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In vivo therapeutic applications of phosphorus dendrimers: state of the art

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Author biographies

Serge Mignani was head of the medicinal chemistry department and scientific director at Sanofi. In 2017, he was nominated as professor in medicinal chemistry at the Centro de Quimica da Madeira, University of Madeira at Fungal, Portugal. He is a member of the scientific advisory boards of Glycovax Pharma (Montreal, Canada) and Sai Phytoceuticals (New Delhi, India). He is a consultant in medicinal chemistry at the Indian Institute of Integrative Medicine (R. Vishwakarma, IIIM, Jammu, India), the 'Laboratoire Chimie de Coordination' (J-P. Majoral, Toulouse, France) and Donghua University (X. Shi, Shanghai, China).

Xiangyang Shi obtained his PhD in 1998 from the Chinese Academy of Sciences. Between 2002 and 2008, he was appointed as a research fellow, research associate II, research investigator and research assistant professor at Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor. In September 2008, he joined Donghua University as a full professor. He has published more than 268 peer-reviewed SCI-indexed journal articles. His current research interests are focused on dendrimer-based nanomedicine and electrospun polymer nanofiber-based technology for applications in regenerative medicine, sensing and therapeutics.

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Jean-Pierre Majoral is emeritus director of research, exceptional class at the CNRS in Toulouse, France. His research interest is focused on the design and the properties of macromolecules such as phosphorus dendrimers and hyperbranched polymers. His main efforts are directed at the use of dendrimers in medicinal chemistry, nanotechnologies, material sciences and catalysis. He is a co-founder and scientific director of the start-up Dendris. He is a member of several scientific academies worldwide, has received 17 international awards and has authored more than 685 publications, seven books, 35 book chapters and 45 patents.(*h* index 72, over 19,000 citations).

Highlights

- *In vivo* therapeutic applications of phosphorus dendrimers as nanocarriers or drugs
- Phosphorus dendrimers as *in vivo* anti-inflammatory agents
- Phosphorus dendrimers as *in vivo* anticancer agents
- Phosphorus dendrimers against infections in *in vivo* experiments
- Phosphorus dendrimers for non-invasive *in vivo* imaging studies

Abstract

Among the different types of dendrimers, those that incorporate phosphorus in various positions in their structures present fascinating properties and have wide applications in pharmaceutical fields such as drug delivery (including gene transfection), diagnosis and imaging. Therapeutic agents can be encapsulated within the dendritic architecture, or chemically attached or physically adsorbed onto the dendrimer surface. Alternatively, phosphorus dendrimers can be developed as drugs themselves, for instance to treat cancer, inflammations, infections and neurodegenerative diseases, although there are fewer examples of this second approach. Here, we review the most relevant *in vivo* biological activities of phosphorus dendrimers (whether as active drugs *per se* or as nanocarriers) across various therapeutic domains, including anti-inflammation, transmissible spongiform encephalopathies, ocular hypertension, infection, gene therapy, ultrasound-targeted microbubble destruction and two-photon *in vivo* imaging. We discuss the success of phosphorus dendrimers in nanomedicine and look ahead to future innovations in the field.

Introduction

Lammers and Ferrari [1] highlight the challenges faced by nanomedicine. High expectations for the field have been stymied by disappointing clinical trial results from BIND Therapeutics and Cerulean Pharma [2] (see *Smarter Analyst*, 'Here's Why Cerulean Pharma Inc (CERU) Shares Are Falling 65%', https://www.smarteranalyst.com/stock-news/heres-cerulean-pharma-inc-ceru-shares-falling-65/), as well as a reduction of funding from the US National Cancer Institute (NCI) for the Centers of Cancer Nanotechnology Excellence (CCNEs) [3]. Nevertheless, Lammers and Ferrari claim that nanomedicine will emerge as a next-generation platform in medicine on the basis of several important successes in both academia and the pharmaceutical industry.

Oncology is the main therapeutic realm in which nanoparticles (NPs) are used. The biggest issue in this field is developing NPs with good tumor penetration; indeed, an analysis found that on average only around 0.7% of an NP dose ends up in tumors [4], although this figure has been challenged [5]. However, more

than 50 drug products containing nanomaterials have been approved by the US Food and Drug Administration (FDA) for clinical use, and more than a dozen of those were approved in the past decade [6].

Various NP types have been developed as nanocarriers of drugs in several therapeutic fields (mainly in oncology) in the form of liposomes, albumin-based particles, biodegradable polymer–drug composites, polyethylene glycol (PEG)ylated proteins, polymeric micelles, polymer–drug conjugate-based particles, inorganic particles, dendrons and dendrimers. A large variety of dendrimers exists [7]. These macromolecules, such as phosphorus dendrimers [8], are a family of nanodevices, and they have been developed as drug-delivery systems, active drugs (in relation to the nature of the terminal groups on their surface) and contrast-bearing agents for imaging purposes. This contextual review describes the *in vivo* applications of phosphorus dendrimers as drugs in several therapeutic areas: as anti-inflammatory and anticancer agents; for treating neurodegenerative diseases, ocular hypertension and infections; and for non-invasive *in vivo* imaging. *In silico* and *in vitro* studies continue to refine the quality of the drugs; and *in vivo* preclinical assays remain the gold standard for assessing both human risk and efficiency.

Drug characteristics during the preclinical process

The process of drug discovery and development takes about 10–15 years and costs in excess of US$1 billion, depending on the therapeutic area (see *Science Translational Medicine* 'Drug Development Costs Revisited', https://blogs.sciencemag.org/pipeline/archives/2017/10/18/drug-development-costs-revisited). Nowadays, the timeline breaks down into six critical steps: (i) disease pathology study, (ii) target identification, (iii) lead identification and optimization (hit-to-lead phase) for investigational new drug (IND) filing, (iv) Phase I–III clinical trials for new drug application (NDA) filing, (v) FDA regulatory approval and (vi) post-market safety monitoring. After the identification and validation of a target (or targets), drug discovery involves several steps, including the discovery of hits (e.g., using phenotypic- and target-based screening or machine-learning approaches) and the optimization of hits using medicinal chemistry to find druggable leads, followed by the selection of the best clinical candidates. The preclinical stage encompasses the use of *in vitro* and *in vivo* studies to evaluate the efficacy and safety of the drug candidates up to the clinical-trial stage.

In 2019, 48 new medicines were approved by the FDA, versus 59 in 2018. Eleven of the drugs approved in 2019 were cancer treatments, and 44% of the total were treatments for rare diseases. In addition, 67% of these 2019 medicines were small molecules, 42% were first-in-class drugs and three were antibody–drug conjugates. Novartis added six drugs to its portfolio in 2019 [9].

The objective of the hit-to-lead stage is to produce more potent and selective compounds with adequate pharmacokinetic (PK) and pharmacodynamic (PD) profiles (PK/PD profiles) and to examine their efficacy in any *in vivo* models relevant to the considered disease. The main challenge of *in vivo* studies is choosing the animal model (e.g., mice, rats, dogs, cats, minipigs, rabbits, baboons or zebrafishes) that best represents the disease systems that drugs will affect in humans. *In vivo* studies are essential to evaluate the physiological characteristics and effects in a living organism; biochemical processes of biologically active compounds that are not present in *in vitro* studies might affect the release of the drug *in vivo*.

It is not clear whether *in vitro* toxicodynamic evaluation can predict the *in vivo* interactions of drugs with organs; furthermore, the absorption, distribution, metabolism, excretion and toxicity (ADMET) parameters cannot provide full quantitative interpretation in animal models and humans [10]. An important point is that *in vitro* studies have a positive impact on the selection of hits and early leads, helping to avoid failures in later steps of the development process. However, *in vitro* and *in vivo* results do not always correlate in terms of, for example, showing bioequivalency [11], toxic effects and the ideal dosage regimen for clinical trials. Consequently, *in vivo* research is necessary to select a drug that is safe and effective in humans. *In vitro* results can provide a snapshot of a particular aspect of the drug (e.g., ADMET), but multifactorial *in vivo* results help to show its combined effects, including its efficiency, PK/PD profile and toxicological effects.

Nanomedicine is an umbrella term, and it is defined as a specific branch of medicine that uses nanotechnology for the prevention, treatment and diagnosis of diseases, as well as for theranostics [12]. Nanodelivery platforms can help to improve the solubility and bioavailability of small molecules, genes and vaccines, as well as reduce their off-target effects. Several approved nanomedicine formulations have been described, encompassing liposomes (e.g., Abelect, a lipid amphotericin B formulation), PEGylated liposomes (e.g., Doxil, PEGylated liposomal doxorubicin), nanocrystals (e.g., Emend), NPs (e.g., Abraxane, paclitaxel albumin-bound particles) [13–15] and dendrimers (e.g., VivaGel from Starpharma). Doxil was the first FDA-approved nanodrug in 1995, and recently the generic doxorubicin hydrochloride liposome injection Lipodox (Sun Pharmaceutical Industries) has been approved by the FDA.

Dendrimers in nanomedicine: a concise overview

Dendrimers are monodisperse, carefully assembled NPs of synthetic monomers that form branch-like structures. Examples include polyamidoamine (PAMAM) dendrimers, poly-L-lysine (PL) dendrimers, poly(propylene imine) (PPI) dendrimers, PEG polyester dendrimers, carbosilane dendrimers [16] and phosphorus dendrimers [8]. As shown in Figure 1, phosphorus dendrimers enable hollow cavities to form within the branching structures; these cavities can be loaded with drugs, imaging agents, and so on. Both the size and the functionalized surface groups can be tightly regulated or modified. The addition of new generations (G1, G2, etc.) increases the dendrimer's diameter, as well the number of surface groups. Dendrimers can be used as nanocarriers or be active drugs themselves in nanomedicine [17–19].

To date, few clinical successes in using dendrimers as nanocarriers or drugs have been reported in the literature. In 2012, Starpharma (Melbourne, Australia) started two pivotal Phase III trials with the G4 PL polyanionic dendron VivaGel (SPL7013 Gel, astodrimer sodium) for the treatment of bacterial vaginosis. This NP has 32 naphthalene disulfonate groups on its surface and demonstrated potent topical vaginal microbicidal activity. Recently, Starpharma and AstraZeneca have moved from Phase I to Phase II with a PL dendrimer-based nanocarrier encapsulating docetaxel (named DEP docetaxel), which shows higher anticancer activities against breast, prostate, lung and ovarian tumors than docetaxel alone.

Among around 100 types of dendrimer, those incorporating phosphorus groups represent a wonderful opportunity to extend the dendrimer functionalization space. The preparation of phosphorus dendrimers starts generally from commercially available hexachloro-cyclo(triphosphazene), and they are easy to synthesize in large-gram-scale and soft-reaction conditions, with high yields. All the by-products can be removed without wordly techniques [8]. In addition, several types of AB3, AB5 and CD3 or CD5 monomers have been prepared, allowing the preparation of a variety of dendrimers (e.g., [20]), as well as hybrid phosphorus–viologen dendrimers [21], onion peel dendritic structures [22] and Janus dendrimers [23].

In drug discovery, one of the leading challenges is the identification of specific biologically active compounds with adequate therapeutic and pharmacological properties to become a medicinal product. In the nanomedicine domain, one important aspect for the development of NPs such as dendrimers is to consider these nanodevices as active drugs themselves. Thus far, the majority of engineered NPs have been developed for drug delivery, mainly in cancer therapy [16]. To explore the huge chemical space with dendrimer collections as nanocarriers and as drugs, we introduced the concept of dendrimer space as a druggable cluster [24]. Notably, the multidimensional limits of drug-dendrimer space can be associated with their biological and pharmacological profile. Within the huge dendrimer space, the development of phosphorus-based dendrimers has been outstandingly performed in several directions, encompassing, for instance, hybrid materials, catalysts, nanotubes for diagnosis, DNA chips, fiber formation and nanomedicine (e.g., [25]).

Huge numbers of therapeutic areas have been well considered with phosphorus dendrimers, both as drugs and nanocarriers [e.g., small interfering RNAs (siRNAs)]. The majority of the PAMAM dendrimers have been used as nanocarriers, mainly in oncology (as depicted by several studies from Tomalia [26], Backer [27] and Newkome [28]), using Fréchet's polyester dendrimer-[poly(ethylene oxide)] ('bow-tie' dendrimer)

[29] and Simanek's triazine dendrimers [30]. In addition, theranostic applications in cancer have been developed by Shi, who used non-invasive functionalized PAMAM-entrapped gold(0) NPs [31], and Tomalia [32]. Few *in vivo* studies of antitumor agents using the dendrimer approach have been published [16].

A remarkable example of the architecture optimization of dendrimers has been reported by the Fréchet group [33]. They prepared an asymmetric, biocompatible, doxorubicin-functionalized bow-tie dendrimer that showed potent *in vitro* and *in vivo* antiproliferative activities against C-26 colon carcinoma cells. In addition, an innovative strategy against difficult cancers such as pancreatic cancer has been presented by Shi *et al.* [34]. This strategy was based on the use of the ultrasound-targeted microbubble destruction (UTMD) technique, which promotes the co-delivery (synergistic effect) of gemcitabine (Gem) and miR-21 inhibitor (miR-21i) from PEGylated G5 PAMAM dendrimer-entrapped gold NPs (Gem-Au DENPs and miR-21i NPs).

Whereas most other types of dendrimer have chiefly been used in classical research experiments, phosphorus dendrimers have tended to be used as drugs, as well as to deliver siRNAs. In this review, we survey and analyze successes in the development of phosphorus dendrimers *in vivo*. These *in vivo* studies represent fundamental pharmacological knowledge in drug discovery and the development of phosphorus dendrimers in animal models, and ultimately in humans. Importantly, the main challenge in *in vivo* studies is choosing an animal model that best illustrates the human disease. The many physiological differences between animals and humans do not allow for completely similar results if the drug testing reaches the clinical-trial stage, even when using primates, which is strongly recommended by the regulatory agencies (the FDA and European Medicines Agency).

Crucial chemical parameters for preclinical therapeutic applications of dendrimers: general aspects

It is generally thought that the *in vitro* and *in vivo* therapeutic applications of dendrimers are mainly dependent on the nature of their surface chemical units (e.g., polycationic, polyanionic and neutral moieties). In this regard, several of us analyzed the different parameters to delineate new 'druggable' clusters within the chemical space (*vide supra*). These clusters can be delimited using 'drug-likeness' parameters, which are fully integrated into the practical drug-discovery process. Both *in vitro* and *in vivo* physicochemical parameters were evaluated for preclinical evaluation of biomedical dendrimers [35,36]. For instance, in the toxicity domain, there are two main strategies to tackle dendrimers' toxicity issues: the preparation of biodegradable and biocompatible scaffolds (for the core and branches), and/or surface modifications. Jain *et al.* proposed an interesting approach involving the development of non-toxic polypropyletherimine (PETIM) dendrimers [37]. Also, to extend the usage scope of dendrimers in nanomedicine, Haag and colleagues have conducted an important analysis of the *in vivo* druggability requirements of multifunctional dendritic polymers [38]. Figure 2 presents an overview of the physical characteristics of NPs, including dendrimers, in terms of *in vivo* biocompatibility profile.

Influence of the size and chemical structure of phosphorus dendrimers on therapeutic applications

It is widely known that, against various infection types, the activation of monocytes and the amplification of functional natural killer cells boost the innate immune system's first line of defense against 'assailants' [39].

Rolland and colleagues conducted a study on the influence of the end phosphonate groups of several phosphorus dendrimers on the cellular activation of human monocytes [40]. In a similar vein, an interesting study has been conducted by the Toulouse team on the key role of the scaffold in the efficiency of dendrimers [41]. Thirteen dendrimers from seven different families were studied using a molecular dynamic simulation technique; the study included PAMAM, PPI, poly(carbosilane) and PL dendrimers along with three phosphorus dendrimer types. The dendrimers had different internal (core) structures but identical azabisphosphonic terminal groups ($N[[CH_2\text{-}P(O)(OH)ONa)]_2$), which activate human monocytes. This study confirmed that the geometry of the scaffold had an important role in therapeutic efficiency. In a

similar way to how the scaffold of a simple drug molecule interacts with, for instance, a receptor, the change in shape and the spatial distribution of the terminal moieties of the dendrimers influenced their biological activity. The most potent dendrimers have their surface functions assembled on one side to potentially maximize interactions with the biological target.

Phosphorus dendrimers as anti-inflammatory agents

Living beings respond to a variety of insults, such as the presence of pathogens or damaged cells, with an inflammatory response that generally includes activation of both the innate and adaptive responses. An inflammatory response is generally acute and resolves after the originating cause has disappeared. However, under certain circumstances, inflammation becomes chronic, causing damage in different organs [42]. Autoimmune diseases are particular cases of chronic inflammation in which the recognition of the body's own cells and tissues as 'self' fails, and the organism generates an immune response against self-antigens, causing damage to tissues and organs. Inflammation has a key role in the pathophysiology of a number of diseases, including arthritis, asthma, multiple sclerosis and inflammatory bowel disease.

Recently, Posadas *et al.* reported that neutral and biocompatible G3 and G4 phosphorous dendrimers, with 48 and 96 terminal biphosphonate groups, respectively, behaved as potent anti-inflammatory agents (Figure 3a). Both compounds were not toxic, up to a concentration of 10 µM, for inflammatory cells such as mouse and human macrophages or lymphocytes, and had good water solubility and stability in aqueous solutions [43]. In mice, purified peritoneal macrophages collected after thioglycolate intraperitoneal injection were stimulated using lipopolysaccharide (LPS), inducing a robust inflammatory response characterized by activation of nuclear factor-κB (NF-κB) induction, inducible nitric oxide synthase (iNOS) expression, an increase in nitric oxide production and secretion of the pro-inflammatory cytokines tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β). Moreover, LPS also induced the expression of CD86, a marker for the macrophage M1 pro-inflammatory population. The presence of either G3 or G4 phosphorous dendrimers markedly decreased all LPS-induced inflammatory markers and decreased CD86 levels, restoring levels of the macrophage M2 anti-inflammatory population marker CD163. Taken together, these results indicate that neutral phosphorous dendrimers can switch the macrophage population from the pro-inflammatory M1 subtype to the anti-inflammatory M2 subtype, helping to resolve inflammation.

Neutral phosphorous dendrimers also showed anti-inflammatory properties *in vivo* against a subchronic model of inflammation: the air pouch. Zymosan injection into the air pouch induced an inflammatory response with increased iNOS expression and nitrite levels in the infiltrating cells, as well as an increase in the macrophage M1 population marker CD86. Administration of G3 or G4 phosphorous dendrimers [10 mg/kg, intravenous (i.v.)] caused a decrease in inflammation markers in the surrounding tissue as well as a decrease in CD86 and an increase in CD163, the marker for the anti-inflammatory M2 macrophage population.

Taken together, these data indicate that neutral G3 and G4 phosphorous dendrimers have anti-inflammatory properties both *in vitro* and *in vivo*, and are able to switch macrophages from a pro-inflammatory M1 population towards an anti-inflammatory M2 population, which would help to resolve inflammation

In the realm of chronic inflammatory disease, and osteoclastogenesis, i.v. injection of the G1 anionic phosphorus dendrimer has been performed in two different murine animal models of autoimmune arthritis: IL-1ra$^{-/-}$ mice and serum transfer from K/BxN mice. These two animal models are most relevant to human rheumatoid arthritis [44]. In the IL-1ra$^{-/-}$ mice model, the animals spontaneously develop autoimmune-like arthritis at 4 weeks old, whereas in the K/BxN serum-transfer arthritis model, analyses of the immunological mechanisms occurring in rheumatoid arthritis and other arthritis types can be evaluated [45,46]. The decrease of disease severity was determined using three different biomarkers: (i) normal synovial membranes, (ii) reduced levels of inflammatory cytokines and (iii) absence of cartilage destruction and bone erosion. In this study, three dendrimer types were compared against inflammatory disease: G1

azabisphosphonate (compound 1 in Figure 3b), G1 azamonophosphonate (compound 2 in Figure 3b) and G1 bisphosphonate PPI dendrimer (compound 3 in Figure 3b).

Arguably, the authors demonstrated that compound 1 inhibited the expression of pro-inflammatory cytokines in monocytes *in vitro* and also activated monocytes, increasing IL-10 secretion by $CD4^+$ T cells. At a dose of 10 mg/kg, only compound 1 demonstrated significant efficacy in the two animal models at 15 weeks of age, on the basis of hind-paw swelling and clinical arthritis score evaluation analysis. Interestingly, histopathological analysis of the ankle joints revealed near-normal synovial membranes versus hyperplasia, inflammation and eroded cartilage in untreated mice. In addition, no osteoclasts corresponding to the multinucleated cells responsible for bone resorption or cells expressing the arthritis-associated cytokine IL-17 were observed in the bone matrix in mice treated with compound 1. Using hematoxylin-eosin (a stain in histology allowing for the localization of nuclei and extracellular proteins), the histological scoring analysis showed a 50% and 80% reduction with compound 1 at 0.1 and 10 mg/kg, respectively. Also, anti-osteoclastic activity on mouse and human cells has been demonstrated with this dendrimer. A recent review highlighted and analyzed the different immunomodulatory effects of compound 1 [47].

The potential use of compound 1 for the treatment of multiple sclerosis has recently been highlighted [48]. Multiple sclerosis is a chronic inflammatory diseases of the central nervous system. Compound 1 displayed potent efficiency in a mouse model of multiple sclerosis, in which myelin oligodendrocyte glycoprotein (MOG35-55) induces experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. The macromolecule compound 1 inhibited the progression of the neuroinflammation by promoting IL-10-producing $CD4^+$ T cells, which produce pro-inflammatory cytokines such as interferon-γ and IL-17. In this *in vivo* study, mice were treated with 10 mg/kg of compound 1 (i.v.) on day 1, day 3 and then every 3 days after that.

Compound 1 has also been tested in a rat model of acute uveitis induced by LPS. The authors showed that at a dose of 2 µg per eye, it was as efficient as 'standard' dexamethasone at 20 µg per eye. This anti-inflammatory activity is mediated through an increase of production of the anti-inflammatory IL-10 cytokine [49].

Repeated intravenous injections of compound 1 (10 mg/kg on days 0, 7, 14 and 21) in male cynomolgus macaques (*Macaca fascicularis*) were demonstrated to be safe, with no specific side effects. During the study, biochemical, hematological and immunological parameters remained within the normal physiological range. The administered dose was tenfold higher than the lowest active dose (1 mg/kg) identified in the mouse model of chronic inflammatory disorder rheumatoid arthritis [50].

Note that recently an interesting analysis of the pro-inflammatory versus anti-inflammatory effects of dendrimers has been conducted by Fruchon and Poupot [39].

For imaging purposes, two different G1 phosphorus dendrimer types were prepared, with 24 terminal azabisphosphonate groups (carried by five main branches) and one main branch bearing a fluorophore probe such as julolidine (green fluorescence) and a specific fluorophore probe emitting near-infrared fluorescence that does not overlap with the auto-fluorescence of tissues [51]. The fluorescent phosphorus dendrimers displayed the same biological profile as compound 1 (*in vitro* in human monocyte models and *in vivo* in a mouse model of arthritis). In mice, the dendrimers were mainly detected in the lungs and liver (10 mg/kg i.v., single injection) rather than the spleen and kidney. Importantly, no bioaccumulation of these macromolecules was detected, because the total fluorescence decreased slowly over time. After intraperitoneal and subcutaneous administration, no distribution was observed, and the dendrimers stayed at the site of injection for several days. The maximum tolerated dose, which defines the dose and schedule for Phase I clinical trials, of compound 1 is 100 mg/kg for a single injection and 60 mg/kg/day for repeated injections over seven consecutive days. These data indicate the favorable therapeutic window for compound 1. Generally speaking, a good safety profile was demonstrated, except in genotoxicity assays that showed potential mutagenicity, but at doses up to 100 times greater than the *in vitro* active dose against human immune cells.

An interesting study by Bohr and Fattal investigated the anti-inflammatory effect of anti-TNFα siRNA G3 cationic phosphorus dendrimers in a murine acute lung injury model after intranasal administration [52]. Asthma, chronic obstructive pulmonary disease and acute lung injury are lung diseases that involve inflammation, and TNFα plays a key part in this inflammation process. In this study, two different phosphorus dendrimers were used as nanocarriers, bearing 48 protonated pyrrolidinium (named DP) or morpholinium (named DM) terminal groups (Figure 3c).

The dendriplexes had mean sizes of 120–190 nm, polydispersity indexes of 0.27–0.44, hydrodynamic diameters of around 3 nm and a positive zeta potential at N/P ratios of 5, 10 and 20. The biological studies were carried out using dendriplexes with N/P of 5 in order to maximize the amount of siRNA associated with the dendriplexes. In cytotoxicity studies against RAW 264.7 cells (monocyte/macrophage-like cells), the DM dendriplexes did not exhibit cellular viability reduction (IC_{50} > 15 µM siRNA), unlike the DP dendriplexes, which showed a reduction of cellular viability with an IC_{50} siRNA concentration of 470 nM (dendrimer concentration: 41 µg/ml). Using flow cytometry, cell-uptake studies demonstrated that DM dendriplexes showed low uptake over time, unlike DP dendriplexes, which displayed significant uptake of siRNA.

Interestingly, using RAW 264.7 cells, flow-cytometry data indicated that DP dendriplexes were mainly taken up by cells, whereas DM dendriplexes mainly adhered to the outer surface of the cell membrane as aggregates. Enhanced *in vitro* silencing efficiency of TNFα in the LPS-activated mouse macrophage cell line RAW264.7 was observed with DP dendriplexes versus DM dendriplexes. In an LPS-induced murine model related to a short-term acute lung injury, DP dendriplexes showed a stronger silencing effect than non-complexed siRNA via nasal administration.

Indeed, this small chemical surface change, the introduction of morpholinium (DM) or pyrrolidinium (DP) groups, induced strong *in vitro* and *in vivo* effects in several inflammatory assays. Isothermal titration calorimetry and molecular dynamics simulations were performed by Deriu and Danani to evaluate the molecular factors influencing the different efficiencies and activities of DP and DM [53]. Chemical surface modification drives the dendrimer–siRNA affinity, as well the flexibility/rigidity ratio. Rigid polycationic dendrimers reorganized their peripheral groups to enhance contacts with the nucleic acid units of siRNA. DP dendrimers displayed better efficiency than DM dendrimers based on: (i) a strong entropic contribution from the free-energy-binding DP–siRNA complex and (ii) a lower number of terminal groups of DP directly interacting with siRNA, inducing additional binding with another siRNA molecule.

Phosphorus dendrimers against transmissible spongiform encephalopathies

The infectious prion protein PrP^{Sc} is the root cause of the transmissible spongiform encephalopathies Creutzfeldt–Jakob disease (CJD), Gerstmann–Straüssler–Scheinker syndrome and fatal familial insomnia in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE) in cows [54].

Solassol and colleagues designed and developed cationic phosphorus-containing dendrimers, which strongly reduced prion replication both in cell culture and in mice infected with scrapie [55]. These G3–5 cationic phosphorus dendrimers have different numbers of diethyl amine end groups: namely, 48 (PD-G3), 96 (PD-G4) and 192 (PD-G5) (Figure 4).

Interestingly, in scrapie-infected neuroblastoma N2a cell assays, PD-G3, PD-G4 and PD-G5 removed PrP^{Sc} rapidly with IC_{50}s of 600 nM, 75 nM and 45 nM, respectively. Importantly, no effect on growth rate or on the morphology of the cells was observed at the concentrations used, indicating that these phosphorus dendrimers are non-cytotoxic agents. PD-G3–G5 macromolecules bind to PrP, and they decreased the amount of pre-existing splenic PrP^{Sc} and BSE prion strains. More importantly, in the C57BL/6 mice scrapie model, PD-G4 decreased PrP^{Sc} accumulation in the spleen by up to 66% and 88% at doses of 50 µg and 100 µg, respectively. In this study, C506M3 scrapie brain homogenate was obtained from terminally ill mice and was intraperitoneally administrated in C57BL/6 mice. Intraperitoneal administration of PD-G4 occurred every 2 days from day 2 to day 30 post-inoculation, and mice were sacrificed 30 days after infection. These

in vivo experiments demonstrated that the phosphorus dendrimers predominantly reached the spleen, which is infected after the brain.

Phosphorus dendrimers against ocular hypertension

Carteolol is a non-selective β-adrenoceptor antagonist with partial agonist activity. It is used topically to reduce elevated intraocular pressure in patients with glaucoma or ocular hypertension [56]. An original monomer complex bearing three carboxylic groups and phosphorus dendrimers bearing 6 or 12 terminal carboxylic groups have been prepared to complex with carteolol through ionic interactions. [57]. These three complexes have been tested *in vivo* in a rabbit model, as (nano)carriers for the ocular delivery of carteolol with the following protocol: G0, G1 and G2 macromolecules were administered in the eyes of rabbits as a solution in Milli-Q water. Importantly, no irritation was observed from these three compounds even after several hours of treatment. Using the G2 dendrimer, the concentration of complexed carteolol that penetrated inside the eyes was 2.5 times higher than expected when compared with carteolol alone.

Phosphorus dendrimers against infections

Mannose-capped lipoarabinomannan (ManLAM) is present in all members of the *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) complexes. It is a high-molecular-mass amphipathic lipoglycan with a defined, crucial role in mycobacterial survival during infection. ManLAM plays the part of both an immunogen and a modulator improving tuberculosis prevention. ManLAM inhibits the release of pro-inflammatory cytokines by LPS-stimulated human dendritic cells (DCs) by targeting the C-type lectin receptor (CLR), which binds to carbohydrates in a calcium-dependent manner. DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) is one unit of transmembrane CLRs, and it is involved in the recognition of several viruses [58].

With the aim of mimicking the bioactive supramolecular structure of ManLAM, an interesting study has been performed by Blattes *et al*. concerning the design and development of several types of phosphorus dendrimers, namely: (i) G1, G2 and G3 phosphorus dendrimers with one mannose unit on the surface, corresponding to 12, 24 and 48 units per dendrimer, respectively; (ii) G1–4 phosphorus dendrimers with dimannose units on the surface, corresponding to 12, 24, 48 and 96 units per dendrimer, respectively; and (iii) a G3 phosphorus dendrimer with a trimannose unit, corresponding to 48 units per dendrimer [59]. Among this phosphorus-dendrimer library, the G3 phosphorus dendrimers grafted with 48 trimannoside caps (named 3T) and the G4 dendrimer bearing 96 dimannosides (named 4D) displayed the highest binding avidity for DC-SIGN. Also, these macromolecules inhibited pro-inflammatory TNFα cytokine production. Importantly, in an acute lung inflammation model in mice exposed to aerosolized LPS, oral administration (1 mg/kg) of a 3T phosphorus dendrimer, bearing 48 end trimannoside units, highly reduced neutrophil influx by targeting DC-SIGN.

Cationic phosphorus dendrimers for tackling cancer *in vivo* using gene therapy

Gene therapy is used to treat diseases caused by missing, defective or overexpressed genes. The key issue in this field is developing a method for introducing a therapeutic gene into target cells. Currently, two approaches are used. One is called gene therapy *in vivo*, wherein the gene of interest is inserted into a vector and then the vector is transferred directly to the patient. The other approach is called *ex vivo*, in which the vector is introduced into cultured cells (which can be taken from the patient), and subsequently these genetically engineered cells are transplanted into the patient.

In both cases, viral or non-viral vectors (such as liposomes, cationic polymers or dendrimers) are necessary for introducing the gene of interest into cells [60,61]. Usually, nucleic acid-based therapies using plasmid DNA (pDNA), siRNA or miRNA are employed, and picking the optimal plasmid or siRNA design is of critical importance to improve the efficiency, safety and therapeutic value of delivery. Nucleic acids are highly hydrophilic: therefore, they are unable to penetrate lipid cell membranes, and additionally they can be degraded by nucleases in the blood.

The delivery vehicle can be a cationic dendrimer that is capable of condensing genetic material containing multiple phosphate residues, forming dendriplexes. The first attempt to use phosphorus dendrimers for DNA separation in microarrays was made by Le Berre *et al*. in 2003 [62]. The authors proposed to use cationic phosphorus dendrimers for the functionalization of glass slides for more sensitive and reliable DNA microarrays. In the same year, Maksimenko *et al*. [63] proposed to use cationic phosphorus dendrimers for gene therapy to optimize dendrimer-mediated gene transfer. To understand the mechanisms of entry of dendrimers into cells, several *in vitro* studies have been performed, such as those by Ionov *et al.* [64,65] and Shcharbin and colleagues [66]. For instance, the use of phosphorus dendrimers to deliver anticancer siRNA cocktails to treat cancer cells has been depicted by Dzmitruk *et al.* [67], as well to deliver siRNAs in HIV-1 infected cells [68]. In addition, Shakhbazau *et al*. described the use of PAMAM and phosphorus dendrimers to modify human and rodent stem cells *ex vivo* so that they overexpress neurotrophic factors [69,70].

Recently, Liang and Shi *et al.* highlighted the development of original polycationic phosphorus dendrimers for the delivery of DNA plasmids to treat tumors [71]. G1–G3 cationic phosphorus dendrimers bearing 1-(2-aminoethyl) pyrrolidine, 1-(3-aminopropyl) piperidine or 1-(2-aminoethyl) piperidine groups on their surface were prepared via straightforward synthetic pathways (Figure 5a).

These protonated polycationic dendrimers were complexed with negatively charged pDNA encoding both enhanced green fluorescence protein (EGFP) and p53. The pDNA-p53–dendrimer polyplexes were prepared with different N/P ratios. For the G1 dendrimers, bearing 12 positive charges on the surface, the DNA condensation capacity followed the order of 1-G1.HCl (N/P = 1 or higher) > 2-G1.HCl (N/P = 2 or higher) > 3-G1.HCl (N/P = 4 or higher). In addition, polyplexes with the pyrrolidinium group displayed a greater positive surface charge than those with the piperidinium group.

Based on a CCK8 viability assay against human cervical carcinoma HeLa cells, the pDNA-p53 polyplexes showed cell viability of at least more than 50%, with the highest concentration of polyplexes at 3000 nM with an N/P ratio of 20. The cell viability decreased in a concentration-dependent manner. The formation of polyplexes from polycationic dendrimers and plasmids decreased the positive charge of the nanosystems, and consequently the polyplexes displayed a lower cytotoxicity than the corresponding dendrimers.

The flow cytometry experiments showed that the best gene-transfection efficiency was achieved with an N/P ratio of 20. At the same N/P ratio, the polyplexes bearing pyrrolidinium termini increased the expression of EGFP more than those with piperidinium termini. Consequently, the 1-G1.HCl–pDNA-p53 polyplex was selected for further studies. The transfection of 1-G1.HCl–pDNA-p53 to HeLa cells induced significant p53 protein expression, as demonstrated by western blotting, and the expression of p53 protein was proven to induce apparent G1/S cell cycle arrest based on the regulation of p21 and cyclin-dependent kinase 4 (Cdk4)/cyclin D1 expression, causing cancer cell apoptosis. The development of the 1-G1.HCl–pDNA-p53 polyplex for anticancer therapies was fully validated based on *in vivo* experiments. As shown in Figure 5b, intratumoral injection of the 1-G1.HCl–pDNA-p53 polyplex into mice bearing xenografted cervical HeLa tumors did not cause systemic toxicity, and the tumor cells displayed increased expression of p53 and p21 protein, and decreased expression of the Cdk4 and cyclin D1, in full agreement with the *in vitro* transfection assay data.

Multivalent copper(II)-conjugate phosphorus dendrimers to take down *in vivo* cancers using UTMD

Recently, in the domain of precision theranostics in nanomedicine, Fan and Shi *et al*. developed a G3 phosphorus dendrimer capped with 48 $CuCl_2$ moieties (denoted 1G3-Cu, Figure 6) for non-invasive UTMD-promoted pancreatic tumor magnetic resonance (MR) imaging and chemotherapy [72]. 1G3-Cu displayed an IC_{50} of 1.24 μM to inhibit the growth of SW1990 pancreatic cancer cell line and an adequate r_1 relaxivity of 0.7024 mM^{-1} s^{-1} for T_1-weighted MR imaging purposes. The chemotherapeutic effect of 1G3-Cu was proven by flow cytometry and western-blot assays. Experimental data revealed that the incubation of

SW1990 cells with 1G3-Cu for 24 h activated the apoptotic process through upregulation of the Bax, p53 and phosphatase and tensin homolog (PTEN) proteins, downregulation of the protein Bcl-2, and a decrease in intracellular adenosine triphosphate (ATP).

Interest has grown in the UTMD technique, which increases the tumor penetration of drugs or NPs through the induced sonoporation effect [34]. In this study, under the application of UTMD, 1G3-Cu enabled enhanced MR imaging of tumors. Biodistribution studies demonstrated that in the absence of UTMD, the majority of the uptake of 1G3-Cu occurred in the liver, lung and kidney, whereas in the presence of UTMD, higher accumulation of 1G3-Cu was observed in SW1990 tumors. 1G3-Cu displayed good anticancer effects on day 14 by decreasing the tumor volume by around 62%, versus around 35% without UTMD. Importantly, 1G3-Cu did not induce a significant hemolysis effect in the concentration range of 0.5–10 µM, and it displayed a good safety profile, without affecting the major organs, blood parameters and bodyweight of mice.

Water-soluble phosphorus dendrimers as two-photon tracers for non-invasive *in vivo* imaging studies

Two-photon excited fluorescence (TPEF) microscopy, invented by Denk *et al.* in 1990 [73], represents a powerful diagnostic tool. The main advantages are (i) the reduction of photo damage, (ii) images with sub-micrometer resolution, (iii) high-sensibility imaging and (iv) the allowance of photochemical reactions within a sub-femtoliter volume inside cells and tissues [74].

Krishna and colleagues reported the design and the synthesis of water-soluble amphiphilic two-photon markers from lipophilic fluorophores [75]. As shown in Figure 7a, three different phosphorus dendrimers were prepared bearing the chromophore Q as a branch. Interestingly, the phosphorus dendrimers, named G1, G2 and G3, showed strong one-photon absorption in the near-ultraviolet spectrum and strong emission in the blue-visible region (Table 1). Their photoluminescence capabilities were similar in ethanol and water, indicating that these water-soluble dendrimers could be used for biological imaging purposes with high photoluminescence efficiency.

Krishna *et al*. performed two-photon imaging of the vascular network in the dorsal part of a rat olfactory bulb. The vessels were labeled after the intravenous injection of a small bolus of 500 µM of G2 dendrimer in water [75]. Imaging of the blood vessels of tadpoles in two and three dimensions was also performed.

In addition, Shakhbazau and colleagues have demonstrated that a G2 fluorescent phosphorus dendrimer (compound I in Figure 7b) can be fully internalized into macrophages. This phosphorus dendrimer can be used as a cytoplasmic tracer both *in vitro* and *in vivo* (Figure 7b) [20,76].

Conclusion and perspectives

Phosphorus dendrimers can be easily synthesized by the incorporation of phosphorus in various positions, such as in the core, internal branches and external surfaces. The construction of several generations of phosphorus dendrimers has allowed specific control of their size, molecular mass, chain types and surface units that show noteworthy therapeutic activities. Their structures can be easily prepared based on the strong versatility of organophosphorus chemistry. Polycationic, polyanionic and neutral phosphorus dendrimers can be prepared by simple chemical modification of the nature of their surface. A large number of synthetic pathways have provided diverse types of phosphorus dendrimers, including: (i) phosphorhydrazone linkages in the internal branches; (ii) viologen dendritic structures; (iii) P=N-P=S linkages in the internal branches; (iv) onion peel dendritic structures; and (v) Janus phosphorus dendrimers. Importantly, these syntheses occurred under mild reaction conditions and produced non-toxic by-products (e.g., HCl and NaCl) that can be removed without elaborate techniques.

Collectively, the major advantages of phosphorus dendrimers in comparison with the widely known PAMAM dendrimers are: (i) several routes of administration can be used; (ii) good manufacturing practices

for clinical use can be developed; (iii) higher lot-to-lot chemical stability was observed; (iv) easily tunable surface synthesis, allowing the development of phosphorus dendrimers as active drugs *per se*; and (v) low generation of phosphorus dendrimers showing potent anticancer and antituberculosis activities.

In the preclinical process, *in vivo* studies are pivotal assets that can enable selection of the optimal drugs and dosages. They can provide relevant information (e.g., PK/PD characteristics, which are not available with *in vitro* studies) for making decisions as well as for avoiding potential hazards. The development of dendrimers in general (and phosphorus dendrimers in particular) is no exception to this rule. Phosphorus dendrimers have been developed as nanomedicines in several important and unmet therapeutic areas; they have been studied as anti-inflammatory agents, anticancer agents and drugs to treat neurodegenerative diseases, ocular hypertension and infections, as well for use in *in vivo* imaging. One of the challenges is the selection of animal models based on the physiological and biochemical similarities between the animal model and humans, including ADMET evaluation. Consequently, further studies are still needed to develop animal models that better represent human physiology.

In the dendrimer realm, few PK/PD studies have been published owing to the mandatory need for validated analytical methods in both preclinical and clinical PK studies. To the best of our knowledge, few preclinical data on phosphorus dendrimers have been published; for instance, there is a lack of data on chemical stability and long-term toxicity under good laboratory practices, as well as on the production of phosphorus dendrimers under good manufacturing practices. Strong recommendations for the design of dendrimers towards translation into nanotherapeutics were recently highlighted by several of the authors of this review [77]. In the dendrimer domain, the lack of robust and validated analytical methods for performing PK studies remains a key obstacle to medical translation. To ensure that we keep on making progress, it is crucial that public-health professionals work with researchers from industry to develop 'druggable' dendrimers as well as more-holistic approaches addressing real-world medical demands in a reliable manner. In addition, strategies should be developed to manage the risk, cost and therapeutic benefits of innovative NPs in general, and dendrimers in particular [78]. The development of dendrimers for human therapeutic applications must not remain such a struggle. Generally speaking, we agree with Antoine de Saint-Exupéry's viewpoint in *The Little Prince*: "The only things you learn are the things you tame."

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Figure 1. 2D chemical structure of an example of a G1 phosphorus dendrimer.

Figure 2. Overview of a schematic representation of physical characteristics of nanoparticles for *in vivo* biocompatibility profile. Adapted from [38].

Figure 3. Phosphorus dendrimers as anti-inflammatory agents. **(a)** 2D chemical structure of neutral phosphorus dendrimers I (G3) and II (G4). **(b)** 2D chemical structure of G1 azabisphosphonate (compound 1), G1 azamonophosphonate (compound 2) and G1 bisphosphonate PPI dendrimer (compound 3). **(c)** 2D chemical structure of G3 phosphorus dendrimers DM and DP.

Figure 4. Chemical structure of PD-G3, PD-G4 and PD-G5.

Figure 5. Cationic phosphorus dendrimers for gene therapy. **(a)** 2D chemical structure of 1-G1, 2-G1, 3-G1, 1-G2 and 1-G3 phosphorus dendrimers. **(b)** Formation of 1-G1.HCl–pDNA-p53 polyplexes for *in vivo* gene delivery experiments. **(c)** Western blot assay indicating the expression of the proteins related to G0/G1 phase in xenografted HeLa tumor cells at 4 days after treatment with normal saline (NS), 1-G1.HCl, free

pDNA-p53 or 1-G1.HCl–pDNA-p53 polyplexes (20 µg pDNA/mouse for pDNA groups). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. **(d)** Quantitative analysis of the G0/G1 phase-related protein expression level from the western blot data *in vivo.* Reproduced, with permission, from [71]. Copyright 2020 ACS Publications.

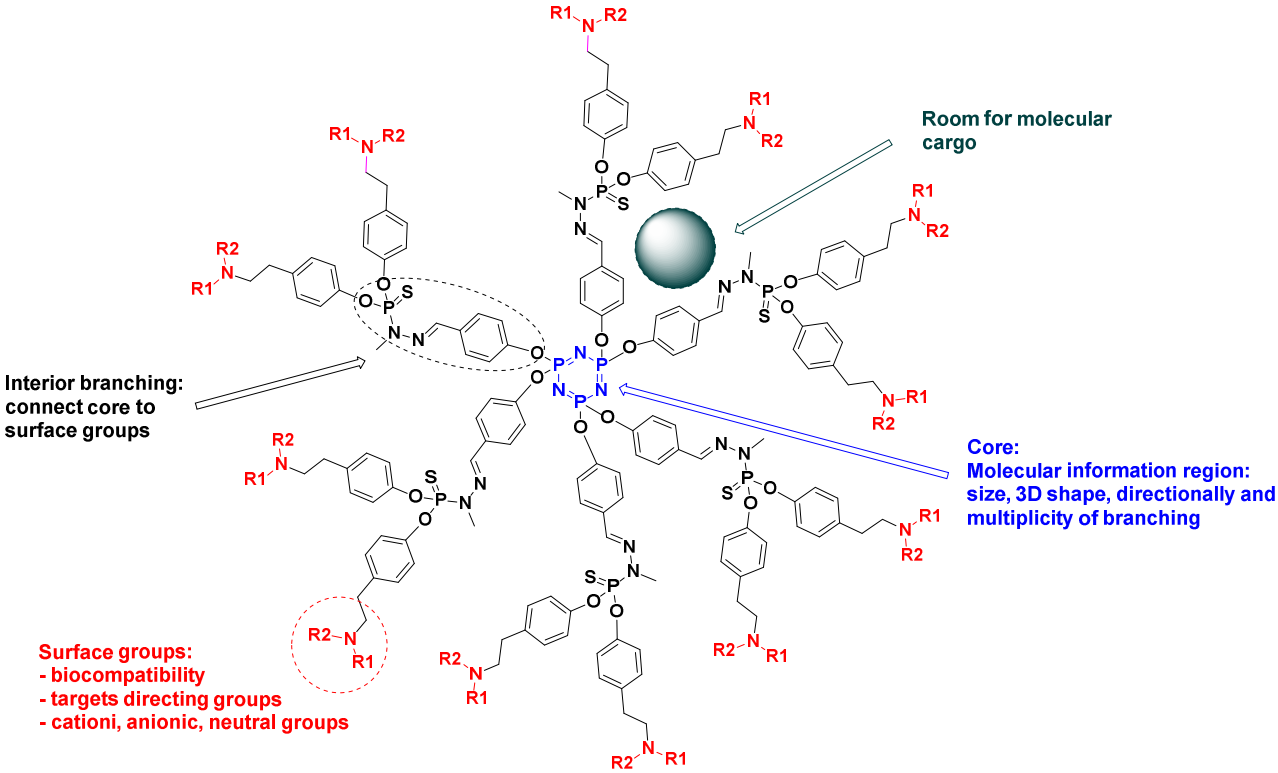
Figure 6. Schematic representation of the development of 1G3-Cu phosphorus dendrimers to tackle tumors using a UTMD-enhanced MR imaging strategy. Reproduced, with permission, from [72]. Copyright 2020 Elsevier.

Figure 7. Phosphorus dendrimers as two-photon tracers for non-invasive *in vivo* imaging studies**. (a)** 2D chemical structure of phosphorus dendrimers G1, G2 and G3. **(b)** Chemical structure of G2 fluorescent phosphorus dendrimers (compound I).

Table 1. Photophysical properties of the chromophore Q and the dendrimers G1–G3

Compound	**Solvent**	**$\lambda_{abs,max}$ (nm)**	**ε_{max} (10^4 $M^{-1}cm^{-1}$)**	**λ_{em} (nm)**	**Φ (fluorescence quantum)**	**σ_2 (GM)[a]**
Q	EtOH	379	7.80	435	0.79	155 (8 in water)
G1	Water	377	7.27	442	0.53	104
G2	Water	381	6.24	444	0.71	119
G3	Water	383	6.31	443	0.66	127

[a]At 705 nm, 1 GM = 10^{-50} cm^4s $photon^{-1}$.


Room for molecular cargo
Interior branching: connect core to surface groups
Core:
Molecular information region: size, 3D shape, directionally and multiplicity of branching
Surface groups:
- biocompatibility
- targets directing groups
- cationi, anionic, neutral groups

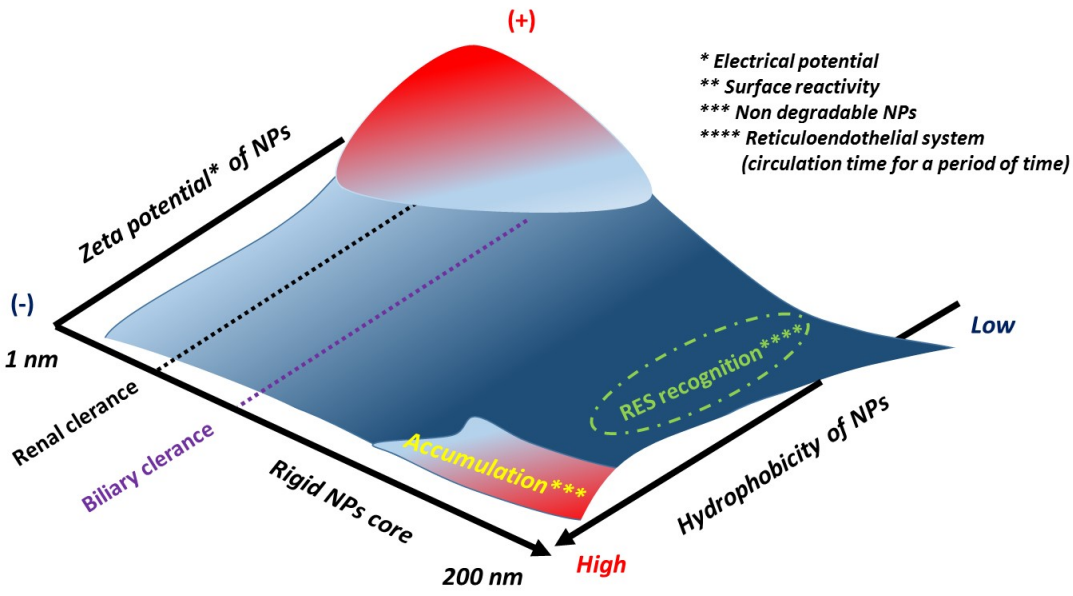

(+)
* Electrical potential
** Surface reactivity
*** Non degradable NPs
**** Reticuloendothelial system
(circulation time for a period of time)
Zeta potential* of NPs
(-)
1 nm
Renal clerance
Biliary clerance
Rigid NPs core
200 nm
Accumulation***
RES recognition****
Low
High
Hydrophobicity of NPs

Figure 3a:

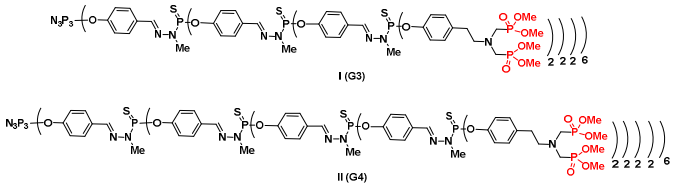


Figure 3c:

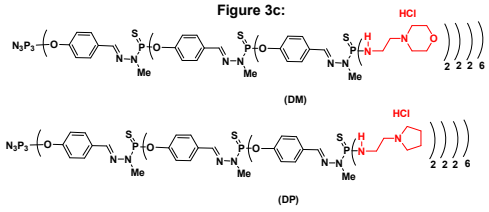
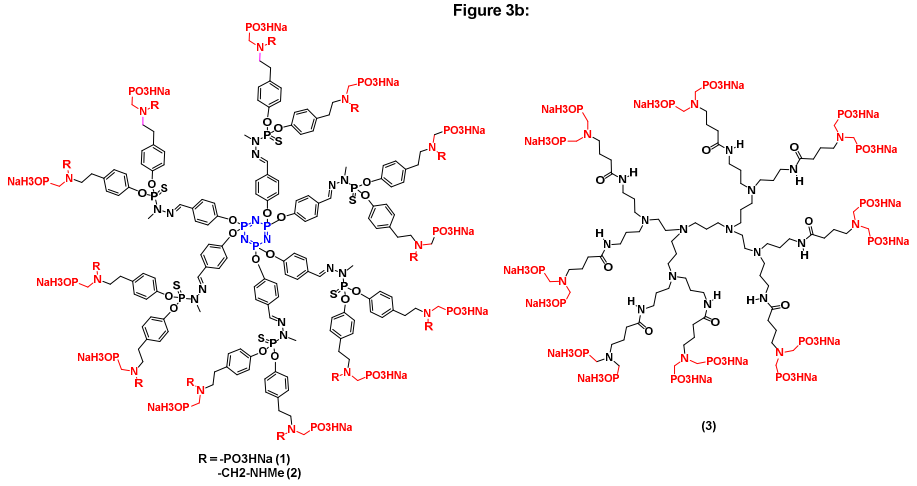
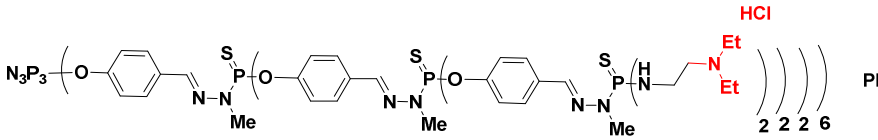


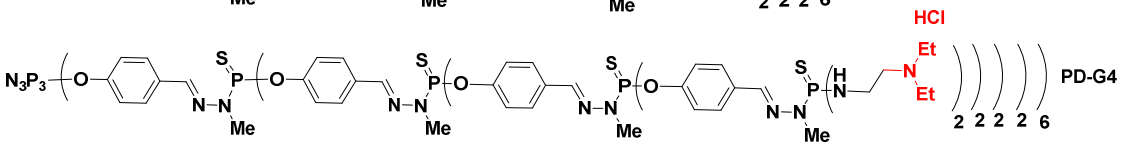
Figure 3b:



R = -PO3HNa (1)
-CH2-NHMe (2)

.


HCl
Et
Et
PD-G3
2 2 2 6


HCl
Et
Et
PD-G4
2 2 2 2 6

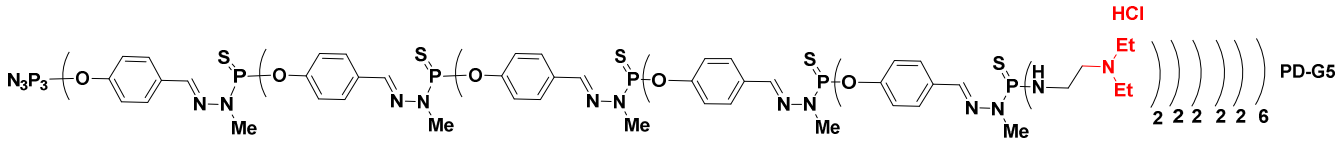

HCl
Et
Et
PD-G5
2 2 2 2 2 6

Figure 5a:

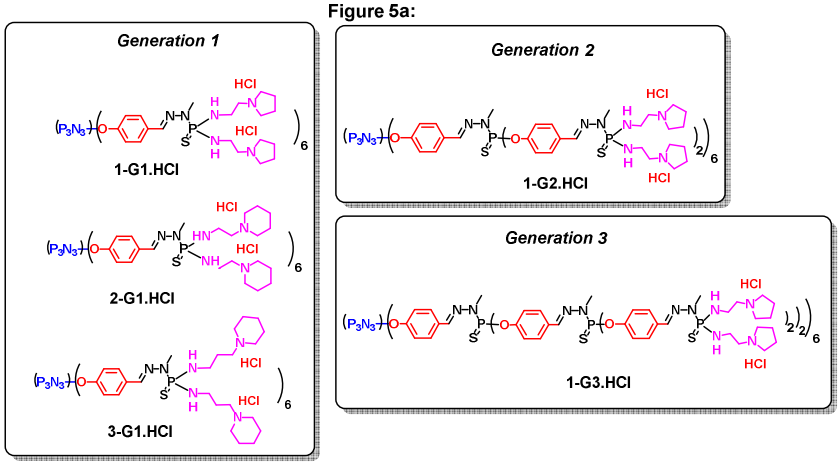
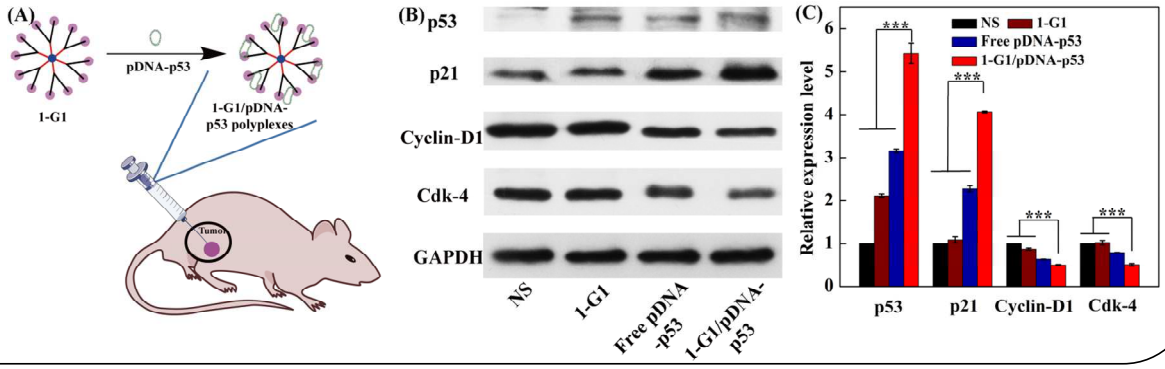


Figure 5b:



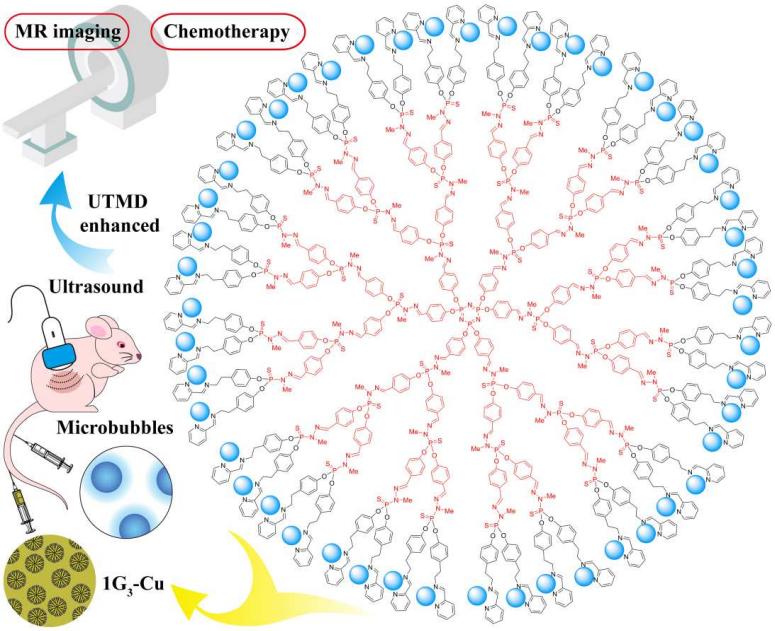

MR imaging
Chemotherapy
UTMD
enhanced
Ultrasound
Microbubbles
1G3-Cu

Figure 7a:

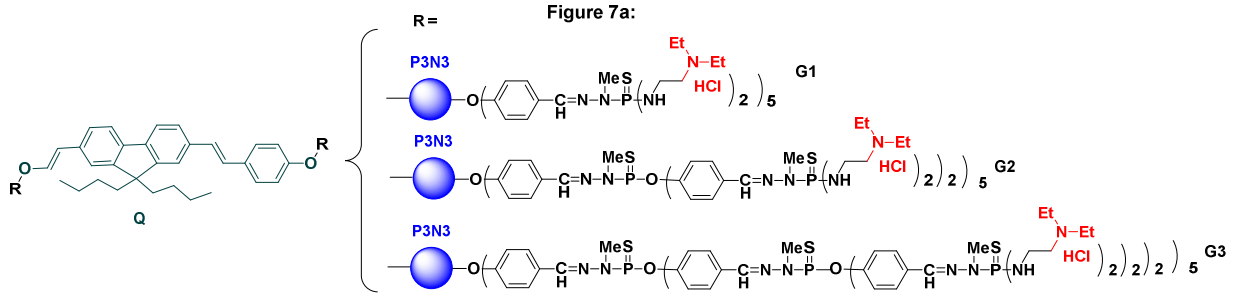
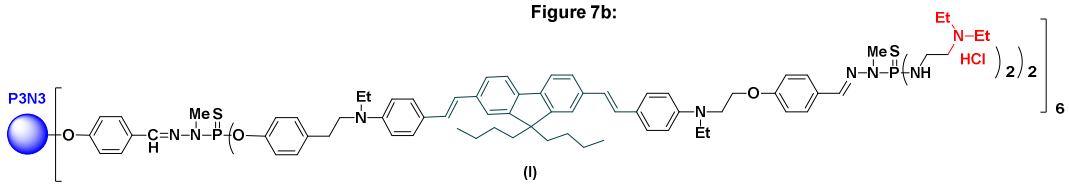
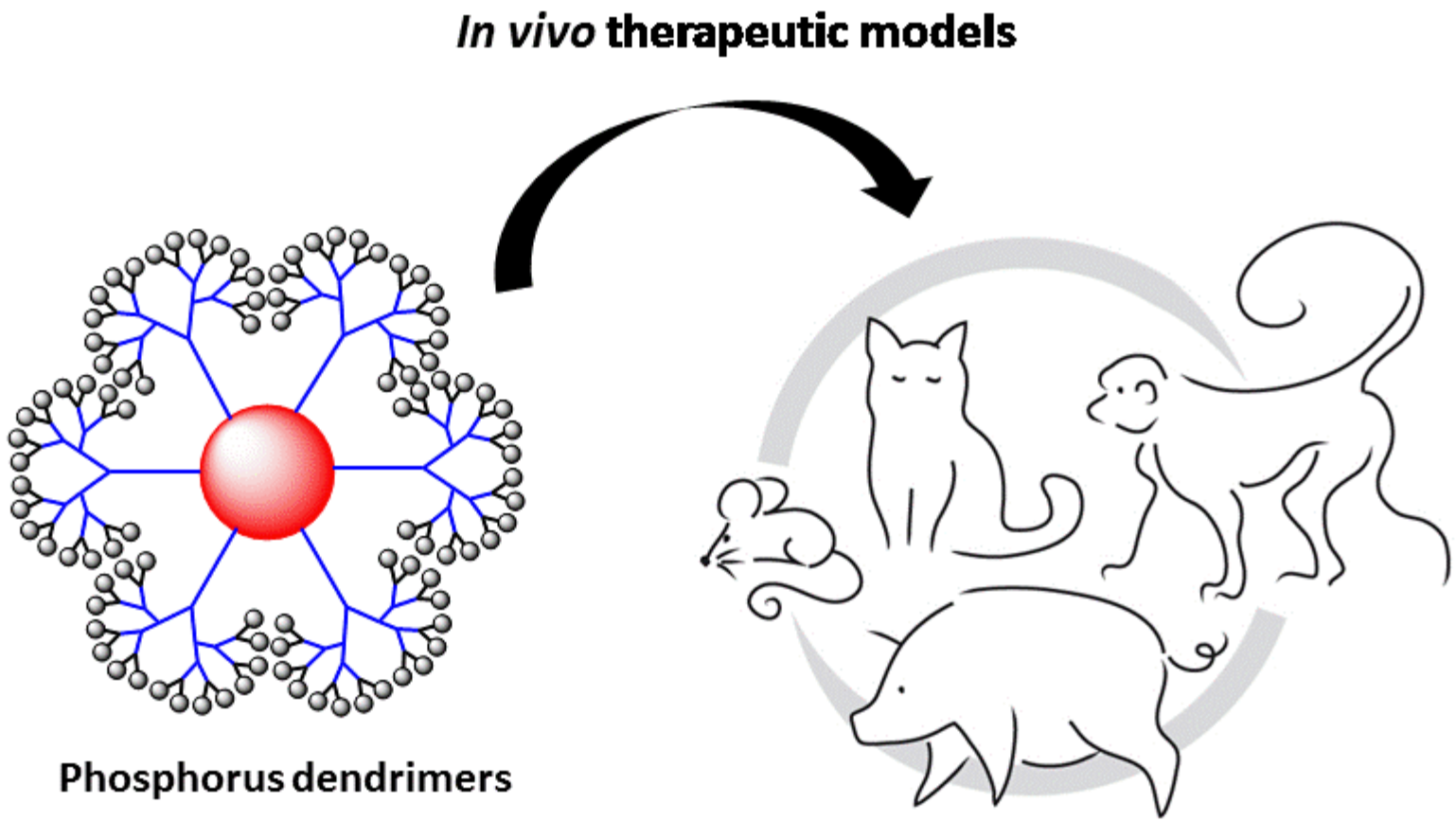


Figure 7b:




In vivo therapeutic models
Phosphorus dendrimers