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Perise-Barrios , A.J., Fuentes Paniagua, M.E., Sánchez-Nieves Fernández, J., Serramía Lobera, M.J., Alonso , E., Reguera , R., Gómez Ramírez, R., Mata De La Mata, Francisco J. De La & Muñoz Fernández, M.A. 2016, "Improved efficiency of ibuprofen by cationic carbosilane dendritic conjugates", *Molecular Pharmaceutics*, vol. 13, no. 10, pp. 3427-3438.

Available at <http://dx.doi.org/10.1021/acs.molpharmaceut.6b00420>

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(Article begins on next page)



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IMPROVED EFFICIENCY OF IBUPROFEN BY CATIONIC CARBOSILANE DENDRITIC CONJUGATES

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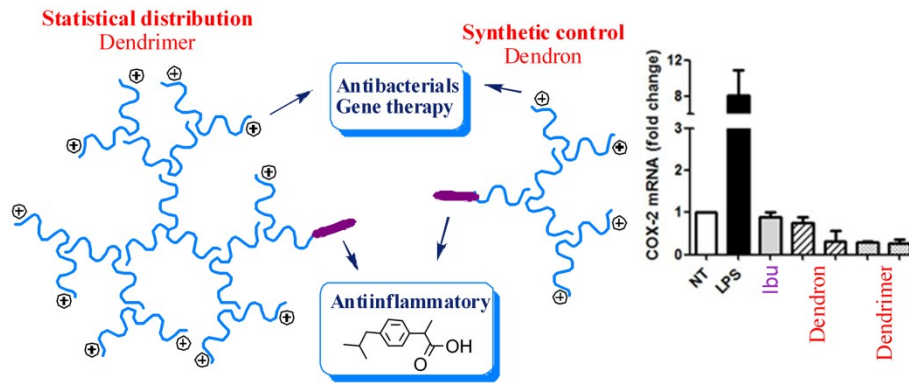
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Ibuprofen-conjugated and non-ibuprofen cationic carbosilane dendritic molecules show important antiinflammatory properties on macrophages.



ABSTRACT

In order to improve the efficiency of the anti-inflammatory drug ibuprofen, cationic carbosilane dendrimers and dendrons with ibuprofen at their periphery or at their focal point, respectively, have been synthesized and the release of the drug was studied using HPLC. Macrophages were used to evaluate the anti-inflammatory effect of the ibuprofen-conjugated dendritic systems and compared with mixtures of non-ibuprofen dendritic systems in the presence of the drug. The cationic ibuprofen-conjugated dendron was the compound that showed higher anti-inflammatory properties. It reduces the LPS-induced *COX-2* expression, decreases the release of several inflammatory cytokines such as $TNF\alpha$, $IL-1\beta$, $IL-6$ and $CCL3$. These results open new perspectives in the use of these compounds as drug carriers.

KEYWORDS

Anti-inflammatory, carbosilane dendrimers, dendrons, ibuprofen, drug release and macrophages.

INTRODUCTION

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) with antipyretic, analgesic and antibacterial activity,¹⁻³ and presents good biocompatibility.⁴ Ibuprofen is a non-selective cyclooxygenase (COX) inhibitor that affects two isoforms, COX-1 (which is constitutively expressed in many cells and tissues) and COX-2 (which is selectively induced by proinflammatory cytokines at the site of inflammation).⁵ The inhibition of COX-2 blocks the prostaglandins biosynthesis, molecules that mediate pathogenic mechanisms as the inflammatory response, and also they are responsible of the pain and fever.⁶ [It has been demonstrated that inhibition of COX-2 reverses inflammation and expression of interleukin \(IL\)-6.](#)⁷ Therefore, treatments of bacterial infections with ibuprofen hasten the healing process in vaginal infections, cellulites or acne.⁸⁻¹⁰ However, its poor solubility joined to protein binding, diminishes its bioavailability.¹¹ This problem can be found in many drugs,¹² so there is a need to develop new platforms for the transport, protection and sustained release of drugs, minimizing its side effects, as well as avoiding a fluctuation of the therapeutic concentration.¹³ Several systems have been reported for their use as drug carriers such as vesicles, nanoparticles, polymers.¹⁴⁻¹⁹

Dendrimers are highly branched globular macromolecules which, due to their multivalency, have been widely employed in biomedical applications.²⁰ The multivalency of dendrimers, as well as the possibility of employing the cavities in their structure, makes them good candidates for obtaining drug conjugates that can act as carriers, giving solubility to the drugs and protecting them.²¹⁻²⁴ However, heterofunctionalization of dendrimers usually implies statistical methods of synthesis, and thus, a loss of their characteristic monodispersity. For this reason, other topologies can be used in order to obtain drug conjugates while maintaining a defined structure. Similarly to dendrimers, dendrons are highly branched cone shaped macromolecules, with a focal point, clearly differentiated from dendritic periphery.²⁵ This extra reactive moiety can be used to obtain more complex structures by anchoring other structures such as polysaccharides, polymers or nanoparticles.²⁶⁻²⁹ Few examples of drug-conjugated dendrons can be found. Bianco et al. reported the synthesis of adamantane based dendrons with ibuprofen moieties at their periphery. These compounds enhanced the anti-inflammatory activity

of ibuprofen due to dendrimer multivalency.³⁰ On the other hand, polyester dendrons have been used to solubilize the hydrophobic dye BODIPY.³¹ Finally, previous works of our research group reported the synthesis of heterofunctionalized cationic carbosilane dendrons with fluorescein.^{32, 33} In this case, biodistribution studies on carbosilane dendrimers showed their capability to cross the blood brain barrier,³⁴ which is an important obstacle in drug delivery to the brain. Regarding ibuprofen modified dendrimers, PAMAM-ibuprofen dendrimers improve the drug's efficacy by enhancing cellular delivery, which may produce a rapid pharmacological response³⁵ depending of the linkage between dendrimer and drug.³⁶ Also has been reported that ibuprofen solubility increases by electrostatic interactions with dendrimers or by conjugation through bond formation.^{37, 38}

The study of the anti-inflammatory effect induced by compounds with ibuprofen requires an appropriate *in vitro* system, being macrophages the suitable model due to their regulatory capacities in inflammatory responses. The immune system is responsible of maintenance the homeostasis on the human body. To do it properly, detects pathogens and trigger different immune responses involving a wide variety of cells with the aim to eliminate the pathogen. Macrophages are one of the most important cells that regulate and trigger different immune responses with the aim to eliminate pathogens.³⁹ To trigger an inflammatory response, macrophages must have a pro-inflammatory phenotype expressing cytokines such as IL-1 β , IL-6 and TNF- α ⁴⁰⁻⁴² and exhibiting microbicidal functions. These cells are defined as M1-polarized macrophages. Once they have accomplished their objective, and therefore an immune response has been successfully activated, macrophages eliminate microorganisms by phagocytosis and also restore tissue homeostasis. Macrophages that are acting in the resolution of the inflammation are M2-polarized macrophages that present an anti-inflammatory phenotype, defined by the production of anti-inflammatory and regulatory cytokines such as IL-10 and TGF- β .^{43, 44}

Herein we deal with the synthesis of cationic carbosilane dendritic macromolecules with one ibuprofen unit bonded to them with the aim to evaluate the effect of this drug in these systems. Previous studies warned about the importance of evaluating the inflammatory effect of molecules with

a future biological application,⁴⁵ as this factor will cause significant problems inducing in a non-controlled way the immune system responses. Moreover, it is well known that some dendritic derivatives failed in biological studies performed *in vitro* due to the inflammatory effect over cells.⁴⁶ On the other hand, since cationic carbosilane dendritic molecules have been proved useful as siRNA delivery vectors against HIV⁴⁷⁻⁴⁹ and as microbicides,^{50, 51} and several types of dendrimers have shown antiinflammatory activities,^{49, 52-54} is very interesting to evaluate them as ibuprofen-carriers, and also to find whether these structures could help to minimize any inflammatory process, effect that could accelerate the healing processes. Finally, the analysis of activity of these compounds would also allow verifying their pharmacological action and thus, their potential use as drug delivery systems or codrugs.⁵⁵

For this purpose, a comparative evaluation of cytokine release by treated macrophages has been carried out for cationic carbosilane dendritic molecules with and without an ibuprofen bonded to them. Macrophages have been treated with cationic dendritic systems (dendrimer or dendron), with combinations of free ibuprofen with cationic carbosilane dendrimers/dendrons, and with ibuprofen-conjugated cationic systems (dendrimer or dendron). The ibuprofen moiety has been bonded through a labile ester bond, using the carboxylic group present in the drug, in order to favor the release of the integral drug in optimum conditions.⁵⁶

MATERIAL AND METHODS

General considerations

All reactions were carried out under inert atmosphere and solvents were purified from appropriate drying agents when necessary. NMR spectra were recorded on a Varian Unity VXR-300 (300.13 (¹H), 75.47 (¹³C) MHz) or on a Bruker AV400 (400.13 (1H), 100.60 (¹³C), 40.56 (¹⁵N), 79.49 (²⁹Si) MHz). Chemical shifts (δ) are given in ppm. ¹H and ¹³C resonances were measured relative to internal deuterated solvent peaks considering TMS = 0 ppm, meanwhile ²⁹Si resonances were measured relative to external MeNO and TMS, respectively. When necessary, assignment of resonances was

done from HSQC, HMBC, COSY and TOCSY NMR experiments. Elemental analyses were performed on a LECO CHNS-932. Mass Spectra were obtained from a Thermo Scientific TSQ Quantum LC-MS and an Agilent 6210. Thiol-ene reactions were carried out employing a HPK 125 W mercury lamp from Heraeus-Noblelight with maximum energy at 365 nm, in normal glassware under an inert atmosphere. Compounds HS(CH₂)₂NH₂·HCl, HS(CH₂)₂NMe₂·HCl, 2,2'-dimethoxy-2-phenylacetophenone (DMPA), MeI, S-(+)-ibuprofen, K₂CO₃, Na₂CO₃, NaOH, porcine liver esterase (PLE, Aldrich), ethylcarbodiimide (EDCI), 4-dimethylaminopyridine (DMAP) were obtained from commercial sources. Compounds G₂O₃V₁₂,⁵⁷ BrG_nV_m,⁵⁸ G₂O₃(NMe₃⁺)₁₂ (**18**)⁵⁷ and HOC₂H₄OG₃(NMe₃⁺)₈ (**19**)⁵¹ were synthesized as previously reported.

HPLC

Release of ibuprofen was monitored at 154 nm (10.39 min) using an Agilent Technologies 1200 series with an Eclipse XDB-C18 5 μM column (Agilent) of 4.6 x 150 mm, with a flux of 1 ml/min and a mixture of acetic acid:acetonitrile (50:50) as eluent. In order to quantify the amount of ibuprofen, a calibration curve was performed between 15 and 275 mg/l of drug (detection limit is 5 mg/ml, quantification limit is 15 mg/ml). Then, PLE (1 unit:1 μmol ester) were added to dendritic aqueous solutions of a known concentration close to 200 mg/l and incubated. Aliquots were taken at several times in order to monitor the release of ibuprofen. The aliquots were measured directly from the reaction systems without quenching. In order to check that complete release was possible, an excess of PLE was added to the incubated mixtures and drug release was HPLC monitored.

Synthesis of compounds

General procedures for the synthesis of compounds are described and a selected number of compounds are collected below. The complete list of compounds is collected in the supplementary information.

Procedure 1: thiol-ene addition

To a dendrimer or dendron solution (THF:MeOH, 1:2) decorated with vinyl groups, 1.05 equivalents of the thiol derivative were added and 2,2'-dimethoxy-2-phenylacetophenone (DMPA)

was used as initiator in a 5 % mol regarding the double bonds. The mixture was deoxygenated and irradiated for 1.5 h ($\lambda_{\text{max}} = 364 \text{ nm}$). After this time, another 5 % mol of DMPA was added and the mixture was again deoxygenated and irradiated for 1.5 h. Afterwards volatiles were removed under vacuum and compounds were purified using nanofiltration (Stirred Ultrafiltration Cells from Millipore) or dialysis in water, using membranes with the adequate molecular weight cut off according to the size of the corresponding dendritic systems (see experimental in supplementary information).

In the case in which two different thiols were employed, the first derivative was added in a stoichiometric amount, according to the number of vinyl groups to be modified, and irradiated for 30 minutes in the same conditions than those described above. Then, the rest of the periphery was functionalized using the procedure above described after addition of the corresponding amount of the second thiol.

Procedure 2: neutralization of $-\text{NMe}_2\cdot\text{HCl}$

To a dendritic solution in a mixture of $\text{H}_2\text{O}/\text{CHCl}_3$ (1:1), an aqueous solution of NaOH or Na_2CO_3 (1.3 equivalents regarding the $-\text{NMe}_2\cdot\text{HCl}$ groups) was added. The reaction mixture was stirred for 15 minutes at room temperature and then, the organic phase was separated and dried with Na_2SO_4 . Finally, volatiles were removed under vacuum to obtain the desired products.

Procedure 3: quaternization of amines with MeI

In a schlenk, the desired amount of $\text{G}_2\text{O}_3(\text{OH})(\text{NMe}_2)_{11}$ or $\text{IbuCO}_2\text{G}_n(\text{NMe}_2)_m$ was solved in a mixture of dry Et_2O and THF (10:1). Afterwards, excess MeI (1.05 equivalents per amine) was added at room temperature. The mixture was stirred at room temperature overnight under inert atmosphere and afterwards volatiles were removed under vacuum and products were washed with hexane.

Procedure 4: Introduction of ibuprofen at the focal point of dendrons

To a DMF solution of BrG_nV_m the stoichiometric amount of S-(+)-ibuprofen was added in presence of two equivalents of K_2CO_3 , 0.1 equivalents of 18-crown-6 and catalytic amounts of NaI . After heating for one night at $80 \text{ }^\circ\text{C}$ in a sealed ampoule, volatiles were removed under vacuum and

Et₂O/NH₄Cl (ac) extraction was performed. The organic phase was dried under MgSO₄ and solvent removed under vacuum to obtain the desired compounds as oils.

G₂O₃(OH)(NMe₂·HCl)₁₁ (2). Procedure 1. Step 1: G₂O₃V₁₂ (0.575 g, 0.46 mmol), 2-mercaptoethanol (0.03 ml, 0.46 mmol) and DMPA (0.012 g, 0.04 mmol); Step 2: 2-(dimethylamino)ethanethiol hydrochloride (0.788 g, 5.28 mmol) and DMPA (0.129 g, 0.50 mmol). Compound **2** was obtained as a white powder (0.127 g, 82 %).

¹H NMR (DMSO-d₆): δ -0.07 (s, 9 H, OC₄H₈SiMe), 0.01 (s, 18 H, SiMeC₂H₄S), 0.58 (m, 30 H, OCH₂CH₂CH₂CH₂SiCH₂ and CH₂SiC₂H₃), 0.85 (m, 24 H, SiCH₂CH₂S), 1.32 (m, 18 H, OCH₂CH₂CH₂CH₂Si and SiCH₂CH₂CH₂Si), 1.67 (m, 6 H, OCH₂CH₂CH₂), 2.57 (m, 24 H, SiCH₂CH₂S), 2.73 (s, 68 H, SCH₂CH₂O and -NMe₂H⁺), 2.87 (m, 22 H, SCH₂CH₂N⁺ and SCH₂CH₂O), 3.19 (m, 20 H, CH₂N⁺), 3.50 (m, 2 H, SCH₂CH₂O), 3.88 (m, 6 H, OCH₂CH₂CH₂), 6.01 (s, 3 H, C₆H₄). ¹³C {¹H} NMR (DMSO-d₆): δ -5.7 and -5.4 (SiMe), 12.7 (OCH₂CH₂CH₂CH₂Si), 13.5 (SiCH₂CH₂SC₂H₄N⁺), 13.8 (SiCH₂CH₂SC₂H₄O), 16.9-17.6 (SiCH₂CH₂CH₂Si), 19.5 (OCH₂CH₂CH₂), 24.3 (SiCH₂CH₂SC₂H₄N⁺), 26.0 (SCH₂CH₂N⁺), 26.4 (SiCH₂CH₂SC₂H₄O), 32.1 (OCH₂CH₂CH₂), 33.2 (SCH₂CH₂O), 41.4 (-NMe₂H⁺), 55.3 (CH₂N⁺), 60.4 (SCH₂CH₂O), 66.6 (OCH₂CH₂CH₂), 93.1 (C₆H₄, C-H), 160.0 (C₆H₄, C-O). Anal. Calc. C₁₁₅H₂₆₄Cl₁₁N₁₁O₄S₁₂Si₉ (2892.93 g/mol): C, 47.75; H, 9.20; N, 5.33; S, 13.30; Obt.: C, 48.21; H, 8.71; N, 5.69; S, 12.01.

G₂O₃(OH)(NMe₂)₁₁ (3). Procedure 2. Data: **2** (0.600 g, 0.21 mmol) and NaOH (0.100 g, 2.50 mmol). Compound **3** was obtained as yellowish oil (0.418 g, 81 %).

¹H NMR (CDCl₃): δ -0.08 (s, 9 H, OC₄H₈SiMe), 0.00 (s, 18 H, SiMeC₂H₄S), 0.54 (m, 30 H, OCH₂CH₂CH₂CH₂SiCH₂ and CH₂SiC₂H₃), 0.87 (t, J_a = 8.7 Hz, 24 H, SiCH₂CH₂S), 1.28 (m, 12 H, SiCH₂CH₂CH₂Si), 1.41 (m, 6 H, OCH₂CH₂CH₂CH₂Si), 1.75 (m, 6 H, OCH₂CH₂CH₂), 2.24 (s, 66 H, -NMe₂), 2.48 (m, 22 H, SCH₂CH₂N), 2.51 (m, 24 H, SiCH₂CH₂S), 2.57 (m, 22 H, SCH₂CH₂N), 2.70 (m, 2 H, SCH₂CH₂O), 3.68 (m, 2 H, SCH₂CH₂O), 3.88 (m, 6 H, OCH₂CH₂CH₂), 6.04 (s, 3 H, C₆H₄). ¹³C {¹H} NMR (CDCl₃): δ -5.2 and -5.1 (SiCH₃), 13.8 (OCH₂CH₂CH₂CH₂Si), 14.7 (SiCH₂CH₂S), 18.3, 18.4 and 18.6 (SiCH₂CH₂CH₂Si), 20.6 (OCH₂CH₂CH₂), 27.7 (SiCH₂CH₂S), 29.7 (SCH₂CH₂N),

33.3 (OCH₂CH₂CH₂), 35.0 (SCH₂CH₂O), 45.3 (-NMe₂), 59.2 (CH₂N⁺), 60.7 (SCH₂CH₂O), 67.7 (OCH₂CH₂CH₂), 93.7 (C₆H₄, C-H), 160.9 (C₆H₄, C-O). Anal. Calc. C₁₁₅H₂₅₃N₁₁O₄S₁₂Si₉ (2491.86 g/mol): C, 55.43; H, 10.23; N, 6.18; S, 11.99. Obt.: C, 55.81; H, 10.23; N, 6.69; S, 11.89.

G₂O₃(OCOibu)(NMe₂)₁₁ (4). A dry DMF solution of **3** (0.114 g, 0.04 mmol), 2 equivalents of S-(+)-ibuprofeno (0.019 g, 0.09 mmol) and 4 equivalents of DMAP (0.047 g, 0.18 mmol) was stirred overnight at room temperature under inert atmosphere in a valved ampoule. Then, 4 equivalents of EDCI (0.035 g, 0.18 mmol) were added and the solution was heated at 60 °C for 2 days. Afterwards, volatiles were removed, THF was added and after filtering, a size exclusion chromatographic column (Bio-Beads SX-1, Bio-Rad) was performed in THF obtaining **4** as yellowish oil (0.115 g, 93 %).

¹H NMR (CDCl₃): δ -0.07 (s, 9 H, OC₄H₈SiMe), -0.01 (s, 18 H, SiMeC₂H₄S), 0.55 (m, 30 H, OCH₂CH₂CH₂CH₂SiCH₂CH₂CH₂Si), 0.86 (m, 30 H, SiCH₂CH₂S and CH(CH₃)₂), 1.26 (m, 21 H, SiCH₂CH₂CH₂Si), 1.42 (m, 6 H, OCH₂CH₂CH₂CH₂Si), 1.45 (d, J = 7.2 Hz, 3 H, CH(CH₃)CO), 1.76 (m, 7 H, OCH₂CH₂CH₂ y CH(CH₃)₂), 2.21 (s, 66 H, -NMe₂), 2.42 (m, 2 H, CH₂Ar), 2.47 (m, 22 H, SCH₂CH₂N), 2.50 (m, 24 H, SiCH₂CH₂S), 2.57 (m, 22 H, SCH₂CH₂N), 2.70 (m, 2 H, SCH₂CH₂O), 3.88 (m, 6 H, OCH₂CH₂CH₂), 4.17 (m, 2 H, CH₂OCO), 6.05 (s, 3 H, C₆H₄), 7.10 and 7.16 (m, 4 H, Ar, C-H). ¹³C{¹H} NMR (CDCl₃): δ -5.3 and -5.1 (SiCH₃), 13.5 (OCH₂CH₂CH₂CH₂Si), 14.6 (SiCH₂CH₂S), 18.3-18.6 (SiCH₂CH₂CH₂Si and (CH₃)CH), 20.5 (OCH₂CH₂CH₂), 22.4 (CH(CH₃)₂), 27.6 (SiCH₂CH₂S), 29.5 (SCH₂CH₂N), 30.1 (CH(CH₃)₂), 33.2 (OCH₂CH₂CH₂), 34.9 (SCH₂CH₂O), 45.0 (CHCO), 45.2 (-NMe₂), 45.4 (CH₂Ar), 59.1 (CH₂N⁺), 63.6 (SCH₂CH₂O), 67.9 (OCH₂CH₂CH₂), 93.6 (C₆H₄, C-H), 127.1 and 129.3 (C₆H₄(ibu), C-H), 137.5 y 140.5 (C₆H₄(ibu), C-H), 160.9 (C₆H₄, C-O), 174.5 (C=O). Anal. Calc. C₁₂₈H₂₆₉N₁₁O₅S₁₂Si₉ (2680.13 g/mol): C, 57.36; H, 10.12; N, 5.75; S, 14.36; Obt.: C, 57.61; H, 9.84; N, 5.29; S, 14.06.

G₂O₃(OCOibu)(NMe₃I)₁₁ (5). Procedure 3. Data: **4** (0.028 g, 0.01 mmol) and MeI (0.01 ml, 0.13 mmol). Compound **5** was obtained as a yellowish powder (0.028 g, 65 %).

¹H NMR (DMSO-d₆): δ -0.06 (s, 9 H, OC₄H₈SiMe), 0.04 (s, 18 H, SiMeC₂H₄S), 0.56 (m, 30 H, OCH₂CH₂CH₂CH₂SiCH₂ and CH₂SiC₂H₃), 0.84 (m, 30 H, SiCH₂CH₂S and CH(CH₃)₂), 1.34 (m, 21

H, SiCH₂CH₂CH₂Si and CHCH₃), 1.43 (m, 6 H, OCH₂CH₂CH₂CH₂Si), 1.75 (m, 7 H, OCH₂CH₂CH₂ and CH(CH₃)₂), 2.63 (m, 24 H, SiCH₂CH₂S), 2.90 (m, 22 H, SCH₂CH₂N⁺), 3.10 (s, 99 H, -NMe₃⁺), 3.53 (m, 22 H, SCH₂CH₂N⁺), 3.86 (m, 6 H, OCH₂CH₂CH₂), 4.13 (m, 2 H, CH₂OCO), 6.01 (s, 3 H, C₆H₄), 7.08 and 7.15 (m, 4 H, Ar, C-H). ¹³C{¹H} NMR (DMSO-d₆): δ -5.7 (SiCH₃), 13.7 (SiCH₂CH₂S), 17.4 (SiCH₂CH₂CH₂Si), 19.6 (OCH₂CH₂CH₂), 21.7 (CH(CH₃)₂), 23.1 (SiCH₂CH₂S), 26.4 (SCH₂CH₂N), 29.1 (OCH₂CH₂CH₂), 43.7 (CH₂Ar), 47.8 (-NMe₃⁺), 64.0 (CH₂N⁺), 126.6 and 128.7 (C₆H₄(Ibu), C-H). Anal. Calc. C₁₃₉H₃₀₂I₁₁N₁₁O₅S₁₂Si₉ (4241.45 g/mol): C, 39.36; H, 7.18; N, 3.63; S, 9.07; Obt.: C, 38.80; H, 7.17; N, 3.16; S, 9.36.

IbuCO₂G₃V₈ (8). Procedure 4. Data: BrG₃V₈ (0.627 g, 0.69 mmol), IbuCO₂H (0.142 g, 0.69 mmol), K₂CO₃ (0.191 g, 1.38 mmol) and 18C₆ (0.017 g, 0.06 mmol). Yellow oil (0.669 g, 94 %).

¹H NMR (CDCl₃): δ -0.12 and -0.11 (s, 9 H, SiMe), 0.11 (s, 12 H, SiMeC₂H₃), 0.42 (t, J_a = 8.4 Hz, 2 H, OCH₂CH₂CH₂CH₂Si), 0.53 (m, 16 H, SiCH₂CH₂CH₂Si, overlapped), 0.68 (t, J_b = 8.1 Hz, CH₂SiC₂H₃), 0.87 (d, J_c = 6.6 Hz, 6 H, (CH₃)₂CH), 1.32 (m, 14 H, OCH₂CH₂CH₂CH₂Si and SiCH₂CH₂CH₂Si), 1.46 (d, J_d = 7.2 Hz, 3 H, CH(CH₃)₃CO), 1.57 (m, 2 H, OCH₂CH₂), 1.80 (m, 1 H, (CH₃)₂CH), 2.42 (d, J_e = 6.9 Hz, 2 H, CH₂Ar), 3.64 (q, J_e = 6.9 Hz, 1 H, CHCO), 4.03 (t, J_f = 6.4 Hz, 2 H, OCH₂), 5.68 and 6.06 (m, 24 H, SiCHCH₂), 7.07 and 7.16 (m, 4 H, Ar, C-H). ¹³C{¹H} NMR (CDCl₃): δ -5.2 and -5.0 (SiCH₃), 13.6 (OCH₂CH₂CH₂CH₂Si), 18.3–18.9 (SiCH₂CH₂CH₂Si and (CH₃)₂CH), 20.2 (OCH₂CH₂CH₂CH₂Si), 22.4 ((CH₃)₂CH), 30.2 ((CH₃)₂CH), 32.4 (OCH₂CH₂CH₂CH₂Si), 45.0 (CHCO), 45.2 (CH₂Ar), 64.5 (OCH₂), 127.1 and 129.2 (Ar, C-H), 132.6 (SiCHCH₂), 137.2 (SiCHCH₂), 137.8 y 140.4 (Ar, C_{ipso}), 174.8 (C=O). ²⁹Si NMR (CDCl₃): δ 1.6 (G₁-Si), 1.0 (G₂-Si), -13.3 (G₃-SiCHCH₂). Anal. Calcd. C₅₈H₁₀₆O₂Si₇ (1032.06 g/mol): C, 67.50; H, 10.35; Exp.: C, 67.12; H, 10.00.

Macrophage differentiation and cell culture

Blood samples were obtained from buffy coats of healthy donors from the Transfusion Center of Madrid respecting national guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated by a standard Ficoll-Hypaque density gradient from buffy coats as already described.⁵⁹ Monocytes were

cultured at 0.5×10^6 cells/mL for 7 days in RPMI medium supplemented with 10% foetal bovine serum (FBS) and 1000 U/mL of recombinant human granulocyte macrophage-colony stimulating factor (rh GM-CSF) (ImmunoTools) or 10ng/mL of recombinant human macrophage-colony stimulating factor (rhM-CSF) (ImmunoTools) to generate M1 and M2 monocyte-derived macrophages respectively. Cytokines were added every 2 days. It was verified that the differentiated macrophages had the proper phenotype (size and morphology) for subsequent experiments (figure S1).

MTT assay

Monocytes were differentiated into M1 or M2 during 7 days as mentioned above. Macrophages were treated with dendrimers or dendrons and mitochondrial metabolism was evaluated by detecting the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) as indicator of toxicity. Macrophages (M1 and M2) were plated (7.5×10^4 macrophages per condition, distributed in 3 wells in a 96 well plate), and treated with cationic carbosilane dendrimers or dendrons at different concentrations (5-20 μ M) for 24 hours. MTT was added at 0.5mg/mL (in Dulbecco's Modified Eagle's Medium) and incubated during four hours at 37°C. The plate was centrifuged, the supernatant removed and the reaction was stopped by adding DMSO (50 μ L per well). The plate was shaken for 5 minutes and analyzed in a plate reader at 570nm and the reference used was absorbance at 690nm. Viability was calculated by normalizing data to untreated cells, and referred to 100%. Conditions which their 2xSEM (confidence interval of 95%) do not include values equal or greater than 80% were rolled out.

CASY® Cell Counter

Viability was evaluated using the CASY® Cell Counter. Monocytes were differentiated into M1 or M2 during 7 days as mentioned above. The 7th day 3×10^5 macrophages per condition (M1 or M2) were counted and turn them to the plate to adhere over night. Cationic carbosilane dendrimers or dendrons were added in the culture plate at 3 μ M or 5 μ M. After 24 hours cells were washed with PBS, trypsinized, and resuspended in PBS. Samples were analyzed with the cell counter. Counts were

corrected by the dilution factor and cells per mL were calculated. Viability was calculated by normalizing data to untreated cells, and referred to 100%. Conditions that their 2xSEM (confidence interval of 95) does not include values equal or greater than 80% were discarded.

Quantitative real-time PCR

Three combinations of different conditions were tested. The first condition was: M1 and M2 were treated with LPS (10ng/ml), ibuprofen, $G_2O_3(NMe_3^+)_{12}$ (**18**), $G_2O_3(OCOibu)(NMe_3^+)_{11}$ (**5**), $HOC_2H_4OG_3(NMe_3^+)_8$ (**19**), or $IbuCO_2G_3(NMe_3^+)_8$ (**17**) at 5 μ M for 24 hours. The second condition was: M1 and M2 were treated with ibuprofen or compounds at 5 μ M for 30 minutes and then treated with LPS (10ng/ml) for 24 hours. The third condition was: M1 and M2 were treated with LPS at 10ng/ml for 30 minutes and then were treated with ibuprofen or compounds for 24 hours. Subsequently, RNA of all samples was extracted by using the RNeasy Plus mini kit (Qiagen). RNA integrity was analyzed with Agilent 2100 bioanalyzer (Agilent Technologies) by using RNA Nano chips (Agilent Technologies). A reverse transcriptase was used to generate complementary DNA (cDNA) from RNA templates (reverse transcription) with the GoScript Reverse Transcription System (Promega). mRNA expression was analyzed by qRT-PCR using specific probe and primers for *COX-2* (designed by using the Universal Human Probe Roche library system, Roche Diagnostics). Results were processed with the iQ5 2.0 software (Bio-Rad). Data was normalized according to the expression levels of a housekeeping gene (TATA-binding protein, TBP), and expressed relative to the mRNA of untreated samples (relative mRNA).

Cytokines release

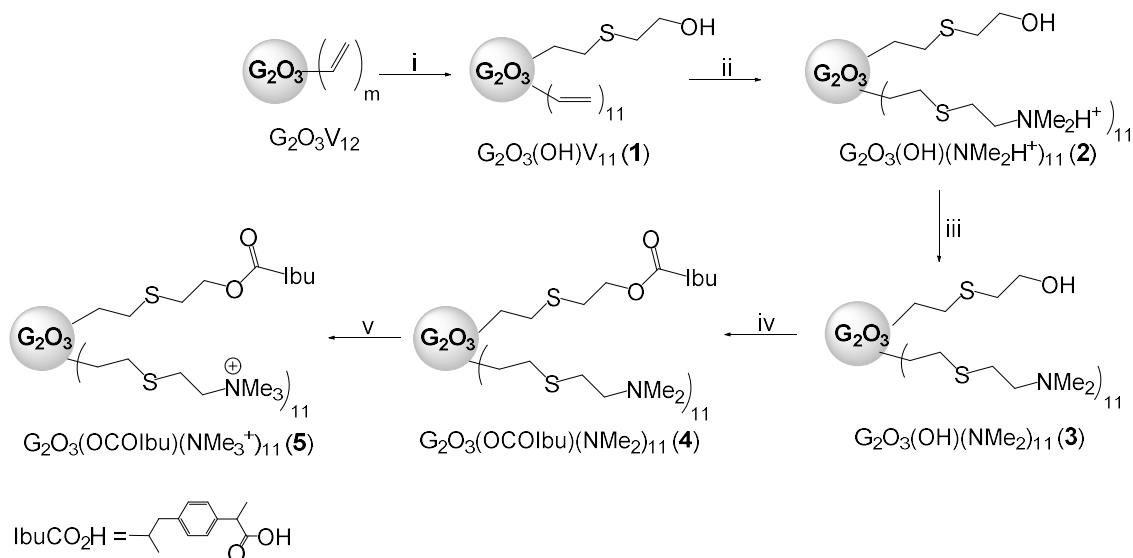
Macrophages (M1 and M2) were treated with LPS (Sigma-Aldrich) at 10ng/ml for 24 hours, or pre-treated with ibuprofen or compounds at 5 μ M for 30 minutes and after treated with LPS for 24 hours; or pre-treated with LPS for 30 minutes and after treated with ibuprofen or compounds for 24 hours. Then, supernatants were collected and amount of cytokines and chemokines were determined (TNF α , IL-8, IL-6, CCL3 and IL-1 β in M1 cultures; and TNF α , IL-8, CCL3, IL-10, CXCL9 and CCL2 in M2 cultures) using the Flow Cytomix system (eBioscience) following the instructions given

by manufacturer. Gallios Beckmann Coulter cytometer was used and data was analyzed by FlowCytomixPro software, version 3.0.

RESULTS AND DISCUSSION

As commented above, we report the synthesis of ibuprofen conjugated cationic carbosilane dendritic molecules through a labile ester bond. The ammonium groups decorating dendritic molecules are of the type $-NMe_3^+$, since carbosilane dendrimers and dendrons with more acidic $-NR_2H^+$ functions become insoluble if the proton is released from their structure, due to the highly hydrophobic framework of these macromolecules. To simplify the nomenclature of dendritic structures they will be named as $G_nO_3Y_m$ for dendrimers and XG_nY_m for dendrons, where G_n stands for the dendritic generation and Y_m for the peripheral functions and its number; O_3 denotes the polyphenoxo moiety used as a core in dendrimers, and X refers to the group placed in the focal point of dendrons.

For the synthesis of a cationic dendrimer with ibuprofen bonded through an ester bond (scheme 1) we required the presence of a hydroxyl function on the periphery for further attached of ibuprofen via Steglich esterification, and amine groups, precursors of cationic ammonium groups. With this in mind, starting from the vinyl dendrimers $G_2O_3V_{12}$, two consecutive thiol-ene addition reactions were carried out, following the synthetic procedure previously described for homofunctionalized dendrimers.⁵⁷ First, the treatment with one eq. of 2-mercaptoethanol and subsequently with excess 2-(dimethylamino)ethanethiol hydrochloride, led to dendrimer $G_2O_3(OH)(NMe_2H^+)_{11}$ (**2**), which after base treatment afforded neutral derivative $G_2O_3(OH)(NMe_2)_{11}$ (**3**). Once obtained dendrimer **3**, addition of ibuprofen was performed in dry DMF using DMAP (4-dimethylaminopyridine) as a base and EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) as a coupling agent, leading to ibuprofen-conjugated dendrimer $G_2O_3(OCOibu)(NMe_2)_{11}$ (**4**), which after reaction with MeI allowed us to obtain the target product $G_2O_3(OCOibu)(NMe_3^+)_{11}$ (**5**) as a water soluble solid in high yields (figure 1). These compounds were characterized by NMR spectroscopy and elemental analyses.



Scheme 1. Synthesis of the ibuprofen conjugated cationic dendrimer $G_2O_3(OCOibu)(NMe_3^+)_{11}$ (5). i) $HS(CH_2)_2OH$, $h\nu$, DMPA; ii) $HS(CH_2)_2NMe_2HCl$, $h\nu$, DMPA; iii) NaOH; iv) S-(+)-ibuprofen, DMAP, EDCI; v) MeI.

Functionalization with first thiol compound to prepare $G_2O_3(OH)V_{11}$ (1) was followed by NMR, which showed the appearance of two signals at around δ 0.9 and 2.5, in 1H -NMR, and 15 and 27, in ^{13}C -NMR, corresponding to the new formed chain SCH_2CH_2Si . The signals due to 2-mercaptoethanol could be observed at c.a. δ 2.7, 2.8 and 3.7, in the 1H -NMR spectrum, and at c.a. δ 35 and 60, in the ^{13}C -NMR spectrum. Also, the 1H -NMR spectrum corroborated the presence of this moiety in the established proportion by comparison of the new signals and the ones corresponding to the non-reacted vinyl groups. The spectroscopic data of compounds 2 and 3, showed the expected signals due to the introduction of the second thiol derivative.⁵⁷ For the heterofunctionalized ibuprofen-dendrimers $G_2O_3(OCOibu)(NMe_2)_{11}$ (4) and $G_2O_3(OCOibu)(NMe_3^+)_{11}$ (5), confirmation of ester bond formation was done by the resonances corresponding to the methylene group directly attached to ibuprofen at c.a. δ 4.1 (from δ 3.7 in compound 3) and 64 in 1H - and ^{13}C -NMR spectra, respectively. Also, the resonances due to the presence of the ibuprofen fragment were observed (figure S3). Finally, a DOSY NMR experiment showed a unique diffusion coefficient for compounds 4 and 5. The same experiment for a mixture of the corresponding homofunctionalized dendrimer and ibuprofen showed one diffusion coefficient for each compound (figure S3).

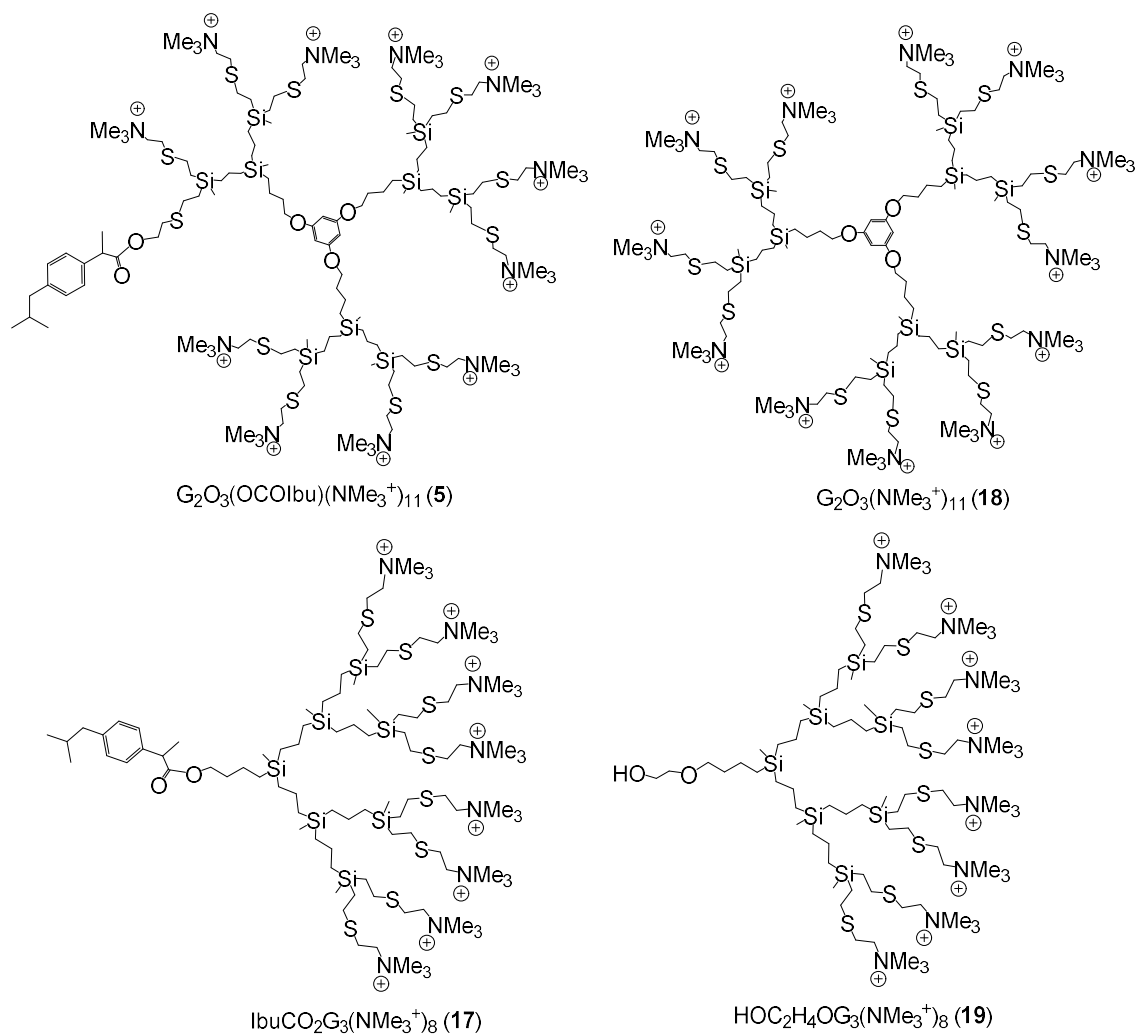


Figure 1. Drawing of selected cationic ibuprofen-conjugated and non-ibuprofen dendritic molecules. Iodide anions are omitted for clarity.

Regarding dendrons, introduction of the ibuprofen moiety at the focal point was afforded by nucleophilic substitution of the bromide atom in BrG_nV_m ($n = 1, m = 2$; $n = 2, m = 4$; $n = 3, m = 8$)⁵⁸ by the carboxylic moiety in ibuprofen (scheme S1), avoiding the use of coupling agents. The new dendrons $\text{IbuCO}_2\text{G}_n\text{V}_m$ ($n = 1, m = 2$ (**6**); $n = 2, m = 4$ (**7**); $n = 3, m = 8$ (**8**)) thus formed were easily purified by simple water/ Et_2O extraction. **The presence of this ibuprofen moiety in dendrons 6-8 was confirmed by NMR spectroscopy. The original resonances of the methylene BrCH_2 disappear (δ 3.40 in the ^1H -NMR spectra) and new resonances belonging to the methylene CO_2CH_2 at the focal point bound to ibuprofen were observed at *c.a.* δ 4.0 and *c.a.* δ 64 in ^1H - and ^{13}C -NMR spectra, respectively.** In these spectra also the appearance of the signals due to the ibuprofen fragment could be

observed. Next, following an identical protocol than for dendrimers (see above), the cationic dendrons $\text{IbuCO}_2\text{G}_n(\text{NMe}_3^+)_m$ (**15-17**, scheme S1, figure 1, figure S2) were obtained.

These compounds were characterized by NMR spectroscopy, mass spectrometry (MS) and elemental analyses. Their NMR characteristics were also analogous to those of dendrimer but for the presence of the focal chain, which resonances were as those described in dendrons **6-8**. That is, the presence of the ibuprofen bound through an ester bond is confirmed by the resonances belonging to the methylene group CO_2CH_2 directly attached to this group at *c.a.* δ 4.1 and *c.a.* δ 64 in the ^1H - and ^{13}C -NMR spectra, respectively. Moreover, in the MS were detected peaks corresponding to the whole cationic dendritic structure containing ibuprofen. On the other hand, HPLC did not show the presence of free ibuprofen in cationic dendrons **15-17**.

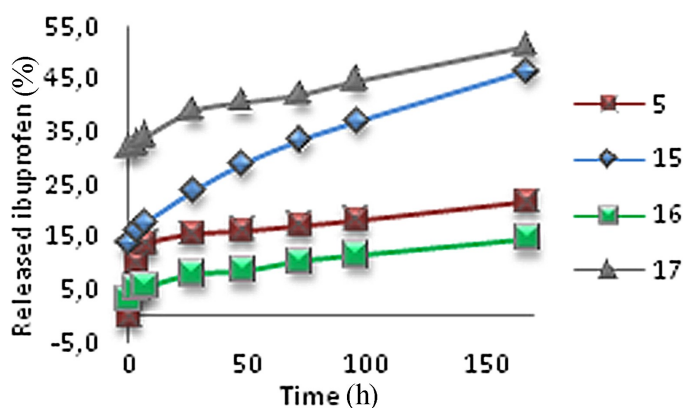


Figure 2. Release of ibuprofen (% HPLC) after addition of 1 unit of porcine liver esterase (PLE) per ester bond at different times (hours, h) for dendrimer $\text{G}_2\text{O}_3(\text{OCOibu})(\text{NMe}_3^+)_{11}$ (**5**) and dendrons $\text{IbuCO}_2\text{G}_n(\text{NMe}_3^+)_m$ ($n = 1, m = 2$ (**15**); $n = 2, m = 4$ (**16**); $n = 3, m = 8$ (**17**)).

The purpose of introducing an ibuprofen moiety in cationic derivatives **5** and **15-17** is obtaining a combined action between the cationic dendritic framework and the anti-inflammatory drug. Hence, the release of ibuprofen in an adequate environment should be produced. Although the synthesized compounds are stable in aqueous media, even at slightly acid pH, it must be considered that in the organism there are hydrolytic enzymes able to break the ester bonds. For this reason, dendrimer $\text{G}_2\text{O}_3(\text{OCOibu})(\text{NMe}_3^+)_{11}$ (**5**) and dendrons $\text{IbuCO}_2\text{G}_n(\text{NMe}_3^+)_m$ (**15-17**) were incubated in the

presence of porcine liver esterase (PLE, 1 μmol ester: 1 unit) and the release of ibuprofen was monitored at several times using HPLC. After addition of esterase, a certain amount of ibuprofen, dependent on the compound, was released (figure 2). Although it turned out impossible to establish a relationship between the structure and the amount of drug released, it must be highlighted that it might be related with the conformation of these compounds in aqueous solutions. **These dendritic systems contain hydrophobic, ibuprofen ligand and carbosilane framework, and hydrophilic, ammonium groups, moieties and therefore, the exposure of the hydrophobic parts in aqueous solution tend to be minimized. For this reason, the accessibility of the ester bond can randomly vary for these compounds.** Addition of extra portions of esterase reactivated the released process.

Next step was to evaluate the effect of cationic systems $\text{G}_2\text{O}_3(\text{NMe}_3^+)_{12}$ (**18**), $\text{G}_2\text{O}_3(\text{OCOibu})(\text{NMe}_3^+)_{11}$ (**5**), $\text{HOC}_2\text{H}_4\text{OG}_3(\text{NMe}_3^+)_{8}$ (**19**), or $\text{IbuCO}_2\text{G}_3(\text{NMe}_3^+)_{8}$ (**17**), bound or not with ibuprofen, on the inflammatory response mediated by macrophages. The selection of the second generation dendrimers and a third generation dendrons for these assays was done on the basis of previous studies on cationic carbosilane dendrimers for gene therapy, which showed that the best relationship between activity and toxicity were for these systems than for lower or higher generation derivatives.^{32, 47}

The mRNA of the inducible cyclooxygenase (COX-2) expression was quantified on M1 and M2 macrophages treated with different molecules at 5 μM as non-toxic doses (establish by an MTT assay and confirmed with the electronic cell sizer CASY® Cell Counter, supplementary figure S5). Separately, macrophages were treated with bacterial lipopolysaccharide (LPS), a complex glycolipid present on the outer membrane of Gram-negative bacteria, which is one of the most potent microbial initiators of inflammation.⁶⁰ For those properties, LPS was used as an inflammatory activator stimulus responsible for the production of prostaglandins and inflammatory processes. Results show that none of the cationic dendritic molecules evaluated induced significantly COX-2 mRNA expression (figure 3).

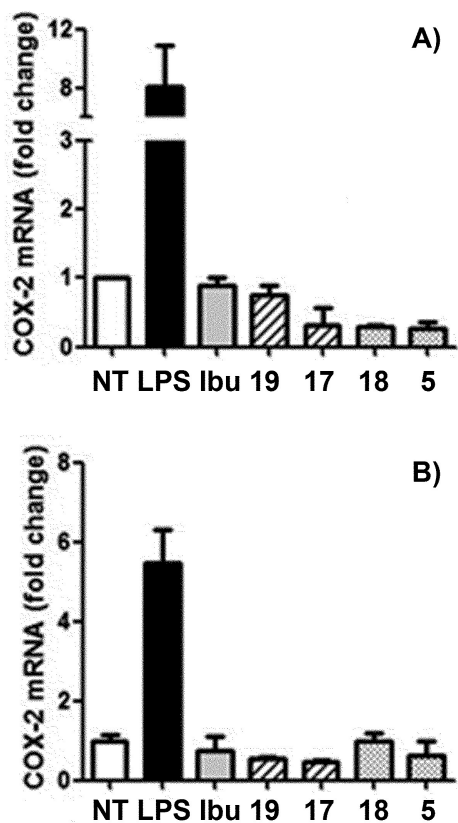


Figure 3. Quantification of gene expression on treated macrophages by Q-PCR. *COX-2* mRNA expression was determined by Q-PCR in M1 (A) and M2 (B) untreated or treated with either lipopolysaccharide (LPS), ibuprofen (Ibu), $G_2O_3(NMe_3^+)_{12}$ (18), $G_2O_3(OCOibu)(NMe_3^+)_{11}$ (5), $HOC_2H_4OG_3(NMe_3^+)_8$ (19), or $IbuCO_2G_3(NMe_3^+)_8$ (17) for 24 hours. Data shows relative mRNA levels; analyzed gene was normalized to TATA box binding protein expression (TBP) and referred to non-treated cells (NT). Mean and SD of triplicates are shown. [One-sample t test, confidence intervals 95% of the mean \(versus NT\).](#) * $p \leq 0.05$.

A lipopolysaccharide (LPS) was used as an activator stimulus over M1 and M2 cells (figure 4). Macrophages were treated with dendritic derivatives previously (pre-treatment; figure 4A and 4B) or subsequently (post-treatment; figure 4C and 4D) at LPS stimulation. Also, to evaluate the effect of ibuprofen released by dendrimer 5 and dendron 17, macrophages were treated with with [free ibuprofen](#) and corresponding dendrimer 18 and dendron 19, simulating the *in vitro* release of the drug. Results show that cells treated with ibuprofen, before or after the LPS stimulus, decrease the LPS-induced expression of *COX-2* mRNA, both on M1 (figure 4A and 4C) and on M2 (figure 4B and 4D), as

expected. For pre-treated M1 cultures, all tested treatments reduced the LPS-induced expression of *COX-2* in a similar way than ibuprofen did (figure 4A). However, post-treated M1 cells with cationic systems show improved reductions on *COX-2* expression compared with ibuprofen (figure 4C). Regarding M2-treated cells, and despite slight differences between pre-treatment and post-treatment, both cationic dendrons **17** and **19** and cationic dendrimer without ibuprofen **18** reduce the LPS-induced *COX-2* expression (figure 4B and 4D). On the contrary, ibuprofen-conjugated dendrimer **5** do not decrease the induction on *COX-2* expression induced by LPS over M2 (figure 4B and 4D) despite it showed great effect over M1 cells (figure 4A and 4C). Our data validate compounds $G_2O_3(NMe_3^+)_{12}$ (**18**), $G_2O_3(OCOibu)(NMe_3^+)_{11}$ (**5**), $HOC_2H_4OG_3(NMe_3^+)_{8}$ (**19**) and $IbuCO_2G_3(NMe_3^+)_{8}$ (**17**) as non-inflammatory molecules, and at least three of them, **17**, **19** and **18**, reduces the LPS-induced *COX-2* expression in both polarization states of macrophages. These are encouraging results since *COX-2* inhibition decreases the synthesis of prostaglandins, molecules involved in mediating inflammation.⁶¹

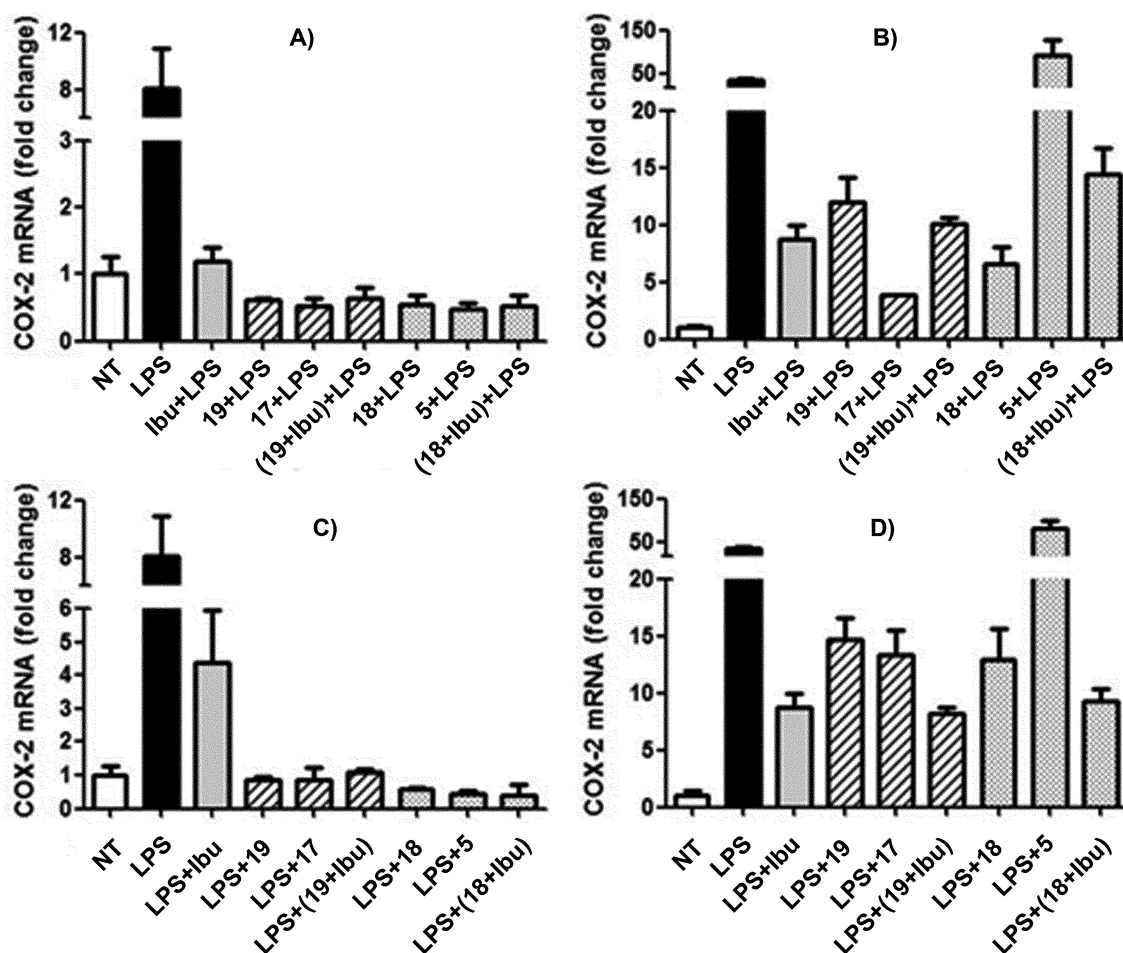


Figure 4. Quantification of gene expression on treated macrophages by Q-PCR. *COX-2* mRNA expression was determined by Q-PCR in M1 (A, C) and M2 (B, D). Results on non-treated cells (NT), stimulated with lipopolysaccharide (LPS) or treated with $G_2O_3(OCOibu)(NMe_3^+)_{11}$ (**5**) or $IbuCO_2G_3(NMe_3^+)_8$ (**17**) previously (A, B) or subsequently (C, D) at stimulating with LPS are shown. Separately, macrophages were treated with free ibuprofen and $G_2O_3(NMe_3^+)_{12}$ (**18**) or $HOC_2H_4OG_3(NMe_3^+)_8$ (**19**) previously (A, B) or subsequently (C, D) at stimulating with LPS. Data shows relative mRNA levels; analyzed gene was normalized to TATA box binding protein expression (TBP) and referred to non-treated cells (NT). Mean and SD of triplicates are shown. One-sample t test, confidence intervals 95% of the mean (versus LPS). * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Concerning inflammatory response, when macrophages are confronted with pathogens, as in case of a bacterial infection, they secrete cytokines and chemokines; these are soluble peptides that play an important role on the development and maintenance of inflammatory process. These molecules have an effect over many signal transduction pathways that regulate cellular differentiation, and further, they are responsible of the recruitment of neutrophils, macrophages, among other cells to specific inflammatory sites. Regarding the study of molecules involved in inflammatory processes, it is important to evaluate the molecule-induction of the inflammatory response triggered by M1 but also to evaluate whether compounds allow the normal resolution of this status. Once the inflammatory response eliminates the pathogen, the anti-inflammatory macrophages (M2) play an important role down-regulating the inflammatory mediators to re-establish a homeostatic environment; this is the resolution phase of an inflammatory response. Therefore, depending on the cellular polarization state, macrophages present different cytokines and chemokines profiles.^{62, 63} It is well known that several cytokines are involved in particle-induced inflammation,⁶⁴ hence, the evaluation of cationic dendritic systems with and without bound ibuprofen over cytokines release by LPS-stimulated M1 macrophages is interesting. Also, the effect of dendrons and dendrimers over M2 cells should be

evaluated to determine whether these treatments could improve the effect of ibuprofen, either downloading proinflammatory or raising anti-inflammatory cytokines.

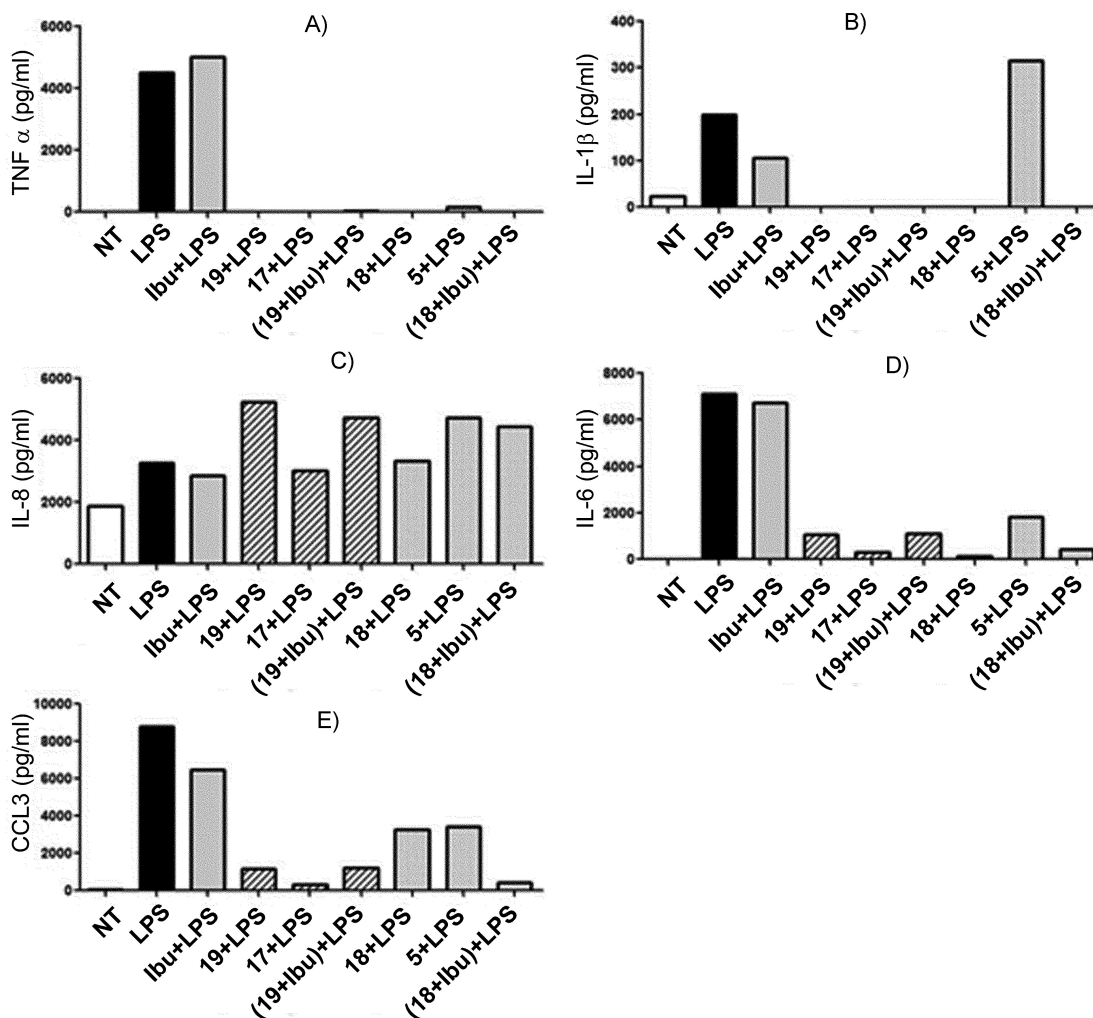


Figure 5. Cytokines release by M1 macrophages. Quantification of TNF α (A), IL-1 β (B), IL-8 (C), IL-6 (D) and CCL3 (E) release in M1 cultures supernatants. Shown non-treated cells (NT), stimulated with LPS (LPS), and pre-treated with ibuprofen (Ibu+LPS), HOC₂H₄OG₃(NMe₃⁺)₈ (19+LPS), IbuCO₂G₃(NMe₃⁺)₈ (17+LPS), G₂O₃(NMe₃⁺)₁₂ (18+LPS) or G₂O₃(OCOibu)(NMe₃⁺)₁₁ (5+LPS) for 30 min previously at stimulating with LPS for 24 h. Also shown macrophages treated with HOC₂H₄OG₃(NMe₃⁺)₈ ((19+Ibu)+LPS) or G₂O₃(NMe₃⁺)₁₂ ((18+Ibu)+LPS) and **free ibuprofen** for 30 min previously at stimulating with LPS for 24 h. Individual values from one donor are shown.

For these assays, macrophages were treated with ibuprofen-conjugated dendrimer (5) and dendron (17), both previously (pre-treatment; figure 5 and figure S6) or subsequently (post-treatment; figure 6

and [figure S7](#)) at LPS stimulation. Macrophages were also treated with ibuprofen in the presence of cationic dendrimer **18** and dendron **19**. As expected and in line with previous results,⁶³ LPS activates M1 macrophages to produce pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α)([figure 5A and 6A](#)), IL-1 β ([figure 5B and 6B](#)), also slightly increases the production of IL-8 ([figure 5C and 6C](#)) and in a higher way affects the IL-6 ([figure 5D and 6D](#)) and CCL3 ([figure 5E and 6E](#)) release.⁶² On the other hand, LPS activates M2 macrophages to produce anti-inflammatory cytokines such as IL-10 ([figure S6 and S7](#)), confirming that these cells are prepared to respond to stimuli releasing anti-inflammatory cytokines.^{42, 65} Ibuprofen-treated macrophages do not modify some cytokines release, since not all of the evaluated cytokines are related with the ibuprofen pathway ([figure S6 and S7](#)). Concerning proinflammatory cytokines, dendrons(**19** and **17**) or dendrimers (**18** and **5**) treatment blocks the TNF- α release by M1 cells ([figure 5A and 6A](#)), showing an important advantage compared with ibuprofen treatment, since it does not affect the TNF- α production. TNF- α promotes COX-2 induction and prostaglandin E₂ (PGE₂) production,⁶⁶ which is the enzymatic product of COX-2.⁶⁷ Given that compounds **19**, **17** and **18** blocks the expression of COX-2 induced by LPS, would be interesting to study whether they have a direct effect on this molecule or whether they are acting upstream in the cell signaling pathway. In this regard, COX-2 mRNA could be induced by IL-1 β and IL-8, and interestingly,⁶⁸⁻⁷² dendrons (**19** and **17**) and dendrimer **18** treatment reduces the IL-1 β production by M1 macrophages, both pretreatment ([figure 5B](#)) and post-treatment ([figure 6B](#)), whilst combination of ibuprofen-conjugated cationic dendrimer **5** with LPS increases the IL-1 β release compared with LPS activation ([figure 5B and 6B](#)). It has been reported that compounds affecting TNF- α and COX-2 expression, also affect the IL-6 and IL-8 expression,^{70, 73} therefore, the evaluation of these cytokines is interesting. The data show that **17** and **18** pre-treatment were not affecting the IL-8 release compared with LPS treatment ([figure 5C and S6C](#)), suggesting an ibuprofen-like profile over M1 and M2 cells, while **19** pre-treatment slightly increase the IL-8 release by M1 regardless it was combine or not with free ibuprofen ([figure 5C](#)). Moreover, dendrons (**19**, **17**) or dendrimers (**18**, **5**) treatments decrease the IL-6 ([figure 5D and 6D](#)) which is usually produced by

viral infection, and also reduce CCL3 release by proinflammatory macrophages (figure 5E and 6E), despite they were activated with LPS (previously or subsequently, figure 5 and 6, respectively). Ibuprofen-conjugated dendrimer $\mathbf{5}$ was the only one of all tested derivatives that increased TNF α (figure S6A and S7A) and CCL3 (figure S6E and S7E) secretion by M2 when combined with LPS, indicating that M2-treated cells acquire inflammatory properties. Given these results together with the induction of *COX-2* expression (figure 4B) and changes in the cytokine profile of the M1 (figure 5 and 6), dendrimer $\mathbf{5}$ was discard as anti-inflammatory treatment, despite induce a higher secretion of IL-10 than LPS (figure S6B and S7B).

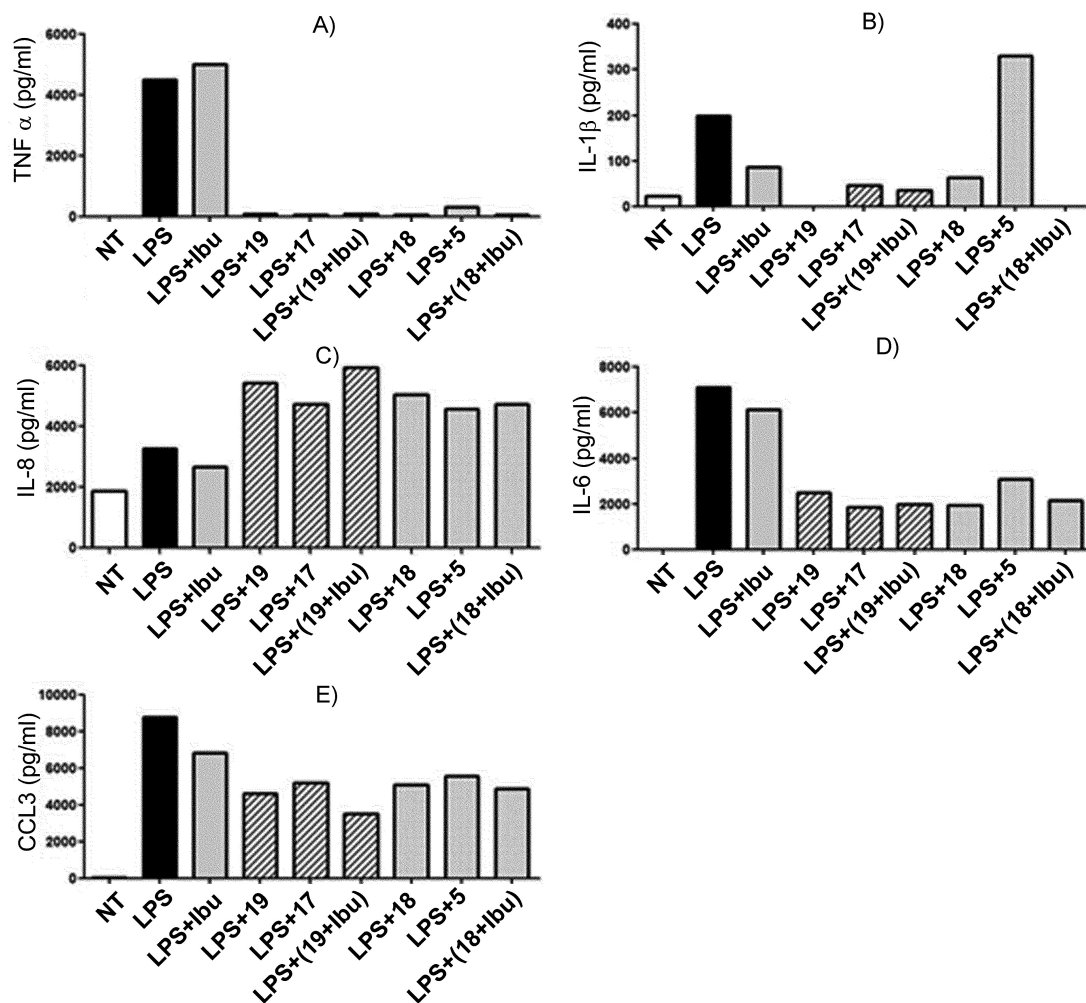


Figure 6. Cytokines release by M1 macrophages. Quantification of TNF α (A), IL-1 β (B), IL-8 (C), IL-6 (D) and CCL3 (E) release in M1 cultures supernatants. Shown non-treated cells (NT), stimulated with LPS (LPS), and treated with LPS for 30 minutes and then with ibuprofen (LPS+Ibu), HOC $_2$ H $_4$ OG $_3$ (NMe $_3^+$) $_8$ (LPS+19), IbuCO $_2$ G $_3$ (NMe $_3^+$) $_8$ (LPS+17), G $_2$ O $_3$ (NMe $_3^+$) $_12$ (LPS+18) or

$G_2O_3(OCOibu)(NMe_3^+)_{11}$ (LPS+**5**) for 24 hours. Also shown macrophages treated with LPS for 30 minutes and after treated with **free ibuprofen** and $HOC_2H_4OG_3(NMe_3^+)_{8}$ ((LPS+(**19**+Ibu)) or $G_2O_3(NMe_3^+)_{12}$ ((LPS+(**18**+Ibu)) for 24 hours. Individual values from one donor are shown.

Summing up, cationic ibuprofen-conjugated dendron **17** presents the best results as anti-inflammatory agent. It reduces the LPS-induced *COX-2* expression and shows an improved effect compared with **free ibuprofen** used as control, since it decreases the inflammatory cytokines in a higher way that ibuprofen do it. Dendron **17** decreases TNF α release (figure 5A, 6A, **S6A** and **S7A**), cytokine involved in systemic inflammation that activates neutrophils, IL-1 β (figure 5B and 6B), IL-6 (figure 5D and 6D) and CCL3 (figure 5E, 6E, **S6E** and **S7E**), that is a cytokine involved in the recruitment and activation of leukocytes in the acute inflammatory state. Further and interestingly, post-treatment with dendron **17** decreases the expression of CCL2 by M2. CCL2 regulates the migration and infiltration of monocytes, memory T lymphocytes, and natural killer (NK) cells at inflammatory sites. {Deshmane, 2009 #525} Besides, dendron **17** do not increase the expression of IL-8 (figure 5C, 6C, **S6C** and **S7C**) nor CXCL9 (figure **S7C** and **S7D**) by M2, indicating that do not induce recruitment of leukocytes to infection sites. These results are very encouraging because they define the dendron $IbuCO_2G_3(NMe_3^+)_{8}$ (**17**) as a drug for a new anti-inflammatory therapy.

CONCLUSIONS

Cationic carbosilane dendrimers and dendrons with ibuprofen bound through an ester bond can be obtained by esterification or nucleophilic substitution, respectively, and further thiol-ene addition. This ester bond can be used to release the antiinflammatory moiety, as has been demonstrated by treating these compounds with esterase (PLE).

The inflammatory response of ibuprofen-conjugated dendritic molecules was evaluated on macrophages (M1 and M2) and the results were compared with those obtained for corresponding non-ibuprofen counterparts, both in the presence or absence of free ibuprofen. These assays highlight that

ibuprofen-conjugated dendron $\text{IbuCO}_2\text{G}_3(\text{NMe}_3^+)_8$ (**17**) reduces inflammatory responses in a better way than ibuprofen does. Taking into account the potential biomedical applications of this type of compounds, for example as microbicides or gene carriers, and that the triggering of inflammatory processes is a handicap for their applications, these results suggest that introduction of ibuprofen to cationic carbosilane dendrimers and dendrons would diminish problems associated to their use.

ACKNOWLEDGMENTS

This work has been supported by grants from CTQ-2014-54004-P (MINECO) and Consortium NANODENDMED ref S2011/BMD-2351 (CAM) to University of Alcalá (UAH). This work has been cofinanced by Instituto de Salud Carlos III (Subdirección General de Evaluación) and Fondo Europeo de Desarrollo Regional (FEDER), RETIC PT13/0010/0028, Fondo de Investigación Sanitaria (FIS) (grant number PI13/02016 and PI12/0014), Comunidad de Madrid (grant numbers S-2010/BMD-2332) to HGUGM. This work has been (partially) funded by the RD12/0017/0037, as part of Acción Estratégica en Salud, Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica 2008-2011 (HGUGM). CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008–2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. E. F.-P. acknowledges MINECO for a fellowship. This work was supported partially by a Marie Curie International Research Staff Exchange Scheme Fellowship within the 7th European Community Framework Program, project No. PIRSES-GA-2012-316730 NANOGENE, co-financed by the Polish Ministry of Science and Higher Education (grant No. W21/7PR/ 2013) and CYTED 214RT0482 to UAH and HGUGM. We want to particularly acknowledge to the Spanish HIV HGM BioBank that is supported by the Spanish Instituto de Salud Carlos III and is integrated in the Spanish AIDS Research Network. The authors thank Dr. Laura Diaz, of the Flow Cytometry Unit (IiSGM, Madrid, Spain) and Dr. M^a Isabel Clemente, of Culture Unit (IiSGM, Madrid, Spain) for their technical assistance.

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For Graphical Abstract

**IMPROVED EFFICIENCY OF IBUPROFEN BY CATIONIC CARBOSILANE
DENDRITIC CONJUGATES**

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Ibuprofen-conjugated and non-ibuprofen cationic carbosilane dendritic molecules show important antiinflammatory properties on macrophages.

