRAmers*. All p values <0.05 were considered significant.

■ ASSOCIATED CONTENT

Supporting Information

Tables on the quantification of cell uptake by flow cytometry and additional confocal images of cell uptake under inhibition conditions. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00270.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: a.bianco@ibmc-cnrs.unistra.fr.

Notes

The authors declare no competing financial interest.

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