Elucidation of the cellular uptake mechanisms of polycationic *HYDRAmers*

Julie Russier, Maxime Grillaud, Alberto Bianco*

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

SUPPORTING INFORMATION

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A

Cellular Uptake	CONTROL MOLECULES	
	With FBS	Without FBS
Without Inhibitors (% of MFI)	100	100
Chlorpromazine (% of MFI)	93.4 ± 4.0	88.0 ± 3.6
Filipin (% of MFI)	81.3 ± 7.9	84.1 ± 18.1
Genistein (% of MFI)	81.9 ± 7.4	81.8 ± 16.1
Amiloride (% of MFI)	60.3 ± 5.1	58.5 ± 8.6
mβCD (% of MFI)	47.0 ± 8.1	37.3 ± 14.4

B

Cellular Uptake	CONTROL MOLECULES	
	With FBS	Without FBS
Without Inhibitors (% of MFI)	100	100
Chlorpromazine (% of MFI)	107.0 ± 4.6	102.2 ± 6.1
Filipin (% of MFI)	104.1 ± 5.8	105.8 ± 17.0
Genistein (% of MFI)	109.4 ± 5.3	105.0 ± 13.9
Amiloride (% of MFI)	122.9 ± 13.9	115.2 ± 17.7
mβCD (% of MFI)	101.6 ± 10.0	95.6 ± 13.7

Table S1. Evaluation by flow cytometry of the effect of selective cellular uptake inhibitors on cellular internalization of fluorescent control molecules by RAW 264.7 (**A**) and HeLa (**B**) cells. Conditions that inhibit different internalization pathways were applied to selectively block the different internalization pathways both in the presence and in the absence of FBS (10 %) in the cellular culture media: clathrin-mediated endocytosis (**Chlorpromazine**), caveolae-mediated endocytosis (**Filipin** and **Genistein**) and macropinocytosis-dependent internalization (**Amiloride** and **mβCD**). The inhibitory effects of each internalization pathway were verified by monitoring the internalization of fluorescent well-known cellular uptake markers: transferrin (30 µg/ml) was used as positive uptake marker in chlorpromazine experiments, Bodipy-LacCer/BSA (0.5 µM) was used as a positive uptake marker in genistein and filipin III inhibitory conditions and dextran (250 µg/ml) was used as a positive uptake marker in amiloride and mβCD inhibition tests. Mean values ± SEM were obtained from at least four experiments run in triplicate. Data are presented relatively to the un-inhibited conditions which correspond to 100 % of the intracellular fluorescence.

A

Cellular Uptake	AMMONIUM		GUANIDINIUM	
	1st Generation Cy5-G1a	2nd Generation Cy5-G2a	1st Generation Cy5-G1g	2nd Generation Cy5-G2g
Without Inhibitors (% of MFI)	100	100	100	100
Chlorpromazine (% of MFI)	70.2 ± 5.2	80.7 ± 7.4	62.6 ± 5.3	70.4 ± 5.3
Filipin (% of MFI)	92.2 ± 4.1	93.4 ± 2.2	92.6 ± 5.0	86.7 ± 3.9
Genistein (% of MFI)	84.0 ± 8.0	132.2 ± 13.7	89.0 ± 10.0	129.3 ± 21.7
Amiloride (% of MFI)	69.0 ± 2.3	86.8 ± 3.9	79.0 ± 3.2	93.1 ± 16.6
mβCD (% of MFI)	64.5 ± 10.0	109.3 ± 11.4	73.4 ± 15.4	177.4 ± 34.5

B

Cellular Uptake	AMMONIUM		GUANIDINIUM	
	1st Generation Cy5-G1a	2nd Generation Cy5-G2a	1st Generation Cy5-G1g	2nd Generation Cy5-G2g
Without Inhibitors (% of MFI)	100	100	100	100
Chlorpromazine (% of MFI)	114.3 ± 4.1	117.8 ± 1.2	114.5 ± 4.6	115.1 ± 6.1
Filipin (% of MFI)	102.6 ± 5.7	111.8 ± 7.6	108.5 ± 8.7	110.7 ± 7.9
Genistein (% of MFI)	107.0 ± 8.6	115.2 ± 13.0	118.4 ± 11.5	118.6 ± 11.9
Amiloride (% of MFI)	105.0 ± 11.7	119.4 ± 10.8	117.4 ± 11.0	146.4 ± 21.8
mβCD (% of MFI)	101.7 ± 25.4	149.1 ± 44.7	104.2 ± 22.5	184.8 ± 52.8

Table S2. Evaluation by flow cytometry of the effect of selective cellular uptake inhibitors on cellular internalization of cyanine 5-labeled first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* (5 µM) by RAW 264.7 (**A**) and HeLa (**B**) cells. The different internalization pathways that were selectively inhibited are clathrin-mediated endocytosis (**Chlorpromazine**), caveolae-mediated endocytosis (**Filipin** and **Genistein**) and macropinocytosis-dependent internalization (**Amiloride** and **mβCD**). Mean values ± SEM were obtained from at least seven experiments run in triplicate in serum free media. Data are presented relatively to the un-inhibited conditions which correspond to 100 % of the intracellular fluorescence.

A

Cellular Uptake	AMMONIUM		GUANIDINIUM	
	1st Generation Cy5-G1a	2nd Generation Cy5-G2a	1st Generation Cy5-G1g	2nd Generation Cy5-G2g
Without Inhibitors (% of MFI)	100	100	100	100
Chlorpromazine (% of MFI)	91.4 ± 10.2	85.2 ± 9.4	91.8 ± 4.0	95.0 ± 3.9
Filipin (% of MFI)	97.1 ± 5.5	99.2 ± 11.3	114.3 ± 3.0	115.3 ± 7.5
Genistein (% of MFI)	119.0 ± 11.4	129.6 ± 8.2	128.4 ± 1.8	137.6 ± 15.6
Amiloride (% of MFI)	91.4 ± 9.8	108.7 ± 16.6	103.5 ± 7.8	131.8 ± 32.2
mβCD (% of MFI)	101.1 ± 13.2	129.7 ± 3.7	110.2 ± 24.8	238.3 ± 50.3

B

Cellular Uptake	AMMONIUM		GUANIDINIUM	
	1st Generation Cy5-G1a	2nd Generation Cy5-G2a	1st Generation Cy5-G1g	2nd Generation Cy5-G2g
Without Inhibitors (% of MFI)	100	100	100	100
Chlorpromazine (% of MFI)	118.5 ± 16.6	119.1 ± 8.8	118.2 ± 7.7	100.3 ± 10.6
Filipin (% of MFI)	118.3 ± 20.0	118.0 ± 7.6	133.3 ± 38.9	125.2 ± 38.1
Genistein (% of MFI)	126.2 ± 39.4	140.9 ± 46.7	133.5 ± 45.8	116.6 ± 27.2
Amiloride (% of MFI)	85.8 ± 10.4	135.2 ± 30.7	108.4 ± 15.5	183.9 ± 62.0
mβCD (% of MFI)	132.8 ± 51.0	248.9 ± 101.6	113.3 ± 26.7	276.4 ± 114.4

Table S3. Evaluation by flow cytometry of the effect of selective cellular uptake inhibitors on cellular internalization of cyanine 5-labeled first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* (5 µM) by RAW 264.7 (**A**) and HeLa (**B**) cells. The different internalization pathways that were selectively inhibited are clathrin-mediated endocytosis (**Chlorpromazine**), caveolae-mediated endocytosis (**Filipin** and **Genistein**) and macropinocytosis-dependent internalization (**Amiloride** and **mβCD**). Mean values ± SEM were obtained from at least seven experiments run in triplicate in media containing FBS (10 %). Data are presented relatively to the un-inhibited conditions which correspond to 100 % of the intracellular fluorescence.

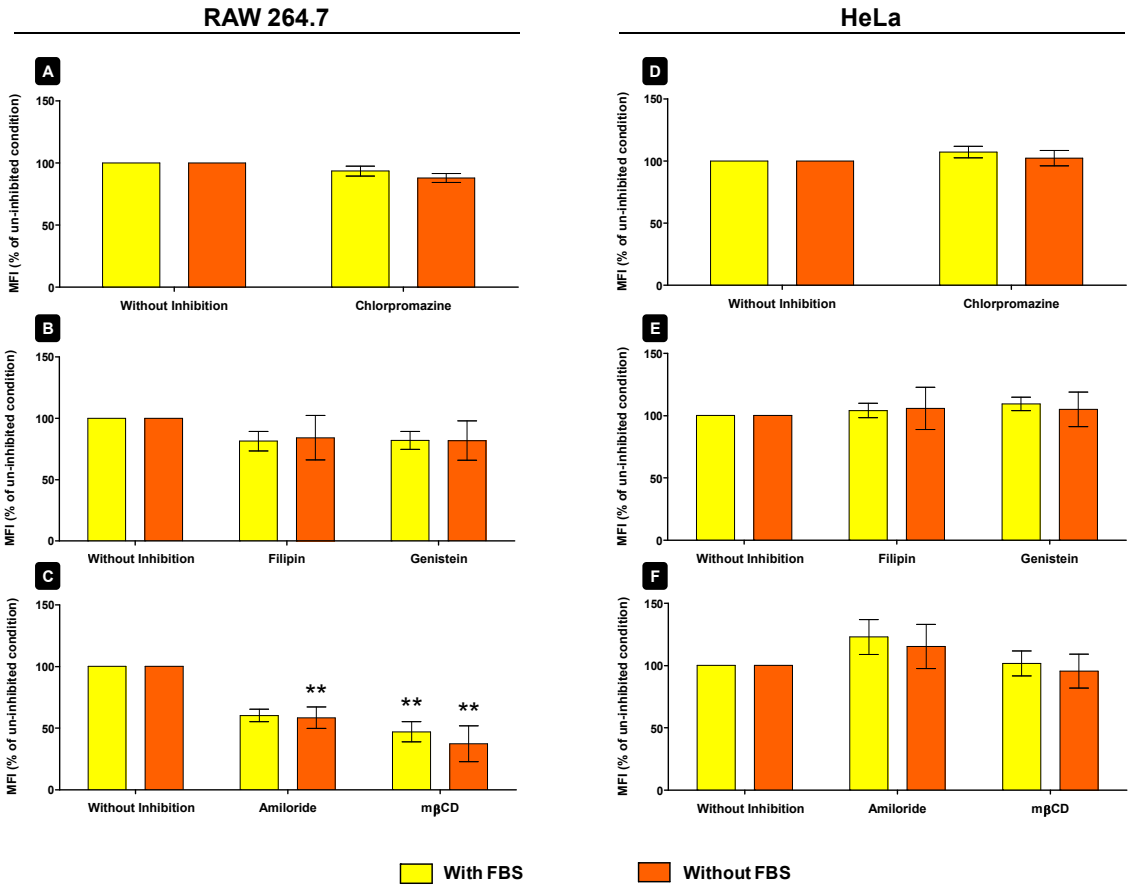

Figure S1. Quantification by flow cytometry of the cellular uptake of fluorescent control molecules in RAW 264.7 and HeLa cells. Conditions that inhibit different internalization pathways were applied to selectively block the different internalization pathways both in the presence and in the absence of FBS (10 %) in the cellular culture media: clathrin-mediated endocytosis (**A** and **D**), caveolae-mediated endocytosis (**B** and **E**) and macropinocytosis-dependent internalization (**C** and **F**). The inhibitory effects in each internalization pathway were verified by monitoring the internalization of fluorescent well-known cellular uptake markers: transferrin (30 µg/ml) was used as positive uptake marker in chlorpromazine experiments, Bodipy-LacCer/BSA (0.5 µM) was used as a positive uptake marker in genistein and filipin III inhibitory conditions and dextran (250 µg/ml) was used as a positive uptake marker in amiloride and mβCD inhibition tests. Mean values ± SEM were obtained from at least four experiments run in triplicate. Data are presented relatively to the un-inhibited conditions which correspond to 100 % of the intracellular fluorescence. One-way ANOVA, followed by Bonferroni's post test was carried out to determine the statistical significance of the data obtained with the different inhibitors compared to the control molecules alone and also between the serum containing and the serum free conditions (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

Overall, the presence of FBS (10 %) in the culture medium did not induce any effect on the cellular internalization of the control molecules by both cell lines. We could also notice that the conditions that we have chosen based on RAW 264.7 results in terms of efficiency of inhibiting cellular internalization while keeping cellular viability, were not efficient in blocking cellular internalization in HeLa cells. This is not surprising as HeLa are non-phagocytic cells. This observation further sustains the interest of using phagocytic together with non-phagocytic cells to eventually highlight the cellular internalization pathways of dendrimers.

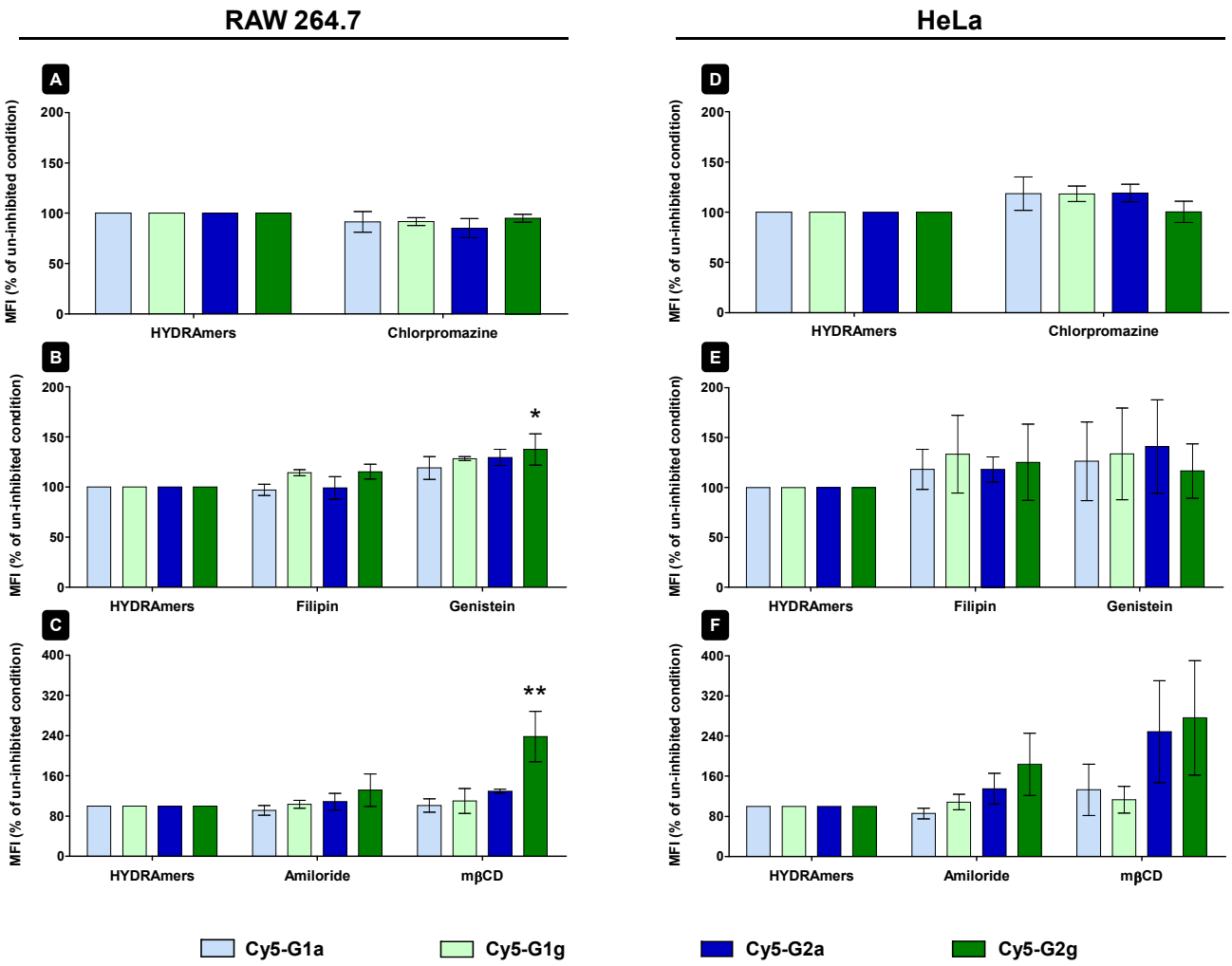

Figure S2. Quantification of the extent of cellular uptake of cyanine 5-labeled first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* (5 μM) in RAW 264.7 and HeLa cells by flow cytometry. Conditions that inhibit different internalization 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 media containing FBS (10 %). Data are presented relatively to the un-inhibited conditions which correspond to 100 % of the intracellular fluorescence**.** 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).

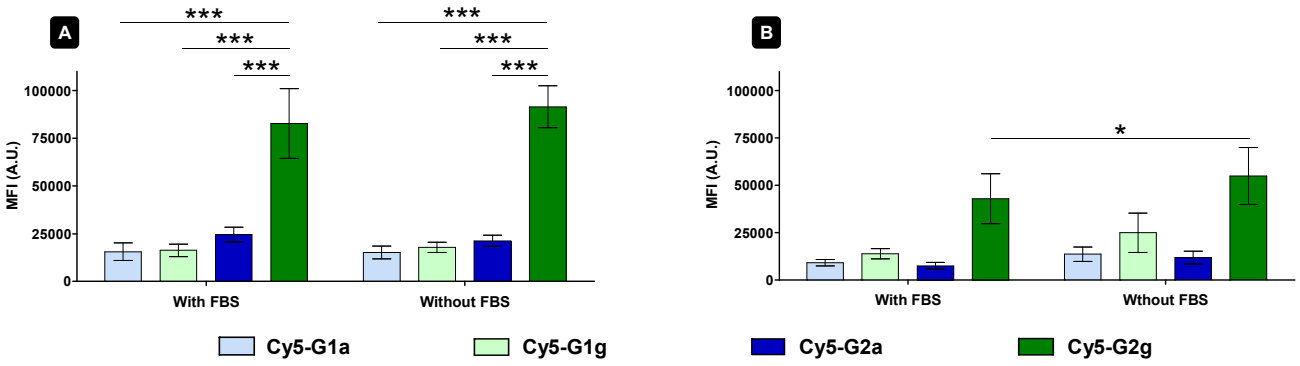

Figure S3. Quantification of the extent of cellular uptake of cyanine 5-labeled first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* (5 μM) in RAW 264.7 (**A**) and HeLa (**B**) cells by flow cytometry. Mean values of the relative mean fluorescence intensity ± SEM were obtained from at least five separate experiments run in triplicate in media containing FBS (10 %) and in serum free conditions. One-way ANOVA, followed by Bonferroni's post test was carried out to determine the statistical significance between the data obtained with the different cyanine 5-labeled first and second generation ammonium and guanidinium *HYDRAmers* and also between the serum containing and the serum free conditions (*p<0.05; **p<0.01; ***p<0.001).

Under un-inhibited conditions, 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. We also observed the other trend showing that guanidinium compounds were more internalized respect to their counter ammonium *HYDRAmers*, especially in RAW 264.7 macrophages. Moreover, the **Cy5-G2g** dendron was much more internalized by both the cell types. This behavior was clearly observed in RAW 264.7 cells even in serum containing or in serum free cellular culture medium. Indeed, it is interesting to notice that the absence of FBS (10 %) did not influence the basal internalization rate of the *HYDRAmers* in RAW 264.7 and HeLa cells. The only difference that was registered regards the internalization of the second generation guanidinium *HYDRAmers* in HeLa cells. Indeed, the internalization of **Cy5-G2g** was significantly enhanced in serum free conditions.

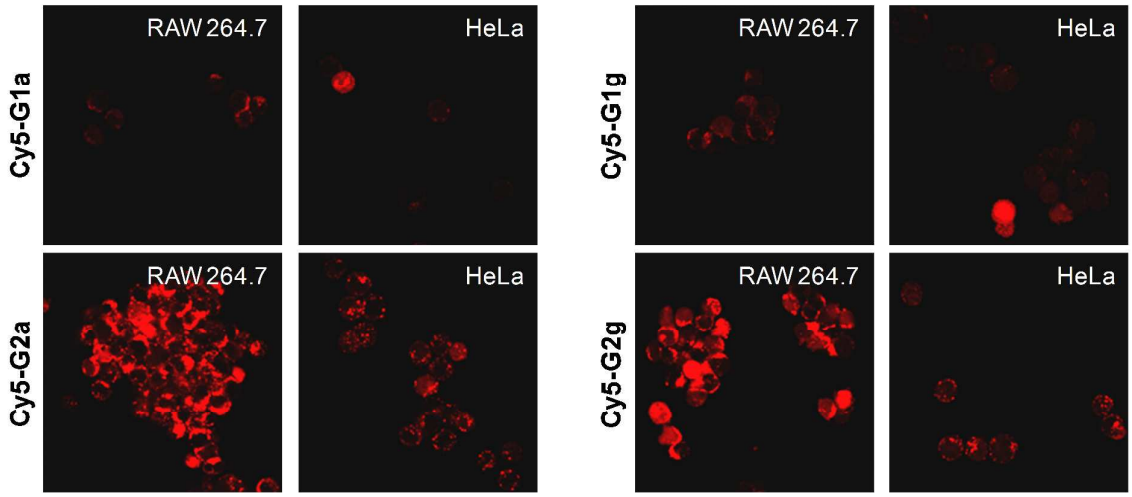

Figure S4. Intracellular localization of the first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* in RAW 264.7 and HeLa cells using laser scanning confocal microcopy. Dendron localization is evidenced in red.

Consistently with what was previously observed by flow cytometry in serum free media, under un-inhibited conditions, 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. We also observed the other trend showing that guanidinium compounds were more internalized respect to their counter ammonium *HYDRAmers*, especially in RAW 264.7 macrophages.

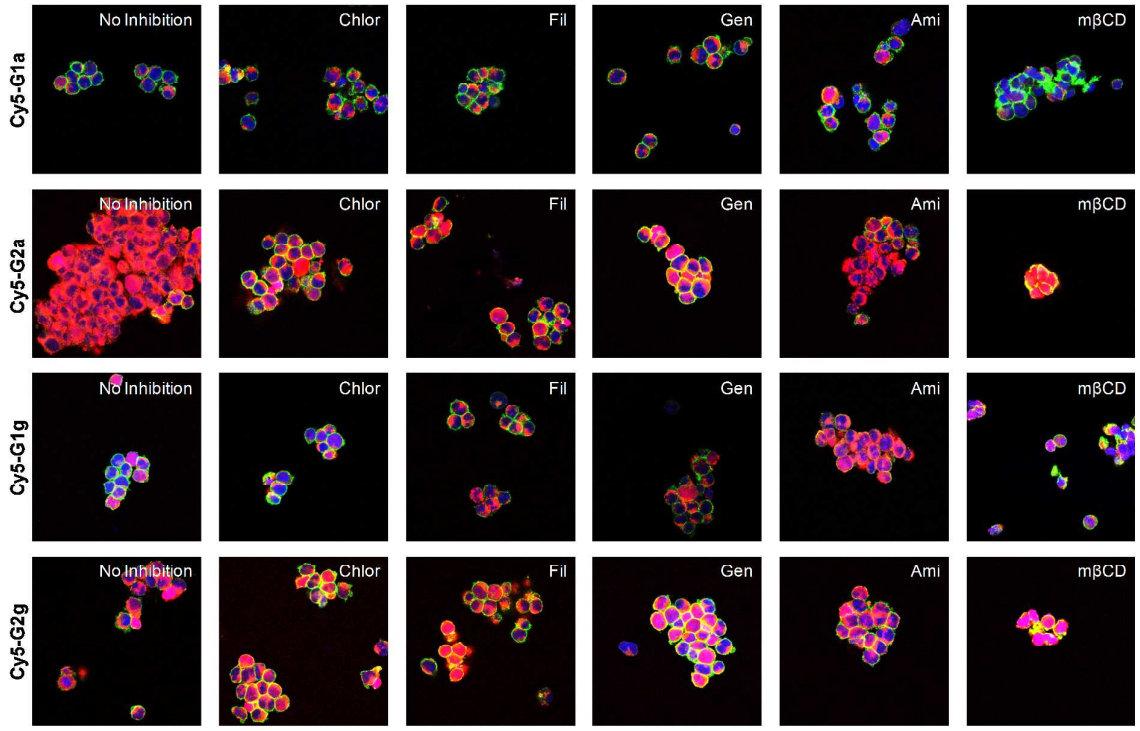

Figure S5. Intracellular localization of the first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* in RAW 264.7 cells using laser scanning confocal microcopy. Cells were incubated with dendrons alone (**No inhibition**) or under conditions 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 while cellular membrane (CD11b labeling) are represented in green and nuclei (DAPI staining) in blue.

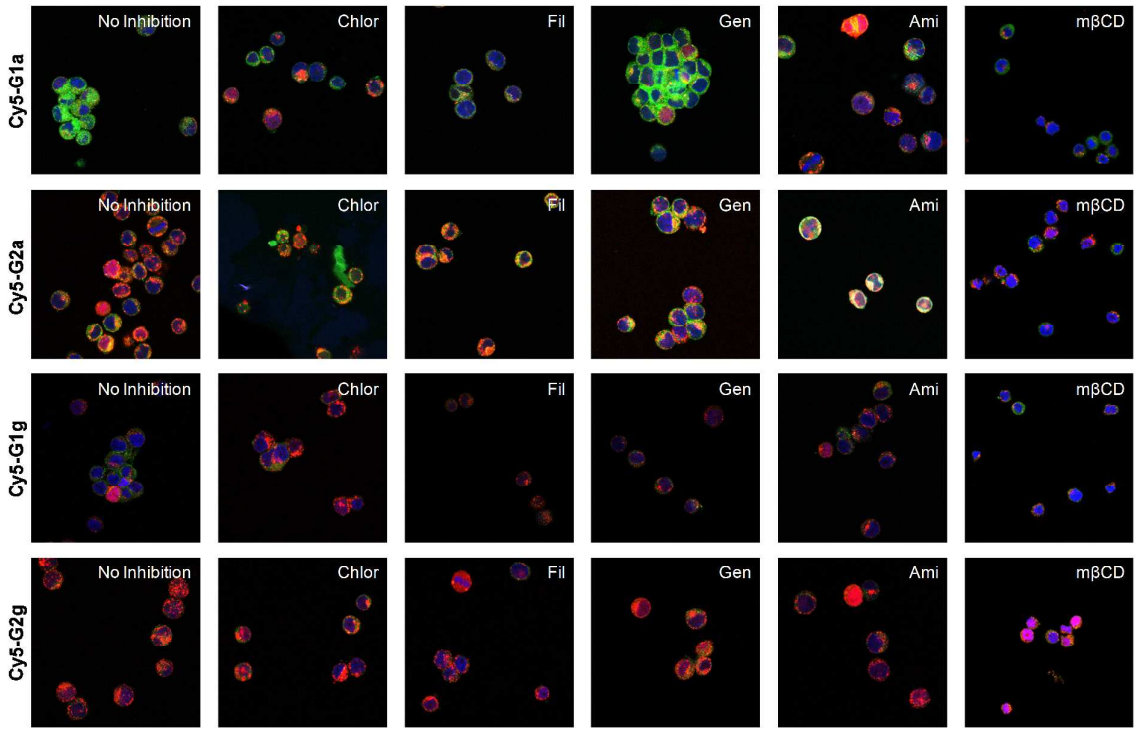

Figure S6. Intracellular localization of the first and second generation ammonium (**Cy5-G1a** and **Cy5-G2a**) and guanidinium (**Cy5-G1g** and **Cy5-G2g**) *HYDRAmers* in HeLa cells using laser scanning confocal microcopy. Cells were incubated with dendrons alone (**No inhibition**) or under conditions 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 while cellular membrane (CD95 labeling) are represented in green and nuclei (DAPI staining) in blue.

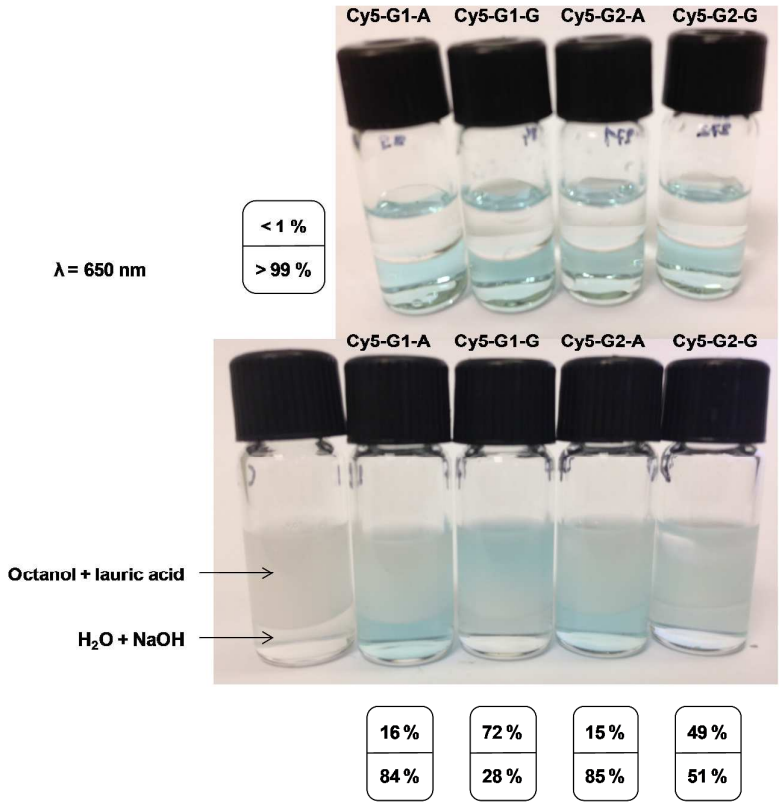

Figure S7. Octanol/water (upper and lower phase, respectively) partitioning of **Cy5-G1a**, **Cy5-G1g**, **Cy5-G2a** and **Cy5-G2g** alone (samples on the top) and after addition of sodium laurate (samples of the bottom; the vials in the left side is the control without *HYDRAmer*). The percentages of partitioning into both phases were calculated after analysis of UV absorbance (at 650 nm) of the organic and the aqueous phases.