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Curing inflammatory diseases using phosphorous dendrimers

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**Authors:**

**Anne-Marie Caminade**

a) Laboratoire de Chimie de Coordination (LCC), CNRS UPR8241, 205 route de Narbonne, 31077 Toulouse cedex 4, France. Email address: anne-marie.caminade@lcc-toulouse.fr. **ORCID ID:** 0000-0001-8487-3578

b) LCC-CNRS, Université de Toulouse, CNRS, Toulouse, France

c) IMD-Pharma, 205 route de Narbonne, 31077 Toulouse Cedex 4, France. E-mail address: turrin@imd-pharma.com

**Cédric-Olivier Turrin**

a) Laboratoire de Chimie de Coordination (LCC), CNRS UPR8241, 205 route de Narbonne, 31077 Toulouse cedex 4, France. Email address: cedric-olivier.turrin@lcc-toulouse.fr. **ORCID ID:** 0000-0001-7187-8070

b) LCC-CNRS, Université de Toulouse, CNRS, Toulouse, France

c) IMD-Pharma, 205 route de Narbonne, 31077 Toulouse Cedex 4, France. E-mail address: turrin@imd-pharma.com

**Rémy Poupot**

a) Institut Toulousain des Maladies Infectieuses et Inflammatoires, CHU Purpan – BP 3028 – 31024 Toulouse Cedex 3, France. Email address: remy.poupot@inserm.fr. **ORCID ID:** 0000-0003-1396-8531

b) Infinity, Université Toulouse, CNRS, INSERM, UPS, Toulouse, France.
Conflict of Interest

C.-O.T. and R.P. are co-founders and shareholders of IMD-Pharma. C.-O.T. is CEO of IMD-Pharma. A.-M.C. is a shareholder of IMD-Pharma.

Abstract

Different types of water-soluble phosphorous dendrimers have been synthesized and display many different biological properties. It has been shown in particular that phosphorous dendrimers of first generation functionalized with azabisphosphonate terminal functions are able to stimulate the human immune system ex vivo. These dendrimers are internalized by monocytes within a few seconds, and induce their anti-inflammatory activation. The presence of the dendrimers induces also the inhibition of the differentiation of monocytes into osteoclasts, the maturation of dendritic cells, and inhibits the proliferation of the pro-inflammatory CD4⁺ T lymphocytes. Finally, after 2-3 weeks of culture of Peripheral Blood Mononuclear Cells (PBMCs), amplifications by several tens of Natural Killer cells is observed. In view of all these properties, the influence of these dendrimers has been tested in vivo in animal models, against several inflammatory diseases, such as multiple sclerosis, rheumatoid arthritis, Uveitis, and psoriasis, but also against myeloid leukaemia, a haematological cancer. The safety of a phosphorous dendrimer functionalized with azabisphosphonate terminal functions has been assessed, in particular with non-human primates as a de-risking pre-clinical test. Other phosphorous dendrimers display anti-inflammatory properties in vivo, in particular dendrimers functionalized with mannose derivatives, which prevent acute lung diseases when given orally (per os) to mice.

Graphical/Visual Abstract and Caption
Phosphorous dendrimers specifically functionalized on their surface positively influence the human immune system ex vivo, and decrease the severity of several diseases, in particular inflammatory diseases, in vivo.

1. INTRODUCTION

Inflammation is a generic but complex biological response of body tissues to harmful stimuli, involving both innate and adaptive immunity (Medzhitov, 2008). The onset of inflammatory response is followed by the onset of a pro-resolutive response that brings the immune system back to its natural balance (Headland & Norling, 2015). Two main types of inflammations are known. Acute inflammation, which occurs immediately upon injury, is due to trauma, microbial invasion (Lowy, 1998), or noxious compounds, and lasts only a few days. Chronic inflammation, which lasts for months or years, can be due to failure of eliminating the agent causing an acute inflammation, recurrent episodes of acute inflammation, or to an autoimmune disorder, which prevents the onset of the pro-resolutive response. Inflammation is associated to numerous conditions, such as cancers (Mantovani, Allavena, Sica, & Balkwill, 2008), diabetes (Wellen & Hotamisligil, 2005), atherosclerosis (Libby, 2002), rheumatoid arthritis (Firestein, 2003), multiple sclerosis (Compston & Coles, 2008), Alzheimer’s disease (Akiyama et al., 2000), and diverse brain diseases (Dantzer, O’Connor, Freund, Johnson, & Kelley, 2008).

Dendrimers are hyperbranched and polyfunctional tree-like polymers, having precisely defined structure and molecular weight (Buhleier, Wehner, & Vögtle, 1978; Tomalia et al., 1985). Indeed, in contrast with polymers, dendrimers are synthesized by iterative step-by-step methods, and not by polymerization reactions. A new “generation” is obtained each time the number of terminal functions is increased. The multiple functions on their surface can be easily modified to fulfil the desired properties. For instance, dendrimers can be used as catalysts, for the elaboration or for the modification of materials, and as tools for diverse biological uses (Caminade, Turrin, Laurent, Ouali, & Delavaux-Nicot, 2011). Multivalency is in particular the driving force for the use of dendrimers in nanomedicine (Rolland, Turrin, Caminade, & Majoral, 2009). Among different biological properties, the role played by some dendrimers as anti-inflammatory agents has to be emphasized (Hayder, Fruchon, Fournie, Poupot, & Poupot, 2011; Kannan, Nance, Kannan, & Tomalia, 2014; Fruchon & Poupot, 2017).
The first use of dendrimers as anti-inflammatory agents concerned a polyamidoamine (PAMAM) dendrimer of generation 4, in which a few carboxylic acid terminal functions were modified with D(+) -glucosamine (Figure 1). This dendrimer displays anti-inflammatory properties, which were measured by the release of proinflammatory chemokines and cytokines in different human immune cells stimulated with lipopolysaccharide (LPS) (Shaunak et al., 2004). Later on, anti-inflammatory properties of unmodified or fully modified PAMAM dendrimers of the same generation, ended with different types of simple functions (e.g. -NH₂, -OH, -CO₂H, etc.) have been reported. The inhibition of the inducible COX-2 enzyme, which is an accurate target of anti-inflammatory drugs, was screened in vitro using the different dendrimers. Tests were also carried out with three different rat models of acute, subacute, and chronic inflammation. PAMAM dendrimers functionalized with amine or hydroxyl surface groups displayed the highest inhibitory activity of COX-2, whereas TRIS, pyrrolidone, and PEG, displayed weak activity, and no activity for carboxylate terminal functions (Chauhan, Diwan, Jain, & Tomalia, 2009).

![Figure 1. PAMAM dendrimers tested for their anti-inflammatory properties.](image)

The covalent grafting of non-steroidal anti-inflammatory drugs (NSAIDs) to the surface of dendrimers has been carried out in several cases. The earliest reports concerned PAMAM dendrimers conjugated
with 5-aminosalicyclic acid, using different spacers containing an azo-bond, suitable for the triggered release by enzyme azoreductase (Wiwattanapatapee, Lomlim, & Saramunee, 2003), naproxen (Najlah, Freeman, Attwood, & D’Emanuele, 2006), or ibuprofen (Kolhe et al., 2006). Two recent paper reported the synthesis of arylether organoiron dendrimers bearing either ibuprofen (Abd-El-Aziz, Abdelghani, et al., 2021) or aspirin (Abd-El-Aziz, Benaisha, et al., 2021) as terminal functions, from the first to the fourth generation. Several examples of dendrimers functionalized with corticoids such as methylprednisolone (Inapagolla et al., 2010), triamcinolone acetonide (Kambhampati et al., 2015; Cho et al., 2021), dexamethasone (Soiberman et al., 2017), have been also reported. Even if several anti-inflammatory dendrimers are based on PAMAM dendrimers, the most important series of dendrimers having anti-inflammatory properties per se concerns polyphosphorhydrazone (PPH) dendrimers, as will be shown in this Advanced Review.

2. SYNTHESIS OF ANTI-INFLAMMATORY POLYPHOSPHORHYDRAZONE DENDRIMERS

2.1 Synthesis of polyphosphorhydrazone dendrimers

The synthesis of polyphosphorhydrazone (PPH) dendrimers consists in the repetition of a two-step process to build one generation. It was first carried out from a trifunctional core (S=PCl₃) up to the fourth generation (Launay, Caminade, Lahana, & Majoral, 1994), then up to the seventh generation (Launay, Caminade, & Majoral, 1995), and finally up to the twelfth generation (Lartigue et al., 1997), which has been for two decades the largest dendrimer ever synthesized. However, in most cases the synthesis is carried out from an hexafunctional core (₃N₃P₃Cl₆) (Slany et al., 1995), as shown in Scheme 1. The synthesis was carried out only up to the eight generation from that core, but it is presumably not the highest generation attainable (Launay, Caminade, & Majoral, 1997). The synthesis necessitates two reagents: 4-hydroxybenzaldehyde, and the phosphorhydrazone H₂NNMeP(S)Cl₂, synthesized by reaction of P(S)Cl₃ with methylhydrazine at about -60°C. Such reagents afford either aldehyde or P(S)Cl₂ terminal functions. Both functions display a large palette of reactivity, which has been used in particular for the synthesis of dendrimers having anti-inflammatory properties. Scheme 1 shows two ways to draw the dendrimers structure, either the full structure or the linear structure, with parentheses after each level of branching points.
2.2 Synthesis of anti-inflammatory phosphorous dendrimers

The first series of phosphorous dendrimers having anti-inflammatory properties was synthesized first to protect metal surfaces against corrosion, and thus was functionalized with phosphonate terminal functions, as phosphonates are known corrosion inhibitors (Touir et al., 2010). However, phosphonates, in particular bisphosphonates have many biological properties, and are widely used as drugs (Lin, 1996). That is why the biological properties of dendrimers bearing phosphonate terminal functions have been assessed. Figure 2 displays dendrimers of first generation bearing as terminal functions diverse types of phosphonate and bisphosphonate sodium salts that have been first synthesized (Poupot et al., 2006). One of them (1a-G1) displayed the most important biological properties, and it is generally coined as ABP (for azabisphosphonate). The name ABP will be used all along this review. Neutral dendrimers bearing the azabisphosphonate functions were also synthesized, up to generation 4 (1aMe-G4) (Posadas et al., 2017). In view of all the biological properties of this first family of dendrimers, a structure / activity relationship was carried out, and numerous other dendrimers having potential anti-inflammatory properties have been synthesized.
In order to determine if the presence of phosphonate groups is mandatory, or if negatively charged dendrimers could be also efficient, first generation dendrimers ended with monocarboxylate groups (2a-G₁, Soler-Illia et al., 2000; Blanzat et al., 2005) or azabiscarboxylate groups (2b-G₁), or azabissulfonate groups (2c-G₁) (Rolland et al., 2009) were also synthesized (Figure 3).
First generation phosphorhydrazone dendrimers functionalized with carboxylate, azabiscarboxylate, and azabissulfonate sodium salts as terminal functions.

Fluorescent analogues of some of the dendrimers shown in Figures 2 and 3 have been synthesized, to help deciphering their biological mechanism. In a first attempt, fluorescein isocyanate was grafted stochastically on ca one terminal function per dendrimer (Poupot et al., 2006). However, it is more desirable to have dendritic compounds with precisely defined structures, thus it appeared more judicious to graft the fluorophore to the core, at the very beginning of the synthesis, taking advantage of the relatively easy synthesis of AB₅ derivatives of the cyclotriphosphazene (N₃P₃) (Zibarov, Oukhrib, Aujard Catot, Turrin, & Caminade, 2021). As shown in Figure 4, three different fluorophores were linked to the core. The first one was a maleimide derivative (3a-G₁), which emits at ca 530 nm in water (Poupot et al., 2006). The second fluorophore was a julolidine derivative (3b-G₁), which emits at 507 nm (Rolland et al., 2008). The third fluorophore has a sophisticated structure (3c-G₁), which induced an emission in the near infra-red (NIR), between 650 and 800 nm (Jebbawi et al., 2020). The julolidine derivative was also grafted to the core of dendrimers having either carboxylate (4a-G₁) (Ielasi et al., 2016) or azabiscarboxylate (4b-G₁) (Ledall et al., 2015) terminal functions (Figure 4).
Figure 4. First generation phosphorous dendrimers bearing a fluorophore at the core.
The grafting of one fluorophore at the core induces the decrease of the number of terminal functions, from 12 for the plain dendrimer (ABP), to 10 for the fluorescent dendrimers shown in Figure 4. In order to detect the importance of the number of terminal functions for the anti-inflammatory properties, the number of inactive (blocked) functions at the core was gradually increased (Figure 5). The reagents used were 4-hydroxymethylbenzoate for odd numbers of blocked functions, and 2,2'-dihydroxybiphenyl for even numbers of blocked functions. In this way, dendrimers bearing 10 (5a-G₁), 8 (5b-G₁), 6 (5c-G₁), 4 (5d-G₁), and 2 (5e-G₁) azabisphosphonate terminal functions were synthesized (Rolland et al., 2008).

**Figure 5.** Different types of first generation dendrimers, having a decreasing (10, 8, 6, 4, 2) or increasing (16, 30) number of terminal functions, compared to ABP (12).
Besides decreasing the number of terminal functions, attempts were made for increasing their number, while keeping almost the same size of the dendrimers. For this purpose, the cyclotetraphosphazene (N₄P₄) was used as core instead of the cyclotriphosphazene (N₃P₃), affording dendrimer 5f-G₁ having 16 azabisphosphonate terminal functions, instead of 12. Another attempt was made using N₃P₃ as branching point instead of P(S). Such method allows to multiply by five the number of terminal functions at a given generation, instead of by 2 for the classical method of synthesis (Maraval, Caminade, Majoral, & Blais, 2003). Dendrimer 5g-G₁ was obtained in this way. It possesses 30 azabisphosphonate terminal functions (Figure 5) (Rolland et al., 2008).

After changing the type and the number of terminal functions, the next step was the modification of the internal structure (Caminade et al., 2015) (Figure 6). Before our work, there was no other example in the literature of a large screening to determine the role of the scaffold of dendrimers on a given property. Indeed, it was believed that the internal structure was only an “innocent” part of the dendrimer. A first modification of the internal structure consisted in lengthening the branches; compound 6-G₁ has an O-C₆H₄ additional linker, compared to ABP. Another type of phosphorous dendrimer was based on a thiophosphate internal structure (Salamonczyk, Kuznikowski, & Skowronska, 2000). The synthesis was carried out up to the second generation 7-G₂ to get 12 azabisphosphonate terminal functions. The carbosilane dendrimer (de Groot, Reek, Kamer, & van Leeuwen, 2002) of first generation 8-G₁ was functionalized with 8 azabisphosphonate terminal functions.

These 3 dendrimers having main group elements as branching entities were functionalized with exactly the same type of terminal functions as for ABP, i.e. on tyramine as support of the bisphosphonate functions. However, the main types of commercially available dendrimers, based on PPI (polypropylene imine) (De Brabander-van den Berg & Meijer, 1993), PAMAM (Tomalia et al., 1985), and poly-L-lysine (Denkewalter, Kolc, & Lukasavage, 1981) branches have NH₂ terminal functions, which cannot be reacted with tyramine. Thus, another method of coupling was used, based on a peptide coupling between the NH₂ terminal functions and carboxylic acid derivatives linked to an azabisphosphonate function through either one or three CH₂. In order to be able to make a suitable comparison between all dendrimers, such method was first applied to the phosphorhydrazone dendrimers, affording compounds 9a-G₁ and 9b-G₁ with CH₃, and CH₂CH₂CH₂ linkers, respectively
The same process was used with the second generations of PPI dendrimers, affording 10b-G2, with PAMAM dendrimers, affording 11b-G2, and with lysine dendrimers, affording 12a-G2, all of them bearing 8 azabisphosphonate terminal functions (Caminade et al., 2015).

Other families of phosphorous dendrimers have displayed anti-inflammatory properties against lung diseases. The first family (13-Gn, Figure 7) was capped with different mannose derivatives. The series with a mono-mannose derivative at each end was synthesized from generation 1 to 3 (13-G1-M to 13-G3-M); the series with a di-mannose derivative at each end was synthesized from generation 1 to 4 (13-G1-D to 13-G4-D); and with a tri-mannose derivative at each end only the third generation was synthesized (13-G3-T) (Blattes et al., 2013). A second family of dendrimers was functionalized with ammoniums as terminal functions, for comparison purposes (14-G2, Figure 7), but it has no anti-inflammatory properties (Loup, Zanta, Caminade, Majoral, & Meunier, 1999).
3. BIOLOGICAL PROPERTIES OF THE PPH DENDRIMER ABP AND ITS DERIVATIVES

3.1 Influence of the PPH dendrimers on the human peripheral blood mononuclear cells (PBMCs)

3.1.1 The case of monocytes

Each dendrimer of the family shown in Figure 2 (1a-G1 (ABP) to 1j-G1) was added to ex vivo cultures of human peripheral blood mononuclear cells (PBMCs), which are white blood cells, issued from healthy donors. These cultures were supplemented with interleukin-2 (IL-2). The very first property discovered for this family of dendrimers concerned the activation of monocytes, which are a key cell population of the innate immunity. Studies by confocal video-microscopy indicated that a dendrimer issued from ABP, tagged with fluorescein, bind to isolated monocytes and was internalized within a few seconds. Monocytes in culture with dendrimer ABP displayed morphological changes within 3 to 6 days of culture, and remained viable over longer periods than untreated monocytes. Monocytes activated by dendrimers underwent phenotypic changes, as analysed by flow cytometry (Poupot et al., 2006). The classical activation pathway of monocytes progresses towards an inflammatory immune response, whereas an alternative pathway progresses towards an anti-inflammatory response. In this first study, it was not possible to choose between both pathways.

Figure 7. Other series of potential anti-inflammatory dendrimers.
Later on, the gene expression profile of human monocytes activated by the dendrimer ABP was analysed by comparison with untreated monocytes. Analysis demonstrated that several genes, coding for proteins characterizing an alternative activation of monocytes, were found to be up-regulated, whereas several other genes, coding for proteins related to the pro-inflammatory activation of monocytes, were found to be down-regulated. Such data indicated an alternative-like, anti-inflammatory activation of the human monocytes. This type of activation was confirmed by analysis of the surface expression of specific markers, using flow cytometry, and by quantitative RT-PCR (Fruchon et al., 2009). Further experiments were carried out on monocytes pre-incubated or not with dendrimer ABP for 1 hour, then incubated with the pro-inflammatory LPS for 38 hours. Despite this context of pro-inflammatory stimulus, a significant decrease in the production of pro-inflammatory cytokines was observed for monocytes treated with dendrimer ABP. Analogous observations were done for experiments carried out with monocyte-derived dendritic cells (Degboe et al., 2014).

Moreover, in the latter experiments, it has also been shown that the ABP dendrimer inhibits the maturation of these dendritic cells.

Experiments were then carried out to determine if the interaction of the dendrimer ABP with monocytes occurs specifically or not, both with model membranes of multi-lamellar vesicles, and with the membrane of monocytes. It was shown that the ABP dendrimer binds both specifically and non-specifically to the membrane of human monocytes, and that the specific interactions induce the internalization of the dendrimer. In contrast, dendrimer 2b-G₁ (a biologically inactive analogue of ABP, with azabiscarboxylate terminal functions) only interacts non-specifically with the membrane of monocytes, thus, it is not internalized. The fluorescent analogues of dendrimers ABP (3b-G₁) and 2b-G₁ (4b-G₁) were particularly useful tools to demonstrate such behaviour (Ledall et al., 2015). Other physicochemical studies were carried out for determining the critical aggregation concentration (CAC) of these dendrimers. The CAC values measured for dendrimer ABP, either in water or in physiological PBS solution, are close to 37 mM. This value is 2000 folds the concentration at which the dendrimer is able to activate monocytes ex vivo (20 μM). Finally, the dose dependant activation of human monocytes was unambiguously observed with dendrimer ABP and its fluorescent analogue 3b-G₁ (Figure 8A) (Ielasi et al., 2016).

The differentiation of monocytes, or bone marrow precursors of monocytes into osteoclasts, which are giant multinucleated cells responsible for bone resorption, can be induced in cultures with M-CSF and
the RANK ligand. As monocytes are the main targets of dendrimer ABP, it was tested in this differentiation. The ex vivo differentiation of monocytes into osteoclasts in cultures of synovial tissue, which were obtained from arthroplastic surgery of rheumatoid arthritis patients, was significantly inhibited by dendrimer ABP at 20 μM. Experiments carried out with arthritic mouse bone marrow precursors of monocytes also demonstrated a statistically significant decrease of osteoclasts, even at a very low concentration of the dendrimer ABP (0.02 μM) (Figure 8B) (Hayder et al., 2011).

Figure 8. A) Dose-dependent activation of human monocytes with dendrimer ABP and its fluorescent analogue 3b-G1. B) Number of osteoclasts in cultures of arthritic mouse bone marrow precursors of monocytes, cultured with M-CSF and RANKL in the presence of different concentrations of dendrimer ABP.

The activation of monocytes was found suitable for assessing the bioactivity of the dendrimers. Thus, it was chosen as test for screening the large library of dendrimers synthesized. The family of dendrimers 5-G1 (5a-G1 to 5g-G1) allowed to determine the optimum number of terminal functions, within a given size range. It was shown that the most efficient immune-stimulating properties of these dendrimers towards monocytes are obtained for dendrimers bearing between 12 and 8 azabisphosphonate terminal functions, that is to say dendrimers of type ABP, 5a-G1, and 5b-G1 (Rolland et al., 2008). Dendrimers bearing the non-symmetrical azabisphosphonate terminal functions (family 1g-G1-1j-G1), were also tested. They were shown able to activate monocytes, but the dendrimer with the longest hydrophobic chain (1j-G1) displayed a cytotoxic effect in long term cultures, not observed with the other dendrimers (Marchand et al., 2009).

The large family of dendrimers having different internal structures, shown in Figure 6, have been tested also for their effect on the anti-inflammatory activation of monocytes. The first screening
demonstrated that only the dendrimers having either phosphorous or silicon at the branching points displayed an activity (dendrimers 6-G1, 7-G1, 8-G1, 9a-G1 and 9b-G1), whereas all the dendrimers having nitrogen atoms as branching points, such as PPI (10b-G2), PAMAM (11b-G2), and P-Lysine (12a-G2) displayed no activity. There was no obvious explanation to this difference. Indeed, dendrimers 10b-G2, 11b-G2, and 12a-G2 have only 8 azabisphosphonate terminal functions, but it is also the case of dendrimer 8-G1, which is active. Besides, the type of terminal functions in dendrimers 10b-G2, 11b-G2, and 12a-G2 is different from that of most of the active dendrimers, but it is the same than for dendrimers 9a-G1 and 9b-G1, which are both active. The explanation to this puzzling biological behaviour came from all-atom molecular dynamics simulations, which demonstrated that the active dendrimers were directional, with all the terminal functions gathered on a part (half) of the surface, whereas the terminal functions of the non-active dendrimers are distributed over their entire surface (Figure 9) (Caminade et al., 2015).

![Figure 9. Molecular dynamics equilibrated snapshots of 9 dendrimers, displaying their shape, and comparison of their efficiency for the activation of monocytes.](image)

3.1.2 The case of Natural Killer (NK) cells

The second biological property was obtained for longer times of ex vivo cultures of PBMCs with the **ABP** dendrimer. A large increase in the number of PBMCs was observed in two-week-old cultures. Phenotyping of the cells multiplied with dendrimer **ABP** indicated a large increase of the number of...
NK cells (with some T cells). Natural Killer (NK) cells are an important population of the innate immunity, which is implicated in early immune responses against infections (viral, bacterial, and parasitic), and above all, they have a central role in anticancer immunity (Blattman, & Greenberg, 2004; Bald, Pedde, Corvino, & Bottcher, 2020). A mean multiplication by 105 of the number of NK cells was obtained after four weeks in culture. Multiplications over 500-fold were even observed with PBMCs from some donors. The proliferation of NK cells known in the literature was extremely tedious to achieve, in particular to produce large batches of NK cells compliant with NK cell-based therapies against cancer. Therefore, it was important to check whether the NK cells generated in cultures with ABP were fully functional, and mature. Thus, the efficiency of NK cells generated with ABP was compared with that of uncultured NK cells. A large panel of 15 cancer cell lines was tested, including seven different leukaemia, and eight different carcinoma cell lines. Both cultured and uncultured NK cells displayed the same killing efficiency. Furthermore, no aggressiveness of the NK cells obtained with the dendrimer ABP was observed against lymphocytes issued from the same donor. A first screening of the efficiency of the dendrimers indicated that the first generation was the most efficient in the series 1a-Gn (n = 0, 1 and 2). Dendrimer 1a-G1 (ABP) was also found by far most efficient than dendrimers 1c-G1 and 1d-G1. Furthermore, dendrimer 2a-G1, bearing carboxylate terminal functions, had practically no activity (Figure 10). Such result demonstrates that the negative charges alone are not sufficient, and that bisphosphonate terminal functions are needed also for the amplification of NK cells, as for the activation of monocytes (Griffe et al., 2007).

Figure 10. Number of NK cells after three weeks of cultures of PBMCs coming from a single healthy donor, with either IL-2 alone, or IL-2 and 20 µm of dendrimers (see Figures 2 and 3 for their structure).
In a more recent experiment, 14 dendrimers, mainly shown in Figure 5, were tested to activate human PBMCs from healthy donors, then the specific proliferation of NK cells has been assessed only for the most active dendrimers. The results shown in Figure 11 indicate that dendrimer ABP is still the most active, even if compounds in which one or two functions at the core were blocked (dendrimers 3a-G1 and 5b-G1, respectively) are also very active. The amplification of NK cells after two weeks cultures was measured for these three dendrimers. It was shown again that dendrimer ABP provided the highest percentage and number of NK cells, and that the proliferation occurred during the second week of culture. In order to determine if there is a relation (or not) between the anti-inflammatory activation of monocytes and the amplification of NK cells, monocytes were removed from PBMCs, to obtain peripheral blood lymphocytes (PBLs, containing essentially NK cells, and B and T lymphocytes). Cultures of PBLs in the presence of the ABP dendrimer show that NK cells did not proliferate in these conditions. Addition of monocytes to PBL cultures in the presence of ABP restored the amplification of NK cells, depending on the dose used. In a next experiment, NK cells and monocytes were co-cultured in membrane-separated chambers to prevent direct contact between the cells, but in the same well. No proliferation of the NK cells was observed in this case. Different other experiments to explore the role of the ABP dendrimer in the complex interactions between NK cells and activated monocytes were carried out. Surprisingly, monocytes activated with dendrimer ABP have an inhibitory effect on the proliferation of NK cells. It was also shown that the NK cells interact much more strongly with monocytes activated with dendrimer ABP than with non-activated monocytes. However, these activated monocytes are finally killed very efficiently by NK cells. Such process is necessary to enable the amplification of NK cells (Poupot et al., 2016).

![Figure 11. Amplification of NK cells in ex vivo cultures of human PBMCs in the presence of various dendrimers.](image-url)


3.1.3 Other types of immune cells

The third cellular subpopulation of PBMCs that interacts with the dendrimers are the CD4+ T lymphocytes. These cells are key players of adaptive immunity to propagate the inflammatory response initiated by monocytes/macrophages. Indeed, they can differentiate in pathogenic Th1 or Th17 CD4+ T lymphocytes upon stimulation and activation by pro-inflammatory cytokines. It was shown that dendrimer ABP inhibits the activation, and thus the proliferation of CD4+ T cells in IL-2 stimulated PBMCs, but does not affect their viability. Such finding offered an additional explanation for the amplification of NK cells, as the quantity of IL-2, which is not used by the CD4+ T cells, can be used by NK cells to increase their development (Portevin et al., 2009). However, some CD4+ T lymphocytes, namely IL-10 producing ones, are involved in the resolution of the inflammatory response. Interestingly, it has been shown that anti-inflammatory monocytes activated by the dendrimer ABP trigger the proliferation of such regulatory CD4+ T lymphocytes in vitro in human Mixed Leukocyte Reactions (Fruchon et al., 2009).

3.2 Influence of the dendrimers on inflammatory diseases in vivo

3.2.1 Systemic inflammatory diseases

In view of the wide anti-inflammatory properties displayed ex vivo by the dendrimers functionalized with azabisphosphonate terminal functions, their influence on several models of inflammatory diseases was tested in vivo. The first example concerns rheumatoid arthritis, an autoimmune inflammatory disorder, which affects ca 1% of the population in the developed countries. This disease induces joint deformation and handicap, as consequences of chronic inflammation of the synovial membrane, together with cartilage degradation, followed by subsequent bone erosion induced by osteoclasts (Klareskog, Catrina, & Paget, 2009). Despite the existence of some drugs, and the advent of biological drugs such as monoclonal antibodies, soluble receptors, and more recently kinase inhibitors, 20 to 40% of the patients are in therapeutic failure (Rubbert-Roth & Finckh, 2009; Arora et al., 2013). Thus, there is an unmet clinical need (Favalli et al., 2017) for innovative molecules able to reduce inflammation response and favour the onset of pro-resolutive response. Three dendrimers have been tested in vivo on IL-1ra−/− mice, as model of rheumatoid arthritis. Dendrimers ABP, 1f-G1, and 10b-G2 were injected intravenously to mice, one time per week, starting at the age of 8 weeks.
Measurement of the arthritic score and of hind paw swelling showed that only dendrimer ABP induced a significant decrease for both criteria at the age of 15 weeks (Figure 12A). Dendrimer ABP completely inhibited inflammation and arthritis, and reduced the level of inflammatory cytokines at doses of 1 and 10 mg/kg. Furthermore, histology of the liver, spleen, kidneys, lungs, and aorta, from mice treated with dendrimer ABP at the 10 mg/kg dose for 12 weeks, indicated that this treatment did not induce any off-target effects. The same treatment was applied to another well-known mouse model, i.e. the K/BxN serum transfer model, induced into wild-type BALB/cA mice. The disease has to be induced in the case of these BALB/cA mice, contrarily to IL-1ra−/− mice, in which it occurs spontaneously. Both paw swelling, due to inflammation, and arthritis were strongly suppressed by the dendrimer in this case also (Hayder et al., 2011). It was shown later on that the dendrimer ABP could be given orally (per os) at the same posology (Hayder et al., 2012).

The dendrimers shown in Figure 5 (Rolland et al., 2008) have been also tested in vivo on nine-week old male BALB/cA mice, which received K/BxN serum. Preliminary tests carried out on the activation of monocytes have demonstrated that the best results were obtained with the dendrimers bearing between 12, 10 (fluorescent analogue) and 8 azabisphosphonate terminal functions (Rolland et al., 2008). The same conclusions were made when considering the expansion of NK cells (Figure 11, Poupot et al., 2016). However, when considering the arthritic score in the K/BxN mouse model, only dendrimers ABP and 5f-G1 (12, and 10 azabisphosphonate terminal functions, respectively) were found efficient in vivo (Figure 12B). All-atom MD simulations in explicit solvent of all these dendrimers have shown that the denser dendrimers, which are non-active, have all their phosphonate functions spread in patches all over the surface, as were the non-active PPI, PAMAM, and P-Lysine dendrimers (Figure 9, Caminade et al. 2015). All the other dendrimers, ABP and its lower analogues are directional, but the number of terminal functions is presumably not sufficient, as only ABP and 3b-G1 (only one function at the core blocked) can efficiently control the arthritic disease (Hayder et al., 2018).
Multiple sclerosis is a chronic inflammatory disease of the central nervous system (CNS). It is believed to be induced by an inflammatory attack due to autoreactive T cells recognizing CNS self-antigens. Such attack triggers additional recruitment of immune cells from peripheral blood, including monocytes (Steinman, 2014). Dendrimer ABP was tested in the classical mouse model of MOG35−55, in which experimental autoimmune encephalomyelitis (EAE) was induced, as a model of multiple sclerosis. In the most relevant protocol, mice were treated from day 18 after EAE induction, and either every 3 days or weekly with dendrimer ABP (10 mg/kg) or vehicle (no dendrimer). The ABP dendrimer inhibited significantly the progression of established disease. The improvement was more significant when mice were treated every 3 days, rather than weekly. Moreover, none of the 32 mice treated every 3 days died, while 15 out of the 34 control (untreated) mice died. The ABP dendrimer was found as efficient as Fingolimod, an approved treatment, administered daily per os (orally). The efficiency of the tested ABP dendrimer unambiguously related to the triggering of the pro-resolutive response by increasing the production of IL-10 (Hayder et al., 2015).

3.2.2 Haematological cancer

Besides the anti-inflammatory properties of dendrimer ABP, the multiplication of NK cells induced by dendrimer ABP enabled testing these amplified NK cells in vivo, in a mouse model of cancer.
xenograft. Immuno-deficient mice received independently but at the same time the K562 myeloid leukaemia cell line (subcutaneously) and the dendrimer-expanded human NK cells (intravenously). Control mice received PBS instead of NK cells. Weighting the solid tumours at necropsy of animals showed a significant decrease in mice which received the NK cells expanded by the dendrimer ABP. One third of the mice treated with the NK cells did not even develop any tumour. Besides, it has long been known that multiple myeloma (MM) cancer cells can be killed by NK cells (Carbone et al., 2005; Godfrey & Benson, 2012). Thus, it was important to know if NK cells from PBMC of patients with multiple myeloma could be expanded upon ex vivo cultures with the ABP dendrimer. On average, most PBMCs of multiple myeloma patients responded to the ABP dendrimer. NK cell amplifications of 11-fold with the dendrimer versus 4-fold without it, were observed in small size prototype cultures. On average, PBMCs from patients before any chemotherapy gave better NK cell multiplications (13-fold) than those which were obtained under chemotherapy (4-fold). In the same conditions, multiplications of 16-fold versus 3-fold, respectively, were obtained with PBMCs from healthy donors. The important conclusion is that the ABP dendrimer enables the ex vivo amplification of NK cells from multiple myeloma patients, before any chemotherapy, as a paradigm of haematological cancer patients, as efficiently as for healthy donors (Poupot et al., 2016).

3.2.3 Loco-regional inflammatory diseases
The same dendrimer ABP was then tested in the treatment of acute Uveitis induced by LPS (lipopolysaccharide) in a robust rat model, considered as clinically relevant for human anterior Uveitis. Such experiment was chosen for expanding the position of dendrimer ABP, and to accelerate the bench to market process. Indeed, this disease necessitates a loco-regional administration (intravitreous) rather than a systemic administration. The ocular tolerance of dendrimer ABP was measured for seven consecutive days, after a single intra-vitreous injection in both eyes of rats. No adverse effect on vision or eyesight was detected in any of the animals treated with ABP. The effect of the ABP dendrimer in this model of acute inflammatory disease was evaluated in comparison with dexamethasone, considered as the "gold standard". It was shown that dendrimer ABP used at 2 μg/eye is as efficient as the "gold standard" dexamethasone used at 20 μg/eye (Fruchon et al., 2013). An example of topical administration of dendrimer ABP concerns the potential treatment of psoriasis. This is "a chronic, non-communicable, painful, disfiguring, and disabling disease for which there is no
Psoriasis is characterized by a general inflammatory background in the skin. The treatment in most cases concerns a local administration of non-steroidal anti-inflammatory drugs (NSAID), or of steroidal cortisone-like drugs (Boehncke & Schoen, 2015). The efficiency of dendrimer ABP and its fluorescent analogue 3c-G₁ has been tested in a preclinical mouse model (male Balb/c mice, at the age of 9 to 10 weeks) of psoriasis induced by imiquimod. The near infra-red fluorescent analogue 3c-G₁ was chosen to visualize the penetration in the diseased skin. The use of a topical application enabled to control both the clinical and histopathological scores, and also to control the infiltration of macrophages in the skin of mice treated with the dendrimer at a dose of 5 mg/kg (Figure 13) (Jebbawi et al, 2020).

![Figure 13](image)

**Figure 13.** A) left part: skin of the back of a control psoriatic mouse; right part: histological picture of the skin of control psoriatic mouse stained with hematoxylin/eosin. B) left part: skin of the back of a mouse treated daily with 5 mg/kg of the ABP dendrimer; right part: histological picture of the skin of a mouse treated daily with 5 mg/kg of the ABP dendrimer stained with hematoxylin/eosin. Single-
headed arrows indicate the granular layer, which is absent in (A), double-headed arrows show acanthosis, single arrow heads show parakeratosis, diamonds show hyperkeratosis, and double arrow heads show immune cells infiltration, all pathophysiological features that are lowered in (B) (Jebbawi et al., 2020, Copyright the authors).

3.2.4 Pre-clinical assessment

Nanosized molecules planned for human use raise the questions of their toxicity (Hoet, Nemmar, & Nemery, 2004) and immunosafety (Dobrovolskaia, Germolec, & Weaver, 2009). The properties displayed above argue in favour of the ABP dendrimer as an innovative drug-candidate. The biodistribution and biosafety were assessed with different types of mice, female C57BL/6J mice and athymic nude mice, for ex vivo and in vivo imaging, respectively, and female hairless SKH1 mice for haematological safety. The haematological safety of the ABP dendrimer in mice has been demonstrated, as there is no platelet aggregation, no haemolysis, and no disturbance in the haematological formula. The micronucleus test has been used for the detection of pro-mutagen activity. The ABP dendrimer has a potential genotoxicity at 215 μM, a value which is 10 to 100 times greater than that of the active concentrations. The Maximal Tolerated Dose (MTD) of the ABP dendrimer, in both mice and rats, is 100 mg/kg for a single injection. Such MTD is 60 mg/kg/day for repeated injections, carried out during seven consecutive days in mice. The early cardiac safety of dendrimer ABP has been also assessed on Purkinje rabbit fibres. All this information taken together indicates that there is a favourable therapeutic window for dendrimer ABP (Fruchon et al., 2019). In a second step, experiments were carried out to detect the biodistribution, using two fluorescent analogues of dendrimer ABP, i.e. dendrimers 3b-G1 and 3c-G1 (Figure 4). The dendrimers were injected (single shot) at the dose of 10 mg/kg, either through intraperitoneal (IP), subcutaneous (SC), or intravenous (IV, in the tail) routes. The fluorescent dendrimer stayed at the site of injection for several days, when using the IP and SC routes. On the contrary, the fluorescent dendrimer can be detected by fluorescence microscopy first in draining lymph nodes, when using an IV injection in the tail. Dendrimer 3c-G1 was mainly detected in the lungs and in the liver, whereas lower quantities were detected in the spleen and the kidneys. In the long-term follow-up performed in this study for 56 days, there is no bio-accumulation of dendrimer 3c-G1 as the total fluorescence decreased slowly with time (Figure 14) (Fruchon et al., 2019).
In the framework of preclinical characterization of a new therapeutic, the biological risk of the ABP dendrimer was tested in a non-human primate model *Macaca fascicularis*, a long-tailed macaque, also known as cynomolgus monkey. Dendrimer ABP was repeatedly injected to 4 cynomolgus macaques (1 injection per week for 4 weeks). All of them survived well. Biochemical, haematological, clotting, and all immunological parameters in peripheral blood remained within a normal physiological range throughout the study. Serum cytokines quantification and histopathological analyses revealed no non-physiological occurrence or noticeable lesion. These preliminary tolerability, safety, and biodistribution data reinforce the potential of the ABP dendrimer as a drug-candidate for future human use (Fruchon et al., 2015).
4. BIOLOGICAL PROPERTIES OF OTHER TYPES OF PHOSPHOROUS DENDRIMERS

The methoxy derivatives 1aMe-G\textsubscript{n} are the precursors of dendrimers 1a-G\textsubscript{n} (Figure 2). The third and fourth generations of the methoxy derivatives are soluble and stable in water, and their anti-inflammatory properties were tested both \textit{in vitro} and \textit{in vivo}. These dendrimers 1aMe-G\textsubscript{3} and 1aMe-G\textsubscript{4} reduced the secretion of proinflammatory cytokines in mice, and also of macrophages derived from human monocyte. \textit{In vivo} experiments were carried out with a model of sub-chronic inflammation, induced by the air pouch method (Selye, 1953), in which zymosan induces the inflammation.

Dendrimers 1aMe-G\textsubscript{3} and 1aMe-G\textsubscript{4} were also injected in the pouch, at the dosage of 10 mg/kg. One day after the start of the experiment, mice were killed, and the exudate in the pouch was collected to be analysed. The results suggest that these dendrimers induced a M2 polarization of macrophages, contributing to the anti-inflammatory effect observed (Posadas et al., 2017).

Dendrimers of the 13-G\textsubscript{n} family (Figure 7) were synthesized for the purpose of mimicking the bioactive supramolecular structure of mannose-capped lipoarabinomannan (ManLAM cluster) (Riviere, Moisand, Lopez, & Puzo, 2004). The ManLAM cluster is exposed in the envelope of \textit{Mycobacterium tuberculosis}, which is the causative agent of human tuberculosis. Indeed, lipoarabinomannan inhibits the production of proinflammatory cytokines, via binding to the C-type lectin dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN). The binding avidity for DC-SIGN of the whole 13-G\textsubscript{n} family was measured, and compared to that of ManLAM monomer and ManLAM cluster. The highest binding avidity was obtained with the third generation dendrimer capped with 48 trimannoside (144 mannose) as terminal functions (13-G\textsubscript{3}-T), and the fourth generation dendrimer capped with 96 dimannoside (192 mannose) as terminal functions (13-G\textsubscript{4}-D); both compare well with the efficiency of the very large ManLAM cluster (ca 7650 mannose) (Figure 15).

Dendrimer 13-G\textsubscript{3}-T was chosen to be tested in mice. C57BL/6 mice were treated \textit{per os} (orally) daily for 15 days with either a saline solution, 13-G\textsubscript{3}-T mannodendrimer, or ManLAM at a dose of 1 mg/kg. They were then exposed (or not) to LPS inhalation to induce lung inflammation. Compared to mice fed with the saline solution, pre-treatment with 13-G\textsubscript{3}-T or ManLAM significantly reduced the recruitment of neutrophils to the lungs in mice exposed to LPS, by ca 42% and 27%, respectively. This shows that \textit{per os} administration of 13-G\textsubscript{3}-T dendrimer can prevent acute lung inflammation (Blattes et al., 2013). Later on, binding experiments were carried out also with Dectin-2, a C-type lectin, which
is involved in the recognition of several pathogens, such as *Candida albicans*, *Aspergillus fumigatus*, *Schistosoma mansonii*, and *Mycobacterium tuberculosis*, using dendrimers 13-G2-M, 13-G2-D, 13-G3-D, and 13-G3-T. The conclusion of this study was that dendrimer 13-G2-M, did not bind to Dectin-2, whereas the three other dendrimers bind, but did not induce signalling (Decout et al., 2018).

**Figure 15.** Binding of dendrimers capped with mannose (13-G1-M to 13-G2-M, 13-G1-D to 13-G3-D, and 13-G3-T) to membrane expressed DC-SIGN, compared to ManLAM monomer and cluster. *N Mann* indicates the number of mannose units in each compound.

**Conclusion**

The versatility of the chemistry of phosphorous enabled the synthesis of a large panel of specifically functionalized water-soluble dendrimers, tested for their biological properties. It is important to note that the internal structure of dendrimers is not at all an innocent scaffold. Indeed, the grafting of identical azabisphosphonate terminal functions to a series of dendrimers has shown that only dendrimers constituted of main group elements as branching points (phosphorous or silicon) are able to activate human monocytes, whereas the “classical” dendrimers such as PAMAM, PPI, and P-Lysine have no activity. The activation of monocytes occurs through an alternative, anti-inflammatory pathway, after the internalization of the dendrimers, which begins after a few seconds. This first activation has several other consequences on the human immune system, in particular the inhibition of the differentiation of monocytes into osteoclasts, the inhibition of the maturation of dendritic cells,
the inhibition of the proliferation of the CD4+ T lymphocytes, and the multiplication of NK cells. In view of all these properties towards the human immune system tested *ex vivo*, phosphorous dendrimers bearing azabisphosphonate terminal functions have been tested *in vivo*, with mouse models suffering from different diseases representative of human inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, Uveitis, and psoriasis. In view of the efficiency of these dendrimers to cure these diseases or to improve health, their innocuity has been assessed with both rodents and non-human primates. Following these researches about the treatment of chronic inflammations, the start-up IMD-Pharma ([http://www.imd-pharma.com/](http://www.imd-pharma.com/)) has been created to “discover innovative molecules that are able to modulate the activity of major cellular actors of the immune system to treat Chronic Inflammatory Diseases (CIDs) related to autoimmune disorders.” Chronic Inflammatory Diseases concern 3 to 5% of the population worldwide, and despite the existence of immune-suppressive treatment, 20% to 40% of patients fail to respond to these treatments or discontinue treatments due to adverse effects. The dendrimers we have designed, synthesized, and tuned have a very innovative way of action, by rebalancing the immune system, by recovering the natural homeostasis between inflammation and anti-inflammation responses. Pre-clinical data are currently accumulated in view of future Phase 1 clinical tests.

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**References**


