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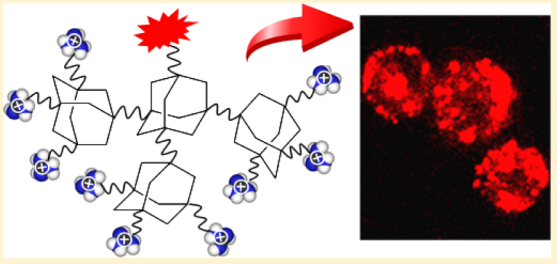
Polycationic Adamantane-Based Dendrons of Different Generations Display High Cellular Uptake without Triggering Cytotoxicity

Maxime Grillaud, Julie Russier, and Alberto Bianco*

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

Supporting Information

ABSTRACT: Dendrons used as synthetic carriers are promising nanostructures for biomedical applications. Some polycationic dendritic systems, such as the commercially available polyethylenimine (PEI), have the ability to deliver genetic material into cells. Nevertheless, polycationic vectors are often associated with potential cellular toxicity, which prevents their use in clinical development. In this context, our research focused on the design and synthesis of a novel type of polycationic dendrons that are able to penetrate into cells without triggering cytotoxic effects. We synthesized first- and second-generation polycationic adamantane-based dendrons via a combined protection/deprotection strategy starting from different adamantane scaffolds. The linker between the adamantane cores is constituted of short ethylene glycol chains, and the periphery consists of ammonium and guanidinium groups. None of these dendritic structures, which we previously called *HYDRAmers*, displayed significant cytotoxicity effects on two different cell lines (RAW 264.7 and HeLa). Conjugation of the fluorescent probe cyanine 5 at their focal point via click chemistry permitted the evaluation of their cellular internalization. All of the dendrons penetrated through the membrane with efficient cellular uptake depending of the dendron generation and the nature of the peripheral groups. These results suggest that the polycationic *HYDRAmers* are potentially interesting as new vectors in biomedical applications, including gene and drug delivery.



INTRODUCTION

Molecular nanostructures with well-defined particle sizes and shapes are widely explored for biomedical applications such as drug delivery, gene transfection, and imaging. Dendrons (wedge-shaped dendrimer sections) have been investigated as ideal nanoscale carrier molecules for the delivery of bioactive materials into cells.[1] Molecular engineering of these hyperbranched, monodisperse, well-defined structures can be easily performed using simple organic synthesis.[2] Multivalency constituted by the multiple surface groups at the periphery of a dendron promotes higher binding affinity for ligand−receptor interactions.[3] By control of their synthesis, it is possible to manipulate both the molecular weight and chemical composition of the dendrons[4] in order to solve problems of biocompatibility, toxicity, pharmacokinetics, and organ-specific targeting.[5] Dendrons consist of three distinct moieties, namely, the focal point, the branching, and the dendritic surface, each of which can play a distinctive role that can be tuned by modifying the characteristics of the functional groups.

Different dendritic architectures have been studied for their ability to deliver genetic material into cells,[6] such as polyamidoamine (PAMAM) globular dendrimers.[7] Polycationic dendrons based on polyamines have been commonly employed for gene delivery. Although they display good transfection activity, they also exhibit problematic toxicity profiles[8] that are strongly dependent on the concentration used.[9] Polyethylenimine (PEI), a commercially available polyammonium dendrimer,[10] is one of the most efficient transfection agents. However, PEI has shown an undesired dose-dependent cytotoxicity that prevents the use of this system for clinical studies. The first burden to overcome is therefore the design of polycationic dendrons that have no potential for toxicity effects. Poly(ethylene glycol) (PEG)-based nanocarriers with low hydrophobicity,[11] for example, displayed low cytotoxicity as well as a reduction in systemic clearance with enhanced permeability and retention (EPR)[12] for effective cancer chemotherapy.

Polyamine-based carriers present positive charges at physiological pH, and they can bind polyanionic molecules such as nucleic acids through electrostatic interactions. In comparison to amines, guanidino groups are highly basic (pK_a = 12.5) and can be fully protonated at physiological pH. This feature renders polyguanidinium carriers[13] highly positively charged and endows them with strong electrostatic interactions. This type of molecule can also form both ionic and hydrogen-bonding interactions with the negatively charged carboxylates, phosphates, and 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.[14] For example, it has been shown that the capacity of a series of guanidinylated dendritic polymers to enter cells is dependent on the number of guanidinium groups at their surface.[15] Furthermore, it has been recently reported

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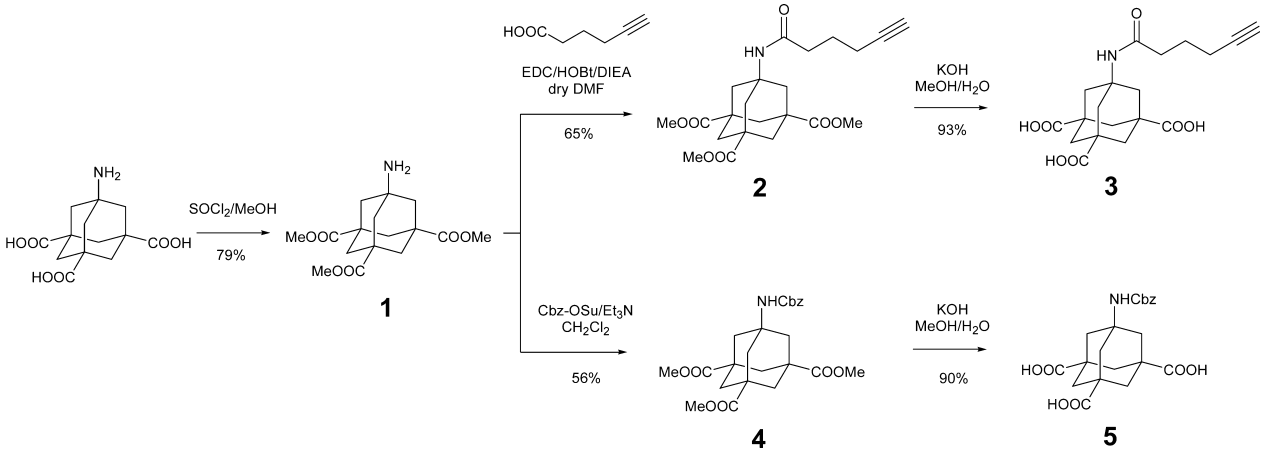
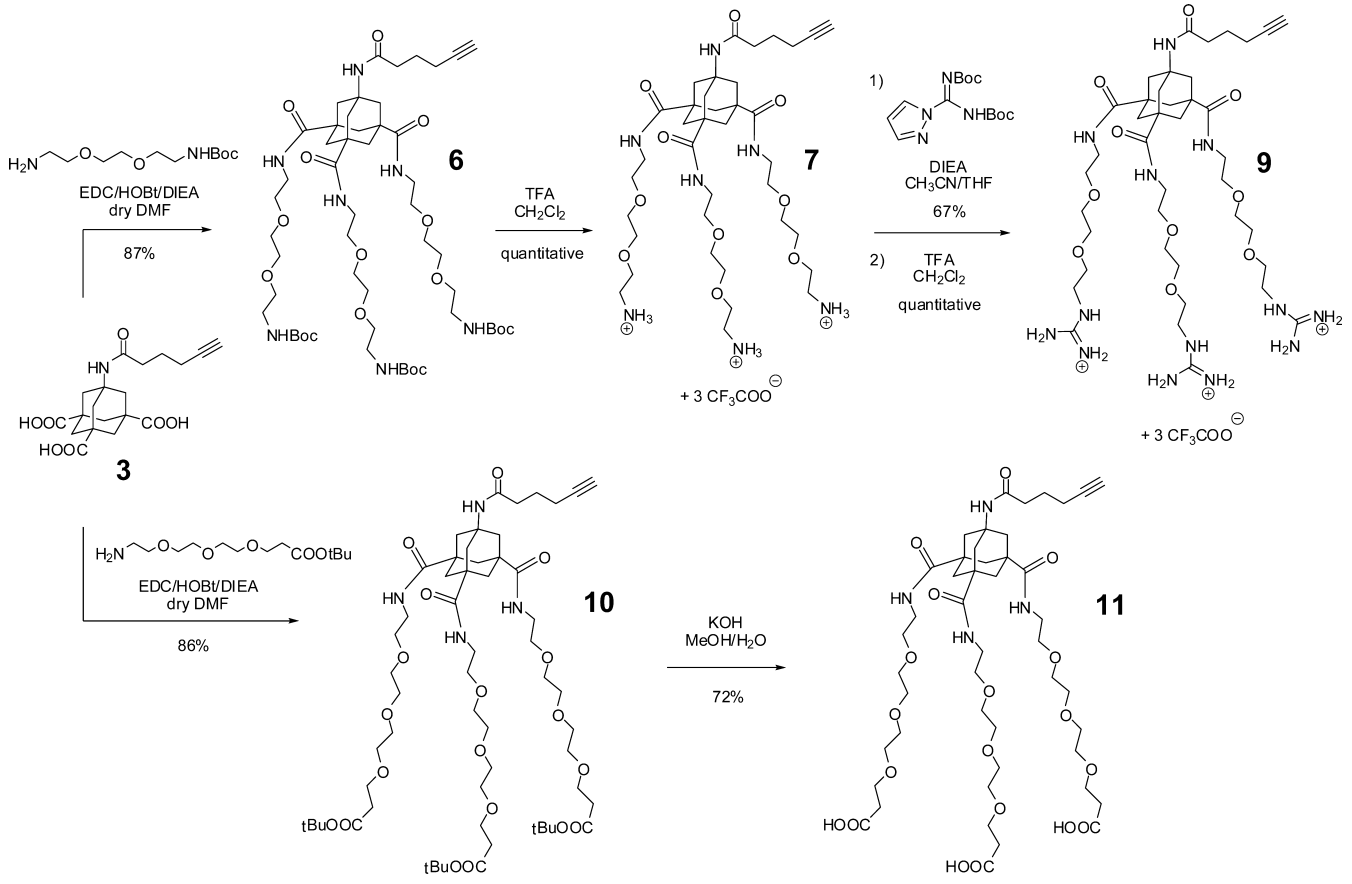
Scheme 1. Synthesis of Building Blocks



Scheme 2. Synthesis of First-Generation *HYDRAmers*



that the internalization of dendrimers into cells is mainly regulated by the functional groups at their surface.[16] Primary amino groups can be easily guanidinylated using 1*H*-pyrazole-1-carboxamidine hydrochloride reagent in one simple step.[17]

We previously synthesized different-generation adamantane-based dendrons that we called *HYDRAmers* for use as multivalent scaffolds.[18] The adamantane core with a well-defined three-dimensional conformation serves as a building block that orients four arms tetrahedrally into space.[19] This tripodal arrangement on a rigid molecule permits the introduction of additional functionality into the fourth bridgehead position without disturbing the geometry of the adamantane-based scaffold. These features give to the arborescent structures less steric hindrance between the attached entities, and we might expect an improvement of multivalent ligand−receptor interactions of this novel type of dendron.[3,18a,19]

Here we report the synthesis and characterization of different-generation polyammonium and polyguanidinium adamantane-based dendrons. Tri- and tetraethylene glycol chains were used as flexible, water-compatible branching units, and an alkyne group was introduced at the focal point to attach a fluorescent probe on the dendrons via click chemistry.

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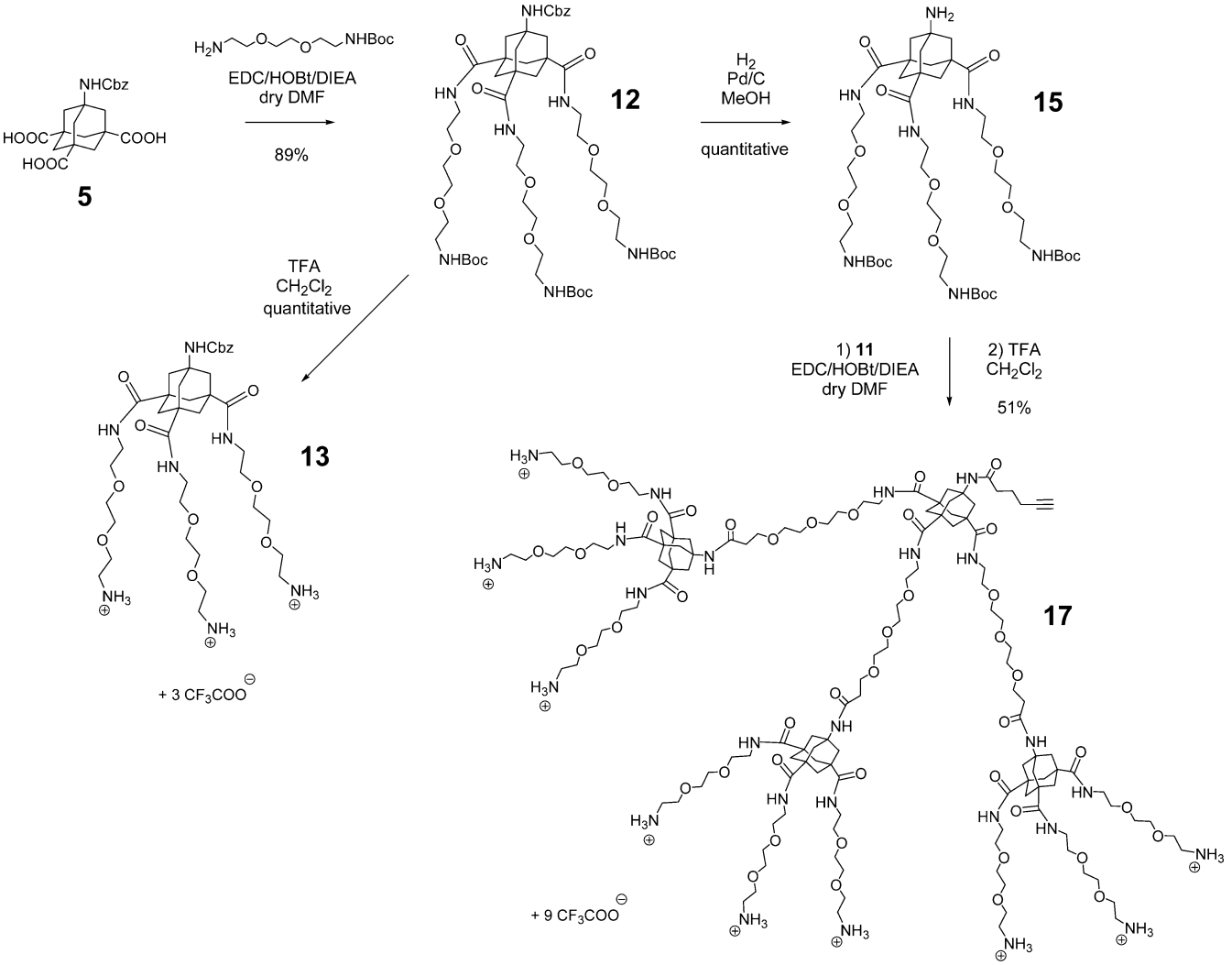
Scheme 3. Synthesis of Second-Generation Ammonium *HYDRAmers*



Cellular investigations were carried out to determine the influence of the nature of the peripheral moieties and their number on the cytotoxicity and cellular internalization of the different first- and second-generation ammonium and guanidinium *HYDRAmers*. As the cellular interactions of dendrons have been shown to be dependent on the cellular substrate in exam,[20] we decided to evaluate the behavior of our different *HYDRAmers* on both phagocytic RAW 264.7 murine macrophages and nonphagocytic HeLa epithelial human cells by flow cytometry and confocal laser scanning microscopy. None of these dendrons displayed significant cytotoxicity effects, and they all exhibited effective cellular uptake that was dependent on the generation and the nature of the peripheral groups.

RESULTS AND DISCUSSION

Syntheses of Building Blocks and *HYDRAmers*. The detailed syntheses and characterization data for all compounds are provided in the Supporting Information (SI). In a previous work, we described a synthetic route to obtain the unsymmetrical tetrasubstituted adamantane starting from the inexpensive compound 1-bromoadamantane.[18c] Following this method, we prepared aminoadamantane-1,3,5-tricarboxylic acid in five steps in high yield. We chose this intermediate to exploit its functional groups for the construction of the dendritic *HYDRAmers* via a protection/deprotection strategy of the amines and carboxylic acids.

We first esterified the tricarboxylic acid intermediate to form trimethyl aminoadamantane-1,3,5-tricarboxylate (**1**) in a yield of 79% (Scheme 1). At this point, two different paths were followed for the protection of the free amine. The first one was the coupling between 5-hexynoic acid, activated with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotriazole (HOBt), and **1** to afford compound **2** (65%). The alkyne moiety was available for click chemistry to attach the desired azide-modified molecules to the dendrons. The second way was the introduction of a carbobenzyloxy (Cbz) protecting group on the amine using *N*-(benzyloxycarbonyloxy)succinimide and triethylamine (Et_3N) to give compound **4** (56%) (Scheme 1).

Basic hydrolysis of compounds **2** and **4** afforded the corresponding tricarboxylic acids **3** and **5** in 93% and 90% yield, respectively. The latter served as building blocks for the construction of the different generations of dendrons. All of the functionalized adamantane molecules were fully characterized by NMR and FT-IR spectroscopy, HPLC, and mass spectrometry.

To synthesize the first-generation (G1) dendrons, we started from compound **3**, on which we introduced the triethylene

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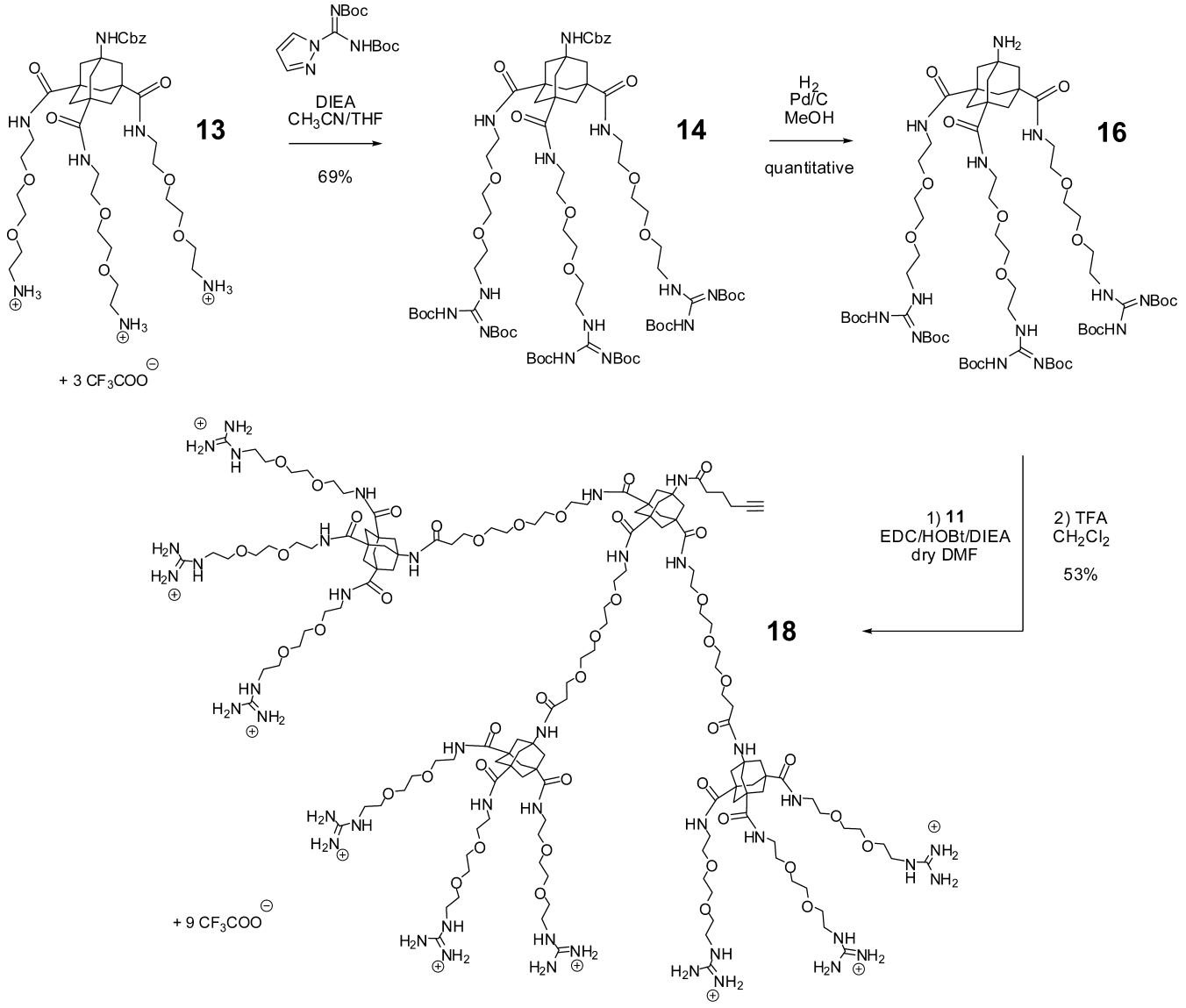
Scheme 4. Synthesis of Second-Generation Guanidinium *HYDRAmers*



glycol chains to improve the flexibility, biocompatibility, and water solubility of the final *HYDRAmers*. These spacers were prepared in large quantity starting from Boc-monoprotected diamines in one easy step.[21] By coupling **3** activated with EDC·HCl and HOBt to {2-[2-(2-aminoethoxy)ethoxy]ethyl}-carbamic acid *tert*-butyl ester, we obtained G1 dendron-$(NHBoc)_3$ (**6**) (Scheme 2). The three amide bonds were formed with a yield of 87%. Then the three Boc protecting groups at the periphery of the dendron were easily removed using TFA to yield the desired compound G1 dendron-$(NH_3^+)_3$ (**7**) in quantitative yield. The next step was the conversion of the ammonium groups into Boc-protected guanidines. We used *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine and *N*-diisopropylethylamine (DIEA) to guanylate the three ammonium groups, and this was followed by purification via column chromatography on silica gel. We obtained G1 dendron-$(NHC(NHBoc)NBoc)_3$ (**8**) in 67% yield, and then deprotection of the Boc groups with TFA afforded the G1 dendron-$(NHC(NH_2)NH_2^+)_3$ (**9**) quantitatively (Scheme 2). The triammonium dendron **7** and triguanidinium dendron **9** were fully characterized by NMR and FT-IR spectroscopy, HPLC, and mass spectrometry (see the SI for details).

We then prepared the building blocks for the second-generation (G2) dendrons starting again from compound **3**. In this case, in order to speed up the synthesis and because a triethylene glycol linker with a terminal ester group was not commercially available, we used the amino ester *tert*-butyl 12-amino-4,7,10-trioxadodecanoate. The amidation reaction between the tetraethylene glycol chains and **3** yielded G1 dendron-$(COOtBu)_3$ (**10**) in 86% yield. Saponification of the *tert*-butyl ester functions produced G1 dendron-$(COOH)_3$ (**11**), which is highly soluble in water. To remove the salts after the reaction, we used a Sephadex G-10 desalting column, and we obtained the tricarboxylic acid dendron **11** in 72% yield (Scheme 2).

Compound **5** with the Cbz-protected amine served as the scaffold for the construction of the "second layer" of G2 dendrons. We initially introduced the Boc-monoprotected diamine spacers on **5** by direct amidation to give Cbz-protected G1 dendron-$(NHBoc)_3$ (**12**) in 89% yield (Scheme 3). Boc deprotection of **12** afforded Cbz-protected G1 dendron-

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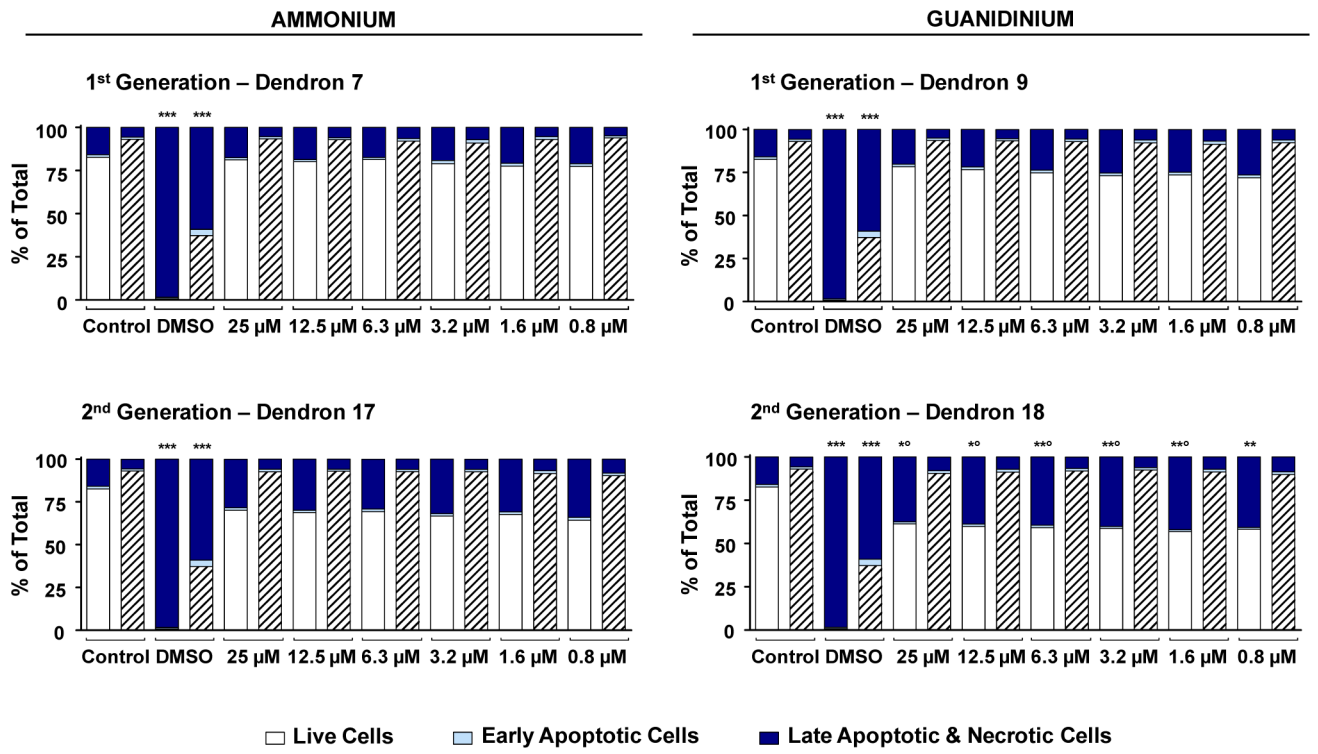

Figure 1. Flow cytometry analysis of cellular viability in RAW 264.7 (open bars) and HeLa (hatched bars) exposed to different concentrations (0.8 to 25 μM) of first- and second-generation ammonium and guanidinium *HYDRAmers*. Two-way ANOVA followed by Bonferroni's post-test was performed to determine the statistical differences vs control cells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) and to compare the four *HYDRAmers* to each other (°, $p < 0.05$ vs G1 ammonium *HYDRAmer*).

$(NH_3^+)_3$ (**13**) quantitatively (Scheme 3), and guanylation of the ammonium groups in **13** gave Cbz-protected G1 dendron-$(NHC(NHBoc)NBoc)_3$ (**14**) in 69% yield (Scheme 4).

In parallel, the Cbz groups at the focal points of **12** and **14** (Boc-protected dendrons) were selectively removed to liberate the amines, making them available to react with the carboxylic acids of the "first layer" dendron **11**. Cbz deprotection of **12** and **14** by hydrogenolysis using H_2 and a catalytic amount of Pd/C produced free-amine G1 dendron-$(NHBoc)_3$ (**15**) (Scheme 3) and free-amine G1 dendron-$(NHC(NHBoc)NBoc)_3$ (**16**) (Scheme 4), respectively, in quantitative yield. Compound **15** was coupled to **11** by amidation, and after the workup the product was directly used for Boc deprotection with TFA to give G2 dendron-$(NH_3^+)_9$ (**17**), which was easily purified by preparative HPLC, in an overall yield of 51% (Scheme 3). The same way was employed to synthesize G2 dendron-$(NHC(NH_2)NH_2^+)_9$ (**18**) in 53% overall yield by coupling of compound **16** to **11** followed by Boc deprotection and HPLC purification (Scheme 4).

The second-generation nonaammonium dendron **17** and nonaguanidinium dendron **18** were fully characterized by NMR and FT-IR spectroscopy, HPLC, and mass spectrometry (see the SI for details). All of the *HYDRAmers* **7**, **9**, **17**, and **18** are highly soluble in water, and their potential cytotoxic effects were subsequently evaluated (vide infra).

To study the cell internalization capacity of our dendrons, we linked the fluorescent probe cyanine 5 (Cy5) at their focal point. Cyanine 5 dye[22] activated with *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) was initially coupled to 5-azidopentan-1-amine[20a] to give the modified compound Cy5-N_3 (**19**), which was purified by preparative HPLC (47%). This azide derivative was then conjugated to the *HYDRAmers* **7**, **9**, **17**, and **18** bearing an alkyne group using "click" chemistry. Compound purifications by preparative HPLC yielded the corresponding fluorescent dendrons **20**–**23** with Cy5 connected at the focal point via a triazole moiety (see the SI for the molecular structures of the four dendrimers). Relatively good yields (38–41%) were obtained after HPLC purification.

Cellular Viability. The assessment of cellular toxicity represents an important issue toward the development of new types of vectors for drug delivery. The first step in our biological investigations was to determine the impact of the first- and second-generation ammonium and guanidinium *HYDRAmers* toward two cell lines in vitro: phagocytic RAW 264.7 murine macrophages and nonphagocytic HeLa epithelial human cells.

For this purpose, both cell types were exposed to increasing concentrations of first- and second-generation ammonium and guanidinium *HYDRAmers* ranging from 0.8 to 25 μM during 24 h. After incubation, the cellular viability was evaluated by flow cytometry using AnnV/PI double staining (Figure 1).

First, we would like to notice that RAW 264.7 and HeLa had different basal viabilities (82.6% and 90.3%, respectively; Table S1 in the SI) and were differently sensitive to our death positive control. Indeed, the remaining viable cells following DMSO exposure were almost zero in RAW 264.7 (0.7%), while HeLa cells showed 37.3% of cells still living under the same conditions. This observation anticipates a major sensitivity of RAW 264.7 in comparison to HeLa cells, as further confirmed after incubation with first- and second-generation *HYDRAmers*. In fact, the first observation was that the cellular viability of murine macrophages was always below the cellular viability of

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human cells under the same conditions (Figure 1; also see Table S1 in the SI).

In more detail, we could see that the RAW 264.7 cellular viability was affected by neither the first- and second-generation ammonium dendrons (**7** and **17**) nor the first-generation guanidinium dendrons (**9**). Only the second-generation guanidinium *HYDRAmer* **18** determined a slight but significant reduction of the viability of these cells (0.8 to 25 μM). Even though this decrease was not dramatic (~60% of residual viable cells), this result leads to the consideration that the second-generation guanidinium *HYDRAmer* affected the macrophage viability more than the counterpart ammonium *HYDRAmer* **17**, which displayed a remaining cellular viability of ~70%. This trend between ammonium and guanidinium dendrons of the same generation was also present to a minor extent between dendron **7** (~80% cellular viability) and dendron **9** (~75% cellular viability). Interestingly, another trend between the first and second generations was also observed. Considering the ammonium or guanidiunium compounds separately, we could observe that second-generation *HYDRAmers* affected RAW 264.7 cellular viability to a greater extent than their related first-generation *HYDRAmers*.

Overall, the cellular viability experiments allowed us to evidence a double trend in the impact of our *HYDRAmers* toward RAW 264.7 macrophages (see Table S1 in the SI). First, the ammonium dendrons **7** and **17** affected the cellular viability to a lesser extent than their counterpart guanidinium *HYDRAmers* **9** and **18**, respectively. Second, considering ammonium and guanidinium dendrons separately, the second generation affected the cellular viability more than the first generation. These two considerations were fully represented by the significant difference of cellular viability between RAW 264.7 treated with dendron **7** (first-generation ammonium *HYDRAmer*) and those treated with dendron **18** (second-generation guanidinium *HYDRAmer*) at almost all of the tested concentrations (1.6 to 25 μM).

In contrast, none of the *HYDRAmers* had an impact on HeLa cell viability (Figure 1; also see Table S1 in the SI). Even at the highest concentration (25 μM), the viability was not affected by either the first- or second-generation ammonium or guanidinium dendrons.

From a more general perspective, our results show that the first- and second-generation ammonium and guanidinium *HYDRAmers* had no remarkable effects on the viability of RAW 264.7 and HeLa cells. Indeed, even though the second-generation guanidinium *HYDRAmer* showed a certain degree of toxicity on murine macrophages, the residual living cells were still above 50%. Moreover, it is important to note that since other guanidinium dendrimers were shown to create reversible poration of the cellular membrane, this phenomenon could also be partly responsible for the augmented percentage of AnnV- and PI-positive cells.[20a]

We would like to point out that the range of concentrations used herein was higher than those reported in the literature by others that evidenced cytotoxicity of polycationic dendrimers.[8] Among polycationic compounds, PAMAM dendrimers are considered good candidates for gene delivery. Studies of the impact on cellular viability of different types of PAMAM dendrimers nevertheless demonstrated that these compounds triggered cellular death in a concentration- and generation-dependent way.[8b] Although some studies evidenced good cytocompatibility of these polycationic systems at low concentrations ranging from 100 nM to 1.5 μM,[16a] other reports showed significant cytotoxicity at concentrations higher than 500 nM.[9d,20a,23,24]

The results of our cellular viability experiments on both RAW 264.7 and HeLa cells, together with the lack of a pro-inflammatory effect on macrophages (i.e., interleukin 6 production; data not shown), allow us to conclude that our first- and second-generation ammonium and guanidinium *HYDRAmers* have good biocompatibility compared with PAMAM dendrimers and thus represent very promising molecules for future development as drug or gene delivery systems.

Study of *HYDRAmer* Cell Uptake. Besides good biocompatibility, another key characteristic for a promising drug/gene delivery system is its capability to reach the intracellular compartments. Polycationic dendrimers have been shown to be internalized differently on the basis of the properties of their peripheral functions and on the type of cells under investigation.[16a,b] In order to follow the cellular uptake of our first- and second-generation ammonium and guanidinium *HYDRAmers*, we used Cy5-conjugated dendrons (see Scheme 2 in the SI). RAW 264.7 and HeLa cells were exposed to the fluorescently labeled first- and second-generation ammonium (**20** and **22**) and guanidinium (**21** and **23**) *HYDRAmers* (5 μM) for 2 h in the absence or the presence of active cellular uptake inhibitors.

We initially assessed that our *HYDRAmers* were present inside both phagocytic RAW 264.7 and nonphagocytic HeLa cells using flow cytometry (Figure 2 and Figure S64 in the SI) and confocal microscopy (Figure 3). Next, we evaluated the effect of two energy-dependent cellular uptake inhibitors, namely, a temperature of 4 °C and NaN_3, on the internalization of our dendrons. Good cellular viability under inhibitory conditions was also ascertained (see Figure S65 in the SI).

As expected, under noninhibitory conditions the phagocytic macrophages internalized a higher amount of *HYDRAmers* in comparison with nonphagocytic cells. The mean fluorescence intensity (MFI) in RAW 264.7 was 1.7−2.6 times higher than that observed in HeLa cells (see Figure S64 in the SI). Figure 2 shows flow cytometry analysis of the cellular uptake of dendrons **20**−**23** under inhibitory conditions, allowing us to further observe some differences of internalization between the two types of cells and the different types of dendrons. In the case of macrophages, we measured a significant reduction of the cellular internalization of the first- and second-generation ammonium *HYDRAmers* (**20** and **22**) both at 4 °C and in the presence of NaN_3, resulting in about 90% and 70% inhibition of internalization, respectively. Similarly, the cellular uptake of first- and second-generation guanidinium *HYDRAmers* (**21** and **23**) was also significantly reduced by the inhibitory conditions. Only 12.9% and 17.8% of dendrons **21** and **23** were found inside the cells at 4 °C, while NaN_3 was less effective in blocking cellular uptake (20.6% for **21** and 39.3% for **23**).

These results suggest that our *HYDRAmers* were mainly internalized via endocytosis. Nevertheless, up to 40% of the fluorescence was still registered under inhibitory conditions, evidencing that both active and passive pathways of internalization likely contribute to the cellular uptake of our dendrons. In addition, it seems that the inhibitory conditions were slightly less effective in blocking the cellular uptake of guanidinium compounds relative to the ammonium *HYDRAmers*, especially considering the second-generation dendrons (**23**).

Similar behavior of our dendrons was confirmed by studies on HeLa cells. Incubation at 4 °C or with NaN_3 determined a

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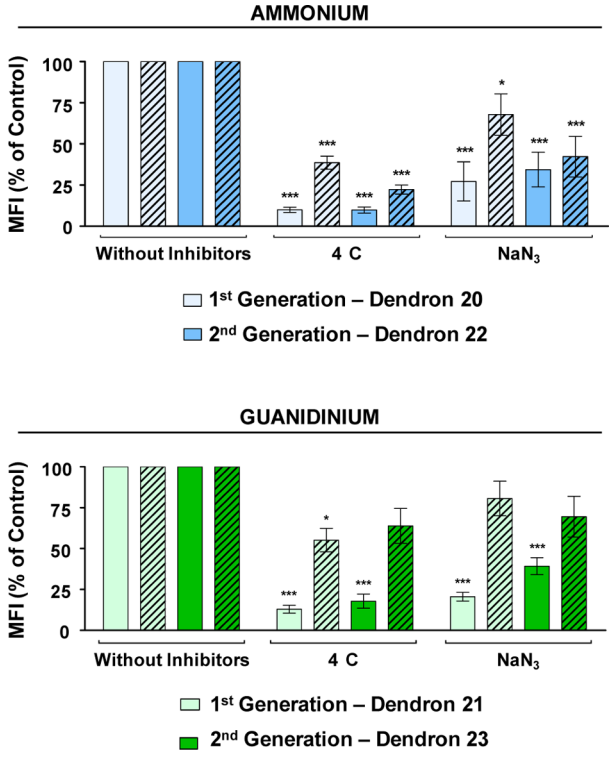

Figure 2. Quantification of the cellular internalization of Cy5-labeled ammonium and guanidinium *HYDRAmers* in RAW 264.7 (colored open bars) and HeLa cells (colored hatched bars) by flow cytometry. The cellular uptake of first- and second-generation *HYDRAmers* was evaluated in the absence and in the presence of the inhibitors of energy-dependent pathways of internalization (4 °C and NaN_3). One-way ANOVA followed by Dunnett's post-test was performed to determine the statistical differences between the data obtained with inhibitors vs normal conditions (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

significant reduction in the uptake of dendrons **20** and **22**, but the decrease was smaller than in the case of RAW 264.7 (see Table S1 in the SI). In fact, cellular uptakes of dendrons **20** and **22** were reduced to 38.6% and 22.2%, respectively, of those under noninhibitory conditions during 4 °C incubation (compared with ∼10% in RAW 264.7 for both dendrons). In the presence of NaN_3, the residual internalizations of dendrons **20** and **22** by HeLa were even greater: 67.8% and 42.3% of those under normal conditions, respectively.

The inhibitory conditions did not significantly influence the cellular uptake of the first- and second-generation guanidinium *HYDRAmers*. Only internalization of dendron **21** was affected at 4 °C, which still allowed the detection of 55.1% of the initial fluorescence inside HeLa cells. Residual uptakes of ∼60% to ∼80% with respect to those under noninhibited conditions were instead registered for dendron **21** in the presence of NaN_3 and dendron **23** both at 4 °C and with NaN_3.

Confocal microscopy studies were performed to further investigate the cellular internalization of the first- and second-generation ammonium and guanidinium *HYDRAmers* (Figure 3). The confocal microscopy observations were in good agreement with the previous flow cytometry analysis under either normal or inhibitory conditions. However, confocal microscopy allowed also to assess other crucial end points such as the intracellular fluorescence intensity and subcellular localization of the different *HYDRAmers*.

Similar to the observations by flow cytometry under noninhibited conditions (see Figure S64 in the SI), it was possible to observe that the second-generation ammonium and guanidinium *HYDRAmers* (**22** and **23**) were more internalized than the corresponding first-generation dendrons (**20** and **21**) in both types of cells. The other trend showing that guanidinium compounds were more internalized than their counterpart ammonium *HYDRAmers* was also observed in RAW 264.7 cells and even better in HeLa cells (Figure 3).

Considering the confocal microscopy analysis under inhibitory conditions, we could draw two main conclusions. First, it was possible to detect that a more intense fluorescence was present close to the cellular membrane, evidencing that our dendrons were somehow blocked at the surface of the cells and could not be internalized. Second, confocal microscopy allowed us to examine in greater detail the subcellular localization of the *HYDRAmers*.

Independent of the conditions, our *HYDRAmers* were present in the cytoplasm of both RAW 264.7 and HeLa cells but were never localized inside the nucleus. 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 (Figure 3, left columns).[25] The endosomes could be better distinguished in HeLa cells, likely because these cells, being nonphagocytic, have a lower endocytic rate, which means that a lower amount of endosomes/phagosomes is formed in comparison with RAW 264.7. In the second case, the extremely high concentration of the phagosomal vesicles resulted in a dense fluorescence instead of well-defined vesicle-localized signals. When inhibitory conditions were applied, the cellular uptake of *HYDRAmers* was reduced, but it was still possible to observe a diffuse fluorescence inside the cells, confirming the previous flow cytometry results and demonstrating the contribution of a passive pathway in the internalization of our dendrons.[26]

Beyond the interest in understanding which mechanisms account for the cellular uptake of the different *HYDRAmers*, it is important to remember that their way 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 the endosomes for their cargoes to exert their therapeutic activity.[13b,21]

Upon comparison of the results on cellular viability and uptake for the ammonium and guanidinium *HYDRAmers* and for the first- and second-generation dendrons, their different behaviors are consistent with those observed in previous studies regarding other polycationic dendrons and polymers.[16a,b,26]

As guanidinium is more basic than ammonium, its degree of protonation is higher. This property is hypothesized to be responsible for its capacity to form strong and stable bidentate hydrogen bonds with negatively charged cellular membrane components, leading to enhanced cellular internalization, a phenomenon called adaptive translocation.[14c,27] Once the dendron is anchored to negatively charged cellular membrane

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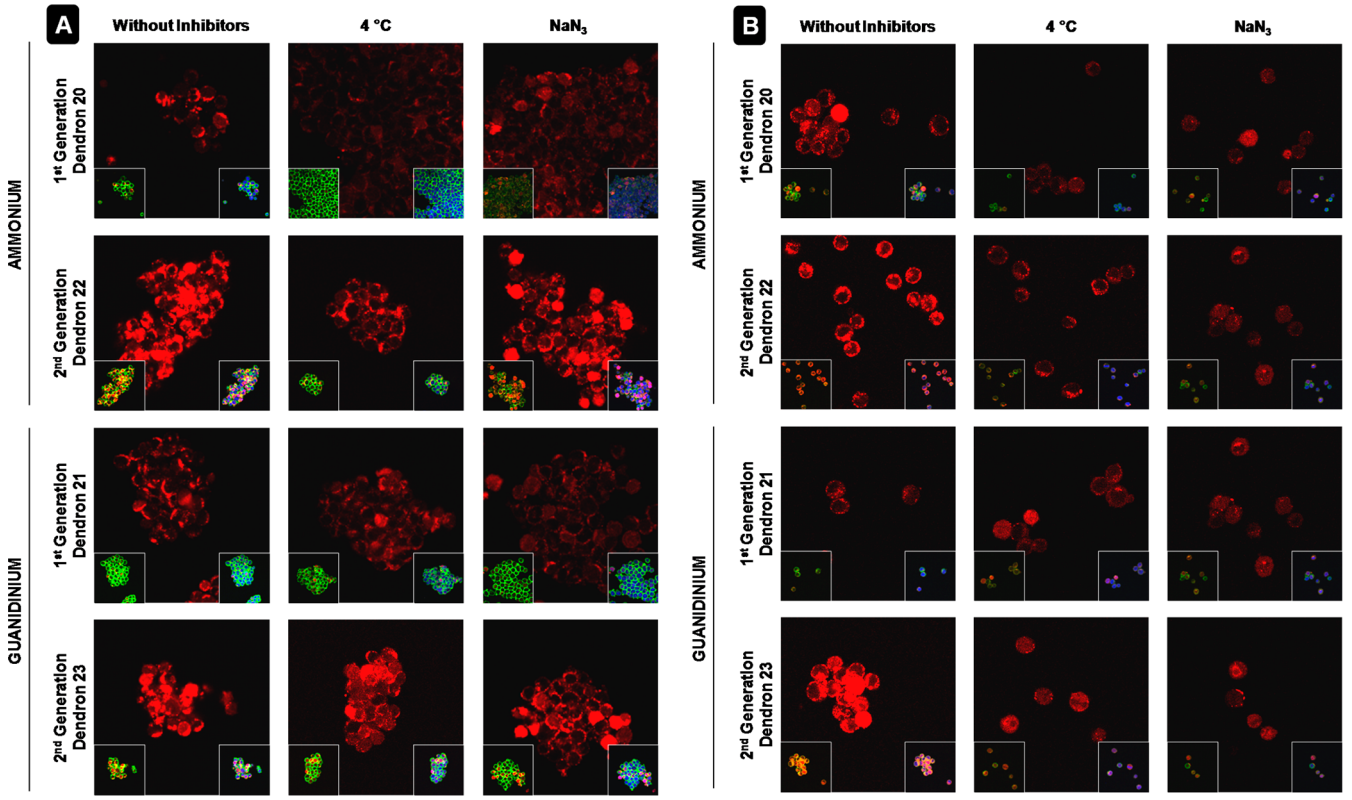

Figure 3. Intracellular localization of first- and second-generation ammonium and guanidinium *HYDRAmers* by (A) RAW 264.7 and (B) HeLa cells using confocal microcopy. Cells were incubated with dendrons alone (left columns) or under conditions that inhibited the energy-dependent internalization, namely, incubation at 4 °C (middle columns) and with NaN_3 (right columns). Dendron localization is evidenced in red, cellular membrane (CD11b labeling in RAW 264.7 or CD95 labeling in Hela cells) in green, and nuclei (DAPI staining) in blue. The left insets display both the dendron- and cellular-membrane-associated signals, while the right insets show the overlay of the dendron-, cellular-membrane-, and nuclei-associated signals. Each image represents one of at least five experiments with similar results.

components (e.g., phosphates, sulfates, carboxylates), the actual polarity of the dendron changes, allowing it to penetrate into the nonpolar membranes through adaptive non-covalent association with membrane constituents and finally to slide into the cytosol under the influence of a membrane potential.[14c] The passive internalization of polycationic dendrons is then correlated to not only the nature of the moieties they bear on their surface (with guanidinium better than ammonium) but also to the properties of the cellular membrane itself, such as its composition and potential.[16a,28]

Moreover, it has also been demonstrated that the architecture of the dendrimer scaffold bearing the positively charged moieties plays an important role in their interaction with cellular membranes, thus influencing the cellular internalization.[14a,b,29] As two negatively charged components of the cellular membrane are unlikely to be close to each other because of electrostatic repulsion, it has been demonstrated that the generation of the dendron also influences its interaction with the cellular membrane.[13b] It has been evidenced that the optimal number of guanidium moieties exposed on the periphery of the dendrons or polymers is 8 to 9,[15b,30] corresponding to our second-generation guanidinium *HYDRAmer* (**23**). Consistent with these earlier observations, this dendron was in fact the one that was the most internalized by the cells, suggesting that these *HYDRAmers* are able to interact strongly with cellular membranes, likely supporting the subsequently enhanced active and passive cellular uptake.

■ CONCLUSIONS

In this study, we have described the synthesis and full characterization of a novel type of polycationic dendrons. They are built from different adamantane scaffolds using a straightforward protection/deprotection strategy. Cyanine 5 dye was covalently linked to the target dendrons via click chemistry to obtain fluorescently labeled molecules for cell imaging. The cellular investigations allowed us to determine the influence of the nature of the peripheral moieties and their number on the cytotoxicity and the cellular internalization of first- and second-generation ammonium and guanidinium dendrons. Our studies revealed that the so-called *HYDRAmers* had an improved biocompatibility relative to other polycationic dendrons proposed as potential drug/gene delivery systems. Furthermore, our *HYDRAmers* were well internalized by both phagocytic and nonphagocytic cells. The cellular uptake analyses provided evidence of a combination of active and passive mechanisms of uptake dependent on the cell type, peripheral groups, and dendron generation.

In comparison with other polycationic carriers, *HYDRAmers* do not show cytotoxicity despite the presence of positive charges and their high cellular uptake. This feature provides to these adamantane-based dendrons a strong potential for biomedical applications. Additional studies to assess the capacity of the *HYDRAmers* to complex nucleic acids and intracellularly deliver this type of therapeutic molecules are currently underway.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental Section, including materials and methods, details of the synthesis and characterization of all derivatives, precursors, and final dendrons, NMR and MS spectra, cell culture protocols, and flow cytometry analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

a.bianco@ibmc-cnrs.unistra.fr

Notes

The authors declare no competing financial interest.

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