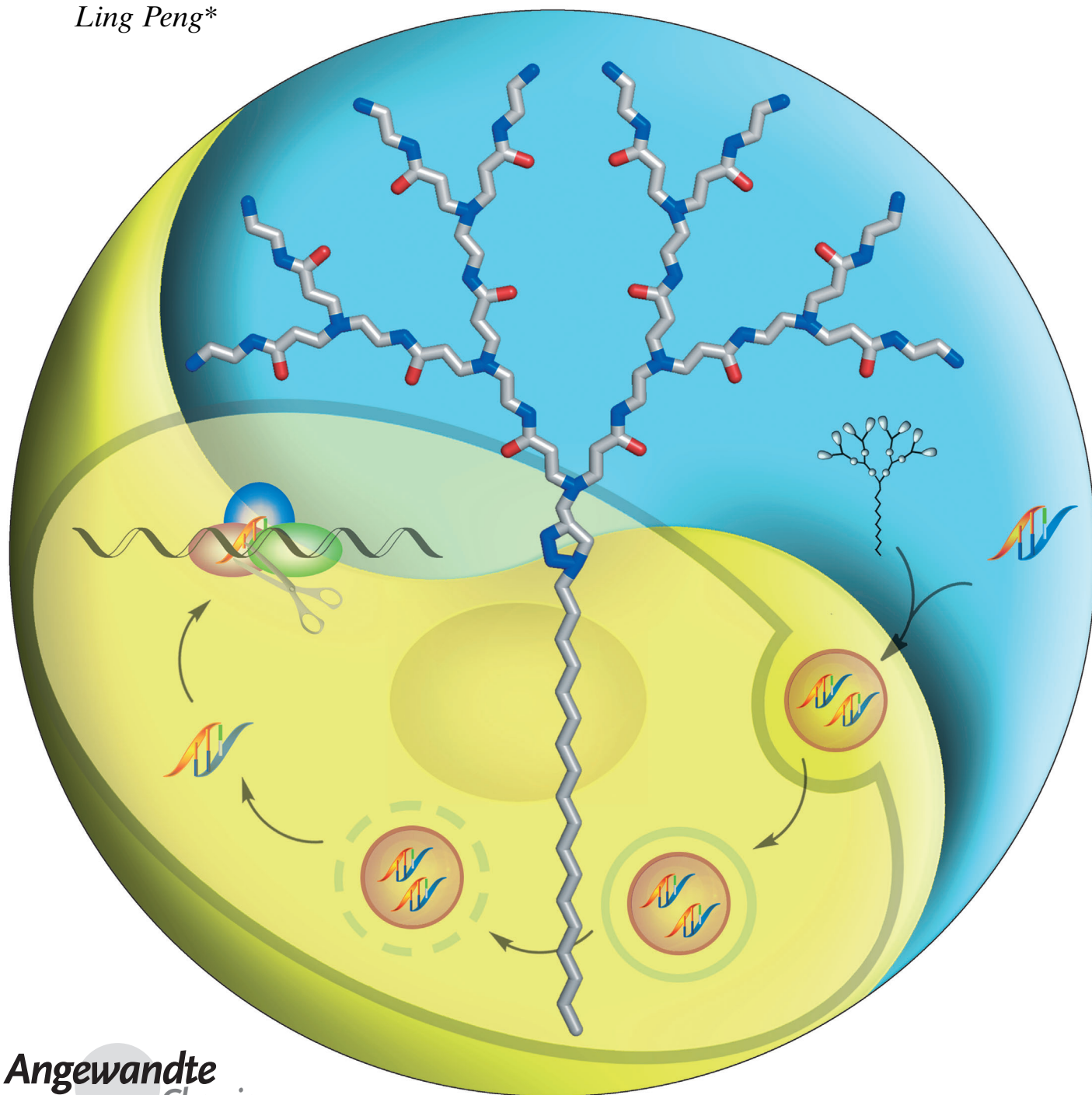


An Amphiphilic Dendrimer for Effective Delivery of Small Interfering RNA and Gene Silencing In Vitro and In Vivo**

Tianzhu Yu, Xiaoxuan Liu, Anne-Laure Bolcato-Bellemin, Yang Wang, Cheng Liu, Patrick Erbacher, Fanqi Qu, Palma Rocchi, Jean-Paul Behr, and Ling Peng*



The potent ability of small interfering RNA (siRNA) to specifically and efficiently inhibit the expression of complementary RNA transcripts offers a promising alternative therapeutic approach for various diseases.^[1] However, the major challenge facing siRNA-based therapy is the requirement of a safe and efficient delivery system for the siRNA.^[2] Although viral vectors are highly efficient, their potential inflammatory, immunogenic, and mutagenic effects make them a safety risk and underline the urgent need for nonviral alternatives. Cationic lipids and polymers are the most common nonviral vectors: both are able to assemble the siRNA, through electrostatic interaction, into stable complexes, which can protect the siRNA from degradation and promote cell uptake.^[3–5] But inadequate release of siRNA into the cytosol often constitutes one of the main obstacles for efficient nonviral delivery. Lipid vectors are presumed to achieve endosome release of siRNA through a membrane fusion mechanism,^[3,4] whereas polymeric vectors commonly use the proton sponge effect^[6] to facilitate endosome escape.^[5] However, lipid vectors have the drawback of being highly toxic for in vivo applications and polymer delivery systems are plagued with undefined structural composition. An ideal nonviral vector would be one that is able to harness

the advantageous features of both lipid and polymer vectors, while at the same time overcome or reduce their limitations.

Cationic dendrimers, a special family of polymers with well-defined molecular architecture, precisely controlled chemical structure, and unique multivalent properties, are emerging as promising nonviral vectors for siRNA delivery.^[7,8] We have recently demonstrated that high generations of structurally flexible triethanolamine (TEA) core poly(amidoamine) PAMAM dendrimers^[9,10] are effective vectors for siRNA delivery^[11] in various disease models.^[12–15] Here we disclose that a small amphiphilic dendrimer acts as an efficient vector to deliver siRNA in a prostate cancer model and achieve potent gene silencing and anticancer activity in vitro and in vivo. This dendrimer, a kind of lipid/dendrimer hybrid bearing a hydrophobic long alkyl chain and a low-generation hydrophilic PAMAM dendron, displays the advantageous delivery features of both lipid and polymer vectors. To our knowledge, this is the first report of an amphiphilic dendrimer able to successfully deliver siRNA and produce a potent gene-silencing effect in vitro and in vivo. It may constitute a promising nonviral system for siRNA delivery in future applications of therapeutic RNAs.

The motivation behind us developing amphiphilic PAMAM vectors for siRNA delivery is based on the reasoning that an improved and safer delivery may be achieved by combining the special features of lipid and dendrimer vectors. We thus designed amphiphilic PAMAM dendrimers **1–3** (Scheme 1) bearing different alkyl chain length and dendron size as hybrid lipid/dendrimer vectors to retain and exploit the properties of both lipid and dendrimer vectors. These dendrimers were synthesized readily using click chemistry (see Scheme S1 in the Supporting Information), and their ability to deliver siRNA and induce gene silencing was first screened on the basis of luciferase gene silencing in A549Luc cells which stably express the GL3 luciferase gene. Among the amphiphilic dendrimers **1–3**, **1a** allowed an exceptionally powerful gene silencing with the specific GL3Luc siRNA in serum-free medium, whereas no gene silencing was observed using the nonspecific GL2Luc siRNA bearing three mismatches (Figure 1A). The observed excellent siRNA delivery capacity of **1a** was further confirmed using **1a** to deliver, respectively, a siRNA targeting heat shock factor 1 (HSF1) (see Figure S1 in the Supporting Information),^[16,17] and a siRNA targeting heat shock protein 27 (Hsp27)^[12] in human castration-resistant prostate cancer PC-3 cells which led to potent down-regulation at both the messenger RNA (mRNA) and protein levels (Figure 1B,C) with strength comparable to the commercial vector Oligofectamine (Figure S2). Collectively, these results demonstrate that **1a** mediated a particularly specific and efficient siRNA-based gene silencing. Moreover, no notable toxicity was observed using either the lactate dehydrogenase (LDH) assay to measure membrane damage (Figure 1D) or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test to assess the metabolic toxicity (Figure 1E and Figure S3), revealing an excellent toxicity/activity profile for **1a**. We further optimized the **1a**-mediated siRNA delivery by varying the siRNA concentration (Figure S4A) as well as the N/P ratio (= [terminal amines in the dendrimer]/[phosphates

[*] T. Yu, Dr. X. Liu, Prof. F. Qu

State Key Laboratory of Virology, College of Chemistry and Molecular Sciences, Wuhan University
430072 Wuhan (China)

Dr. X. Liu, Dr. P. Rocchi

Centre de Recherche en Cancérologie de Marseille
INSERM U1068, CNRS UMR 7258, Institut Paoli-Calmettes
Aix-Marseille Université, 13009 Marseille (France)

Dr. A.-L. Bolcato-Bellemin, Dr. P. Erbacher

Polyplus-transfection SA, Bioparc
Boulevard S. Brandt, BP90018, 67401 Illkirch (France)

Dr. J.-P. Behr

Laboratoire de Chimie Génétique, Faculté de Pharmacie
Université de Strasbourg, CNRS UMR7199
74 Route du Rhin, 67401 Illkirch (France)

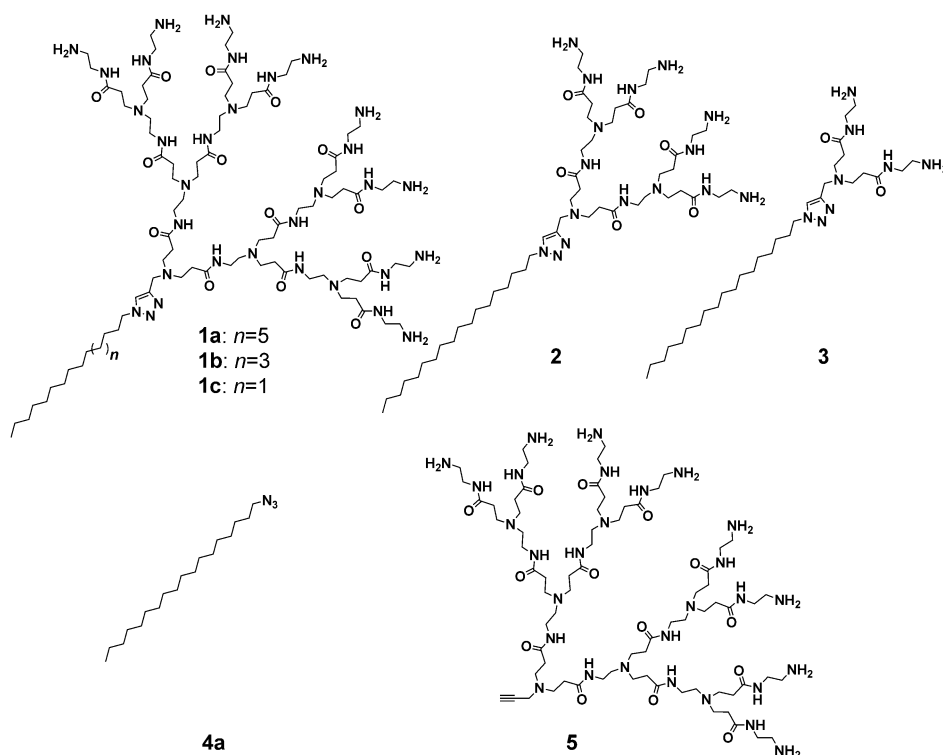
T. Yu, Y. Wang, Dr. L. Peng

Centre Interdisciplinaire de Nanoscience de Marseille
CINaM CNRS UMR 7325, Aix-Marseille Université
163 avenue de Luminy, 13288 Marseille (France)
E-mail: ling.peng@univmed.fr

[**] Financial support from the National Mega Project on Major Drug Development (grant number 2009ZX0930-014), the Ministry of Science and Technology, the international ERA-Net EURONA-NOMED European Research project DENANORNA, the Agence National de la Recherche, the Association Française contre les Myopathies, the Association pour la Recherche sur les Tumeurs de la Prostate (L.X.X.), La bourse Eiffel (Y.T.Z.), the China Scholarship Council (W.Y.), the Wuhan University, CNRS, INSERM, Cancéropôle PACA and the European COST Action (grant number TD0802, "Dendrimers in Biomedical Applications"). We are grateful to Dr. Juan L. Iovanna for his kind hospitality while performing this work, Dr. Claudia Andrieu for her help with the ethidium bromide exclusion assay, Ms. Marie-Noëlle Lavaut and Prof. Stéphane Garcia for the immunohistochemistry and relevant analyses. Y.T.Z. and L.X.X. contributed equally to this work.



Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201203920>.



Scheme 1. Amphiphilic PAMAM dendrimers **1–3** and their precursors **4a** and **5**.

in the siRNA]) between **1a** and the siRNA (Figure S4B). Effective gene silencing was achieved even in 10 % serum-containing medium using chloroquine together with increasing N/P ratios (Figure S4C) and was maintained after seven days (Figure S4D), demonstrating the superiority of **1a** over the structurally flexible TEA core PAMAM dendrimers working at a concentration of 50 nm.^[11,12] In addition, the preparation of the amphiphilic dendrimer **1a** bearing a low-generation dendron is convenient and reliable and thereby constitutes another obvious advantage over high generations of TEA core PAMAM dendrimers, the synthesis of which is time-consuming and tedious.^[10,11]

We next confirmed that the excellent delivery ability of **1a** was indeed because of the combined effect of the hydrophobic alkyl chain and the hydrophilic PAMAM dendron, since neither the alkyl chain **4a** nor the dendron **5** alone led to any gene silencing effect (Figure S5). Only the combined effect of the hydrophobic alkyl chain and the cationic hydrophilic PAMAM dendron was able to impart **1a** with a strong capacity to form stable self-assembled complexes with siRNA and completely retard the migration of siRNA in a gel at N/P ratios over 2.5; dendrimer **5** devoid of the alkyl chain could not completely retard siRNA migration even at a N/P ratio of 10 (Figure 2A). This was further confirmed by ethidium bromide exclusion assay which demonstrated that **1a**, but not **5**, was effective at displacing ethidium bromide from its siRNA complexes (Figure S6). Altogether, these data indicate that the long alkyl chain profoundly favors the siRNA/vector assembly and increases complex stability through hydrophobic interaction.^[18–20] The amphiphilic dendrimers with either shorter alkyl chains (**1b** and **1c**) or lower-

generation dendrons (**2** and **3**) failed to elicit significant gene silencing (Figure S7). This indicates that both PAMAM dendron size and alkyl chain length are important factors impacting the delivery efficiency. One explanation could be that the hydrophobic properties imparted by shorter alkyl chains may be insufficient, and similarly that lower-generation dendrons might not provide sufficient multivalency and cooperativity to ensure a stable electrostatic interaction with siRNA molecules.^[21] The molecular composition of **1a** seems therefore to offer an optimal balance between the alkyl-chain-induced hydrophobicity and the dendrimer-structure-induced hydrophilicity and cooperativity, resulting in superior siRNA delivery activity.

A clear and transparent stock solution of **1a** can be prepared at a concentration reaching 13 mM in phosphate saline buffer, although it has a critical micelle concentration of 15 μ M. In addition, **1a** is simple to mix with siRNA in buffer and is easy to handle in terms of siRNA delivery. This is a major advantage over the conventional lipid vectors, which often require special liposome formulation techniques for siRNA incorporation/encapsulation. Furthermore, the siRNA/**1a** complexes formed uniform and compact nanoparticles as revealed by transmission electron microscopy (Figure 2B). These nanoparticles had diameters of around 100 nm, as was further confirmed by dynamic light scattering (DLS, Figure 2C). Moreover, ζ -potential measurement gave positive values of around +20 mV for the siRNA/**1a** complex at a N/P ratio of 10, demonstrating that **1a** was able to form stable colloidal nanoparticles with siRNA. These nanoparticles could protect siRNA from enzymatic degradation (Figure 2D) and promote effective cell uptake (Figure 2E). As the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) is often employed as a helper lipid to enhance the delivery ability of lipid vectors,^[22] we wished to test its capacity to enhance **1a**-mediated siRNA delivery. To this end, we added DOPE to the **1a** delivery system. In the absence of DOPE, 10 nm siRNA with **1a** did not generate strong gene silencing (Figure 2F); whereas in the presence of DOPE, the corresponding gene silencing was significantly increased. Altogether, these findings strongly support our motivation to introduce a long alkyl chain in **1a**.

Since PAMAM dendrimer vectors are presumed to release their nucleic acid cargo and escape from endosomes through the proton sponge effect because of the protonation of their interior tertiary amines,^[23] we wanted to check

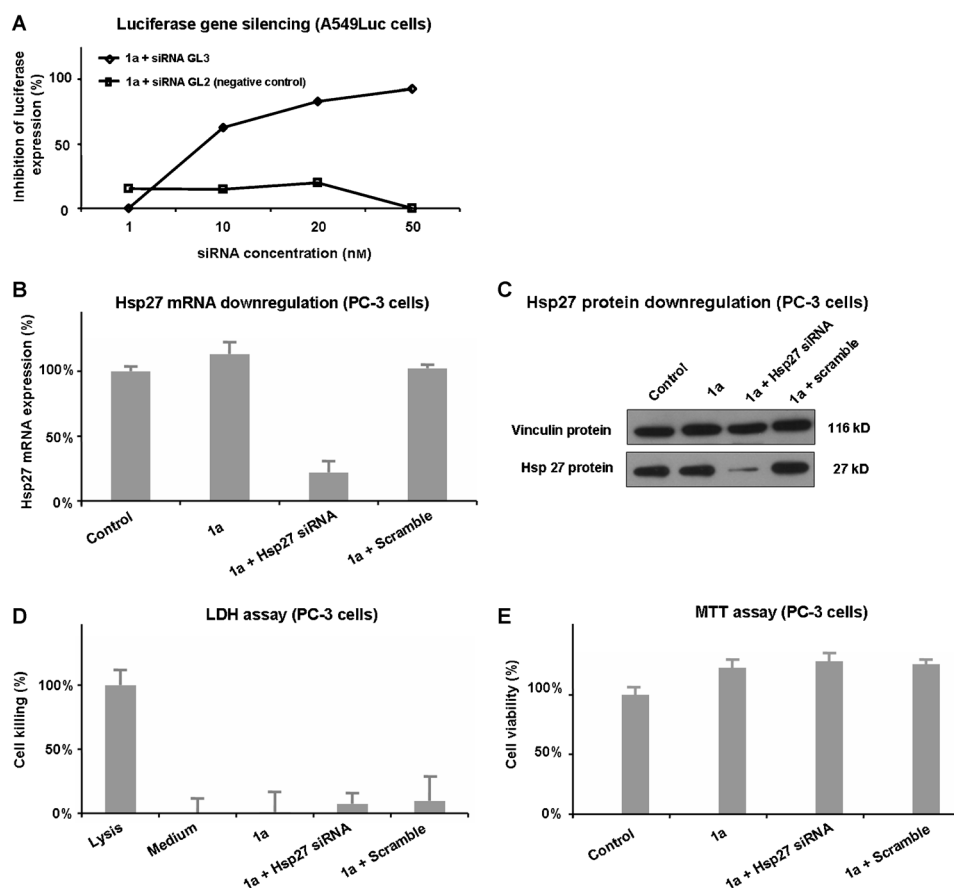


Figure 1. The amphiphilic dendrimer **1a** mediated siRNA delivery and gene silencing of luciferase in A549Luc cells (A) and heat shock protein 27 (Hsp27) in human prostate cancer PC-3 cells (B and C). No toxicity was observed using D) the LDH assay and E) MTT test. A) Inhibition of luciferase gene expression determined using luciferase assay system 48 h post-treatment with different siRNA concentrations and **1a** at a N/P ratio of 10. B) Hsp27 mRNA expression evaluated using quantitative real-time qRT-PCR 48 h post-treatment, and C) Hsp27 protein expression quantified using Western blot 72 h post-treatment with 20 nM siRNA and **1a** at a N/P ratio of 10. D) Cell killing through membrane damage was measured using the LDH assay, and E) cell viability was assessed using the MTT assay for **1a**-mediated siRNA delivery to PC-3 cells with 50 nM siRNA and **1a** at a N/P ratio 10, compared to no treatment control, **1a** alone, and scramble siRNA/**1a** 24 h post-treatment.

whether this mechanism was operating in **1a**-mediated siRNA delivery. We hence examined the gene silencing effect in the presence of bafilomycin A1. Bafilomycin A1 is a proton pump inhibitor which selectively inhibits vacuolar H^+ -ATPase and prevents the acidification of endosomes, and can be used to study the proton sponge effect. As shown in Figure 3A, bafilomycin A1 was able to significantly decrease the gene silencing efficiency, suggesting that the **1a**-mediated siRNA delivery was dependent on the endosomal acidification process and that the proton sponge effect was indeed involved in **1a** mediated siRNA delivery. This can be reasonably understood by the dendron structure of **1a**, which harbors seven tertiary amines in the interior and eight primary amines at the surface, able to act as a buffer collectively. Involvement of the proton sponge effect was further supported by the pH titration profile of **1a** shown in Figure 3B: at physiological pH 7.4, 55% amine nitrogen atoms (8 out of 15 total nitrogen atoms) of **1a** are protonated,

corresponding presumably to the eight primary amines at the dendrimer ends; whereas, at acidic endosomal pH 5.0, 83% amine nitrogen atoms (13 out of 15 total nitrogen atoms) of **1a** are protonated, implying that a further five tertiary amines in the dendrimer interior are protonated in addition to the eight terminal primary amines. Consequently, **1a** is able to perform the proton sponge effect for realizing endosomal release. All in all, these data provide strong evidence that the amphiphilic dendrimer **1a** indeed harnesses the characteristic and beneficial properties of both lipid and dendrimer vectors, the combination of which contributes towards ensuring an efficient siRNA delivery and gene silencing.

Encouraged by these results, we next evaluated the capacity of **1a**-mediated siRNA delivery to silence Hsp27 and its resulting anticancer activity in prostate cancer models in vitro and in vivo. Hsp27 is a molecular chaperone playing an important role in drug resistance and has been recently considered as a novel target for treating drug-resistant prostate^[24,25] and other cancers.^[26,27] Following treatment with Hsp27 siRNA/**1a** complexes, an effective gene silencing (Fig-

ure 1B,C) and the resulting antiproliferation effect on human castration-resistant prostate cancer PC-3 cells were achieved (Figure 4A), alongside the observation of apoptosis (Figure 4B) induced by caspase activation (Figure 4C). In vivo gene silencing and anticancer activity was further assessed using nude mice bearing PC-3 xenograft tumors: Hsp27 was effectively down-regulated at mRNA and protein levels (Figure 4D,E) as revealed by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot, respectively. Further immunohistochemistry with Ki67 staining showed considerable inhibition of tumor proliferation following treatment with Hsp27 siRNA/**1a** as compared to a **1a**/scramble treatment (Figure 4F). Collectively, our results demonstrate that the amphiphilic dendrimer **1a** can successfully mediate the effective delivery of siRNA to down-regulate Hsp27 and produce potent anticancer activity in vivo. To our knowledge, this is the first report of an amphiphilic dendrimer able to effectively deliver siRNA and

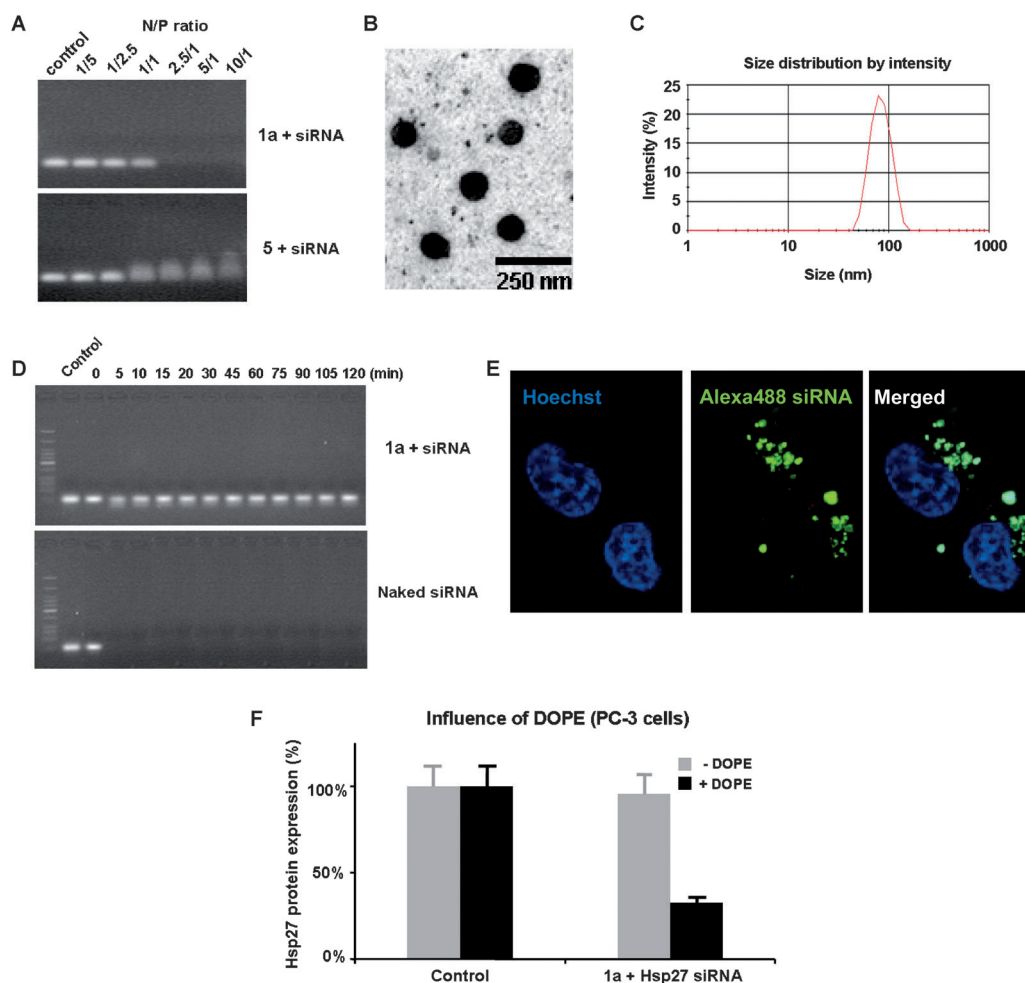


Figure 2. A) Gel retardation of 200 ng siRNA in agarose gel with **1a** and **5**, respectively, at N/P charge ratios ranging from 1:5 to 10:1. Formation of nanosized siRNA/**1a** complexes at a N/P ratio of 10 revealed by **B**) transmission electron microscopy and **C**) dynamic light scattering. **D**) The resulting siRNA/**1a** complexes protect siRNA from RNase degradation. **E**) Uptake of Alexa488-labeled siRNA/**1a** complexes at a N/P ratio of 10 in PC-3 cells analyzed by live-cell-confocal microscopy. **F**) Dioleoylphosphatidylethanolamine (DOPE), a fusogenic lipid, enhances the effective gene silencing of Hsp27 in PC-3 cells with 10 nM siRNA and **1a** at a N/P ratio of 10.

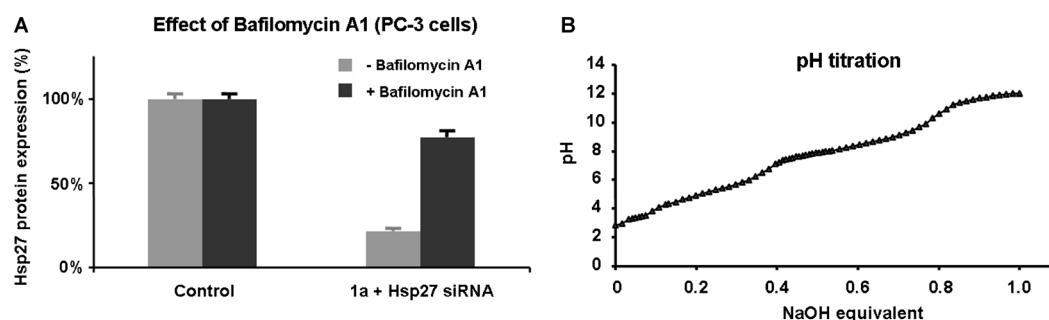


Figure 3. A) Bafilomycin A1, a proton pump inhibitor, decreases the **1a**-mediated gene silencing of Hsp27 observed with 20 nM siRNA and **1a** at a N/P ratio of 10. **B**) Potentiometric pH back titration of the completely protonated form of the amphiphilic dendrimer **1a**.

silence the corresponding gene in vitro and in vivo. Although amphiphilic dendrimers have been previously developed for effective DNA delivery in vitro,^[18–20,28–31] none have been explored and reported for delivery of short synthetic siRNA for gene silencing in vitro and in vivo. Delivery of siRNA differs from that of DNA in several aspects such as the site of

action in the cell, molecular stability, and molecular size. Consequently, the vectors suitable for DNA delivery may not be ideal for siRNA delivery. The amphiphilic dendrimer **1a** disclosed in this work therefore constitutes a significant novelty for effective siRNA delivery and potent gene silencing.

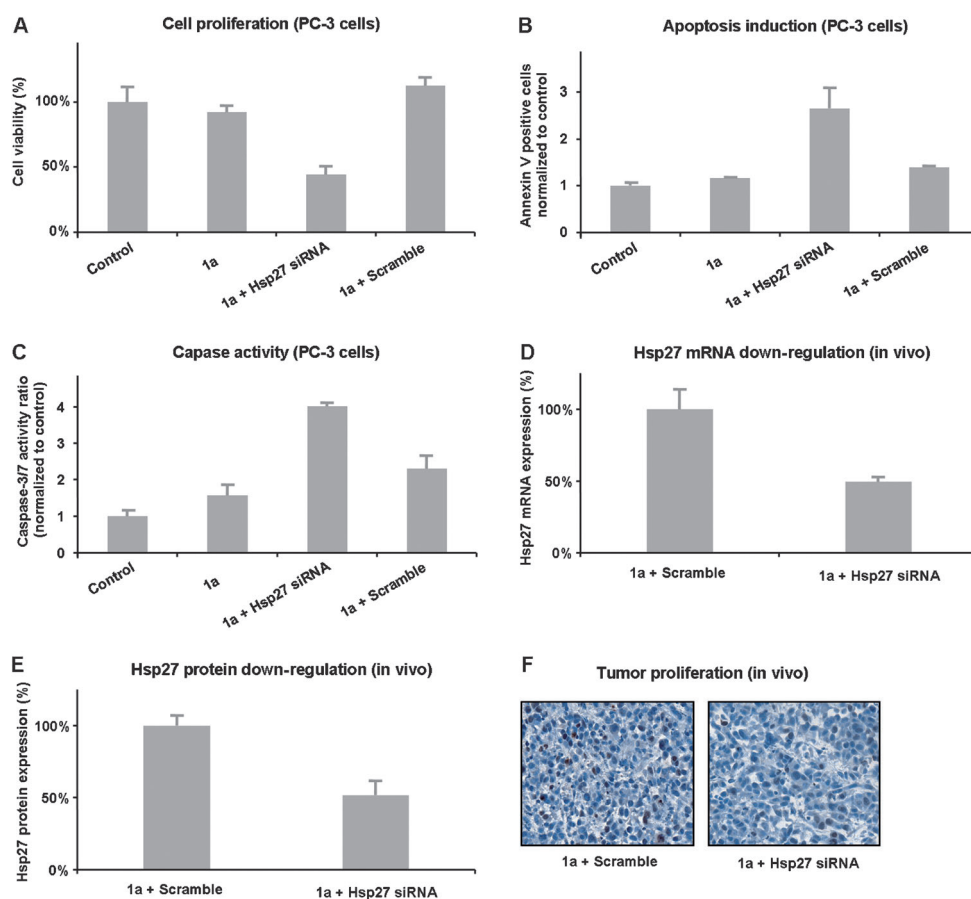


Figure 4. In vitro and in vivo anticancer activity in prostate cancer models following gene silencing of Hsp27 with Hsp27 siRNA/1a complex. Human castrate-resistant prostate cancer PC-3 cells were used for the in vitro assay (A, B, C) with 50 nM siRNA and 1a at a N/P ratio of 10, and PC-3 xenografted nude mice with intratumoral injection of siRNA/1a (3 mg kg⁻¹ siRNA and 1a at a N/P ratio of 5) were used for in vivo evaluation during a period of one week with an injection frequency of twice a week (D, E, F). A) Cell proliferation of human prostate cancer PC-3 cells assessed by MTT assay six days post-treatment. B) Apoptotic cells quantified with FACS flow cytometry using Annexin V staining four days post-treatment. C) Caspase-3/7 activation evaluated using a colorimetric assay three days post-treatment. Effective gene silencing of Hsp27 at both the mRNA level (D) and the protein level (E) as well as the antiproliferation activity (F) in tumors after one week of treatment were assessed using quantitative real-time qRT-PCR, Western blot, and Ki-67 immunohistochemistry staining, respectively.

In conclusion, we have developed an efficient amphiphilic dendrimer vector 1a for siRNA delivery and gene silencing. This vector bears a hydrophobic alkyl chain and a hydrophilic PAMAM dendron with seven tertiary amines in the interior and eight primary amines at the terminals, and is capable of combining the advantageous features of lipid and dendrimer vectors. Most importantly, this vector is able to deliver Hsp27 siRNA in vitro and in vivo in a castration-resistant prostate cancer model and produce significant gene silencing and potent anticancer activity and therefore offers a new alternative for treating castration-resistant prostate cancer, for which there remains no efficacious treatment.^[32] We are currently focusing our efforts on performing a detailed structure/activity relationship analysis to gain a better insight and understanding of this amphiphilic dendrimer vector and will apply it to other disease models based on siRNA therapeutics.

Received: May 21, 2012
Revised: June 25, 2012
Published online: July 24, 2012

Keywords: dendrimers · drug design · gene silencing · prostate cancer · siRNA

- [1] D. Castanotto, J. J. Rossi, *Nature* **2009**, 457, 426.
- [2] K. A. Whitehead, R. Langer, D. G. Anderson, *Nat. Rev. Drug Discovery* **2009**, 8, 129.
- [3] Y. C. Tseng, S. Mozumdar, L. Huang, *Adv. Drug Delivery Rev.* **2009**, 61, 721.
- [4] A. Schroeder, C. G. Levins, C. Cortez, R. Langer, D. G. Anderson, *J. Intern. Med.* **2010**, 267, 9.
- [5] E. Wagner, *Acc. Chem. Res.* **2011**, DOI: 10.1021/ar2002232.
- [6] O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, *Proc. Natl. Acad. Sci. USA* **1995**, 92, 7297.
- [7] X. Liu, P. Rocchi, L. Peng, *New J. Chem.* **2012**, 36, 256.
- [8] M. Ravina, P. Paolicelli, B. Seijo, A. Sanchez, *Mini. Rev. Med. Chem.* **2010**, 10, 73.
- [9] J. Wu, J. Zhou, F. Qu, P. Bao, Y. Zhang, L. Peng, *Chem. Commun.* **2005**, 313.
- [10] X. Liu, J. Wu, M. Yammine, J. Zhou, P. Posocco, S. Viel, C. Liu, F. Ziarelli, M. Fermeiglia, S. Pricl, G. Victorero, C. Nguyen, P. Erbacher, J. P. Behr, L. Peng, *Bioconjugate Chem.* **2011**, 22, 2461.
- [11] J. Zhou, J. Wu, N. Hafdi, J. P. Behr, P. Erbacher, L. Peng, *Chem. Commun.* **2006**, 2362.
- [12] X. X. Liu, P. Rocchi, F. Q. Qu, S. Q. Zheng, Z. C. Liang, M. Gleave, J. Iovanna, L. Peng, *ChemMedChem* **2009**, 4, 1302.
- [13] J. Zhou, C. P. Neff, X. Liu, J. Zhang, H. Li, D. D. Smith, P. Swiderski, T. Aboellail, Y. Huang, Q. Du, Z. Liang, L. Peng, R. Akkina, J. J. Rossi, *Mol. Ther.* **2011**, 19, 2228.
- [14] X. Liu, C. Liu, E. Laurini, P. Posocco, S. Pricl, F. Qu, P. Rocchi, L. Peng, *Mol. Pharm.* **2012**, 9, 470.
- [15] M. F. Lang, S. Yang, C. Zhao, G. Sun, K. Murai, X. Wu, J. Wang, H. Gao, C. E. Brown, X. Liu, J. Zhou, L. Peng, J. J. Rossi, Y. Shi, *PLoS One* **2012**, 7, e36248.
- [16] M. Åkerfelt, R. I. Morimoto, L. Sistonen, *Nat. Rev. Mol. Cell Biol.* **2010**, 11, 545.
- [17] Y. Xia, Y. Liu, P. Rocchi, M. Wang, Y. Fan, F. Qu, J. L. Iovanna, L. Peng, *Cancer Lett.* **2012**, 318, 145.
- [18] T. Takahashi, C. Kojima, A. Harada, K. Kono, *Bioconjugate Chem.* **2007**, 18, 1349.
- [19] A. Barnard, P. Posocco, S. Pricl, M. Calderon, R. Haag, M. E. Hwang, V. W. Shum, D. W. Pack, D. K. Smith, *J. Am. Chem. Soc.* **2011**, 133, 20288.

- [20] S. P. Jones, N. P. Gabrielson, C. H. Wong, H. F. Chow, D. W. Pack, P. Posocco, M. Fermeglia, S. Pricl, D. K. Smith, *Mol. Pharm.* **2011**, 8, 416.
- [21] X. C. Shen, J. Zhou, X. Liu, J. Wu, F. Qu, Z. L. Zhang, D. W. Pang, G. Quelever, C. C. Zhang, L. Peng, *Org. Biomol. Chem.* **2007**, 5, 3674.
- [22] H. Farhood, N. Serbina, L. Huang, *Biochim. Biophys. Acta.* **1995**, 1235, 289.
- [23] N. D. Sonawane, F. C. Szoka, Jr., A. S. Verkman, *J. Biol. Chem.* **2003**, 278, 44826.
- [24] P. Rocchi, A. So, S. Kojima, M. Signaevsky, E. Beraldi, L. Fazli, A. Hurtado-Coll, K. Yamanaka, M. Gleave, *Cancer Res.* **2004**, 64, 6595.
- [25] P. Rocchi, E. Beraldi, S. Ettinger, L. Fazli, R. L. Vessella, C. Nelson, M. Gleave, *Cancer Res.* **2005**, 65, 11083.
- [26] A. P. Arrigo, S. Simon, B. Gibert, C. Kretz-Remy, M. Nivon, A. Czekalla, D. Guillet, M. Moulin, C. Diaz-Latoud, P. Vicart, *FEBS Lett.* **2007**, 581, 3665.
- [27] J. Acunzo, M. Katsogiannou, P. Rocchi, *Int. J. Biochem. Cell Biol.* **2012**, DOI: 10.1016/j.biocel.2012.04.002.
- [28] D. Joester, M. Losson, R. Pugin, H. Heinzelmann, E. Walter, H. P. Merkle, F. Diederich, *Angew. Chem.* **2003**, 115, 1524; *Angew. Chem. Int. Ed.* **2003**, 42, 1486.
- [29] T. Takahashi, K. Kono, T. Itoh, N. Emi, T. Takagishi, *Bioconjugate Chem.* **2003**, 14, 764.
- [30] H. K. Bayele, T. Sakthivel, M. O'Donnell, K. J. Pasi, A. F. Wilderspin, C. A. Lee, I. Toth, A. T. Florence, *J. Pharm. Sci.* **2005**, 94, 446.
- [31] K. Kono, R. Ikeda, K. Tsukamoto, E. Yuba, C. Kojima, A. Harada, *Bioconjugate Chem.* **2012**, 23, 871.
- [32] T. A. Yap, A. Zivi, A. Omlin, J. S. de Bono, *Nat. Rev. Clin. Oncol.* **2011**, 8, 597.