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Targeting cyclin B1 through peptide-based delivery of siRNA prevents tumour growth

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ABSTRACT

The development of short interfering RNA (siRNA), has provided great hope for therapeutic targeting of specific genes responsible for pathological disorders. However, the poor cellular uptake and bioavailability of siRNA remain a major obstacle to their clinical development and most strategies that propose to improve siRNA delivery remain limited for *in vivo* applications. In this study, we report a novel peptide-based approach, MPG-8 an improved variant of the amphipathic peptide carrier MPG, that forms nanoparticles with siRNA and promotes their efficient delivery into primary cell lines and *in vivo* upon intra-tumoral injection. Moreover, we show that functionalization of this carrier with cholesterol significantly improves tissue distribution and stability of siRNA *in vivo*, thereby enhancing the efficiency of this technology for systemic administration following intravenous injection without triggering any non-specific inflammatory response. We have validated the therapeutic potential of this strategy for cancer treatment by targeting cyclin B1 in mouse tumour models, and demonstrate that tumour growth is compromised. The robustness of the biological response achieved through this approach, infers that MPG 8-based technology holds a strong promise for therapeutic administration of siRNA.

INTRODUCTION

Although the *in vivo* delivery of siRNA has been successfully achieved thanks to several non-viral delivery systems, systemic administration of siRNA remains a major challenge for their therapeutic application (1–5). Over the last

decade, cell-penetrating peptides (CPPs) have been shown to improve intracellular delivery of various biologically active molecules into living cells quite efficiently (6,7) and have more recently been applied to siRNA delivery (8,9). Although conjugation strategies with either Transportan, Penetratin or Tat certainly improve the delivery of siRNA into cultured cells, non-covalent strategies are more appropriate for both *in cellulo* and *in vivo* delivery of siRNA (8,10–17). Short amphipathic cell-penetrating peptides forming non-covalent, yet stable complexes with their cargo have been successfully applied to the delivery of different macromolecules into mammalian cells (18,19). In particular, the peptide carrier MPG efficiently delivers siRNA in a fully biologically active form into a wide variety of cell lines, including embryonic stem cells (10,11,20). In the present study we describe a novel strategy which promotes efficient delivery of siRNA *in vivo*, using functionalized peptide-based nanoparticulate formulations. We have developed a shorter version of MPG, MPG-8, that forms stable particles with siRNA, and that can be functionalized. MPG-8 improves siRNA delivery *ex vivo* and *in vivo* without activating the innate immune response. We have validated the potential of this technology by targeting cyclin B1, a non-redundant mitotic partner of cyclin-dependent kinase 1 (cdk1) (21) and report that MPG-mediated administration of siRNA targeting cyclin B1 prevents tumour growth in xenografted tumour mouse models. Cyclin B1 constitutes a key target for anti-proliferative strategies and both anti-sense and siRNA-based-approaches targeting cyclin B1 have been proposed as an anti-proliferative strategy (22–24). Cyclin B1 together with Cdk1 kinase, forms the ‘mitosis promoting factor’, whose activity is required for entry into and progression through mitosis (21). Cdk1 is essential for mammalian cell division, its knockout is lethal and several small molecule inhibitors targeting Cdk1 have been reported to induce arrest in G2 and to reduce tumour growth (22,24). Likewise, altered expression of cyclin B1 has been reported in numerous cancers

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MATERIALS AND METHODS

Peptides and siRNAs

All peptides were synthesized and purified as previously described (10,25). Cholesterol conjugation to MPG-8 was performed at the primary amino group of the N-terminal residues, then cholesterol-MPG-8 was further purified by Reverse Phase-HPLC and analysed by electro-spray ionization mass spectroscopy (25). siRNAs and 5' Alexa⁷⁰⁰ fluorescently labelled siRNA were synthesized by Eurogentec according to the following sequences.

Cyc-B1 sense 5'-GGCGAAGAUAACAUGGCATT-3'
 Cyc-B1 anti-sense 5'-UGCCAUGUUGAUCUUCGCTT-3'
 Cyc-B3 sense 5'-GGUGAAGAUCAGCAUGGCATT-3'
 Cyc-B3 anti-sense 5'-UGCCAUGUCGAUCUUCACCTT-3'
 GAPDH sense 5'-CAUCAUCCUGCCUCUACUTT-3'
 GAPDH anti-sense 5'-AGUAGAGGCAGGAUGAUG-3'.

Cyc-B1 siRNA targeting cyclin B1, and a derived siRNA harbouring two mismatches, Cyc-B3, was used as control. An siRNA targeting GAPDH was used as control to validate target specificity and to monitor associated non-specific interferon response.

Cell culture and MPG-mediated transfection

Adherent HS68 fibroblasts, HeLa, PC3, MCF-7 and SCK3-Her2 cell lines [from American Type Culture Collection (ATCC)] were cultured in Dulbecco's Modified Eagle's Medium supplemented with 2-mM glutamine, 1% antibiotics (streptomycin 10 000 µg/ml, penicillin, 10 000 IU/ml) and 10% (w/v) fetal calf serum (FCS), at 37°C in a humidified atmosphere containing 5% CO₂. Stock solutions of MPG-8/siRNA and MPG^{ΔNLS}/siRNA particles were prepared by complexing 100 nM siRNA with MPG peptides at a molar ratio of 1/20 for 30 min at 37°C. Lower concentrations of MPG-carrier/siRNA (from 20 nM to 0.125 nM) were obtained by serial dilution of the stock complexes in PBS, in order to preserve the same MPG-carrier/siRNA ratio. A total of 150 000 cells seeded in a 35-mm dish the day prior transfection, were grown to 60% confluence and overlaid with 200 µl of preformed complexes, incubated for 3–5 min, then 400 µl of DMEM were added. After a 30-min incubation at 37°C, 1 ml of fresh DMEM containing 16% FCS was added in order to reach a final FCS concentration of 10%, without removing the overlay of MPG-8/siRNA complexes. Cells were returned to the incubator for 24 h. Cyclin B1 mRNA and protein levels were determined 12 and 24 h following transfection, using Quantigen (Panomics Inc.) and western blotting, respectively. Mouse monoclonal anti-Cyclin B1 antibodies (SC-245) and rabbit polyclonal anti-Cdk2 antibodies (SC-163) were obtained from Santa Cruz Biotechnology Inc. Data reported are an average of three or four distinct experiments.

Mouse tumour models

Athymic female nude mice (6–8 weeks of age) were subcutaneously inoculated into the flank with 1×10^6 PC3 or SCK-3-HEK2 cells in 100 µl PBS. Two to three weeks after tumour implant, when tumour size reached about

100 mm³, animals were treated by intra-tumoral or intravenous injection, every 3 days, with a solution of 0.1 ml of either free Cyc-B1 siRNA (50 or 100 µg), control siRNA Cyc-B3 or Cyc-B1 siRNA (1, 5, 10 µg) complexed with MPG-8 or MPG-8/Chol-MPG-8 at a 1/20 molar ratio. Formulations containing 15% Chol-MPG-8 were prepared in a stepwise fashion by first forming a precomplex of MPG-8/siRNA at molar ratio of 1/20, followed by addition of Chol-MPG-8 so as to increase the ratio of siRNA/carrier to 1/25. Tumour diameter was measured in two directions at regular intervals using a digital calliper and tumour volume was calculated as length × width × height × 0.52. Curves show the mean value of tumour size in a cohort of six animals and neither animal death nor any sign of toxicity was observed. Experiments were performed according to national regulations and approved by the local animal experimentation ethical committee. The statistical significance of the results was calculated by Student's *t*-test and *P* < 0.05 considered to be statistically significant.

Characterization of peptide-based nanoparticles

Mean particle size distribution was determined with a Coulter N4 Plus (Coulter-Beckman) at 25°C for 3 min per measurement and zeta potential was measured with Zetasizer 4 apparatus (Malvern Ltd.) as previously described (24).

Cytotoxicity

Toxicity of MPG-8/siRNA complexes was investigated on HeLa and HS-68 cell lines. A total of 30 000 cells seeded in 24-well plate the day prior transfection, were incubated with increasing concentrations of siRNA complexed with MPG-8 at a 20/1 molar ratio ranging from 0.1 to 5 µM (100 µM MPG-8), for 30 min prior to addition of medium to reach a final 10% concentration of FCS. Cytotoxic response was measured 12 h or 24 h later by monitoring the housekeeping gene cyclophilin mRNA level (Quantigen, Panomic Inc.) and by colorimetric MTT assay (Sigma, Germany), respectively. For MTT assay, cell culture medium was removed and replaced with PBS containing 2.5 mg/ml of MTT for 4 h. Results correspond to the average of three separate experiments.

Interferon assay

Interferon response was evaluated on cultured cells by quantitative RT-PCR monitoring induction of INF-β and of Interleukin 8 (IL8). HeLa, SCK3-Her2 and MCF7 cells were mock transfected or transfected with MPG-8 MPG-8/Chol-MPG-8/siRNA complexes (100 nM) or poly(I:C) (5 µg/ml). After 24 h, RNA was isolated using Trizol (Sigma), and analysed by quantitative RT-PCR, standardized to GAPDH mRNA. Quantitative PCR was performed on a Biorad iCycler using primer sequences for IL8 (forward) 5'-GTGCAGTTTTGCCAAGGACT3' and INF-β (forward) 5'-CTGGAGCAGCTGAATGGA AAG-3'. *In vivo* inflammatory response was monitored in the plasma, 6 h after injection of either MPG-8/Chol-MPG-8/siRNA complex, MPG-8, Chol-MPG-8 (0.5 mg/kg) or poly(I:C) (200 µg). The level of mouse cytokine

tissue tumor necrosis factor (TNF- α). Interferon α (INF- α) and interleukin 6 (IL6) were quantified using sandwich ELISA assay kit (BD Biosciences) according to the manufacturer's instructions. The reported results correspond to the average of two separate experiments with four animals per group.

***In vivo* imaging of siRNA biodistribution**

In vivo fluorescence imaging was performed as previously described (26,27). Mice were injected intravenously with 10 μ g (200 μ l) of Alexa⁷⁰⁰ fluorescently labelled siRNA either naked or complexed with MPG-8 or MPG-8Chol/MPG-8 ($n = 3$ animals per group). Anaesthetized mice, using 2% Isoflurane, were illuminated by 663-nm light emitting diodes equipped with interference filters and movies were acquired over the first 15 minutes and fluorescence images were taken every hour for 5 h and then after 24 h, with a back-thinned CCD cooled camera as previously described (26,27). At 24 h, mice were euthanized and different organs were removed for quantification of Alexa fluorescence.

RESULTS AND DISCUSSION

Design and evaluation of MPG-8-based nanoparticles for siRNA delivery

MPG (ac-GALFLGFLGAAGSTMGAWSQPKKKRKV-Cya) is a primary amphipathic peptide consisting of an N-terminal hydrophobic motif derived from the fusion sequence of the HIV protein gp41, a hydrophilic domain derived from the nuclear localization sequence of SV40 large T antigen, and a short linker which separates these domains (25). MPG was previously reported to deliver siRNA into cultured mammalian cells efficiently and to promote potent downregulation of the target at the protein level, thanks to rapid release of siRNA into the cytoplasm (10,28). The sequence of MPG was optimized to improve its interactions with siRNA as well as the stability of MPG/siRNA complexes. For this purpose, MPG parent peptide was shortened by six residues: Gly¹, Leu³, Ser¹³, Ala¹⁷, Glu²⁰ and Val²⁷, and the two hydrophobic Phe⁷ and Ala¹¹ residues were mutated into Trp so as to favour interactions with both siRNA and lipid phase of the membrane. We previously demonstrated that the cysteamide group at the C-terminus of MPG is a prerequisite for its cellular uptake (10), therefore a β -alanine was added to the N-terminus to allow further functionalization of the peptide, thereby yielding MPG-8 a 21-residue amphipathic peptide: β AFLGWLGAWGTMGWSPPK KKK-Cya.

We first characterized the efficiency of MPG-8 to deliver siRNA targeting cyclin B1 (Cyc-B1), compared to the MPG ^{Δ NLS} parent peptide, and found that target silencing was directly correlated to the ratio, and to the size of MPG-8/siRNA particles (Figure 1A). An optimal reduction of cyclin B1 protein level (was achieved for an MPG-8/siRNA molar ratio of 20/1, corresponding to stable MPG-8/siRNA particles with a diameter of 120 ± 50 nm, as determined by dynamic light scattering, with a zeta potential of $+16 \pm 3$ v. At lower ratios, MPG-8/siRNA

particles are unstable and poorly taken up by cells; at higher ratios, a net increase in the size of particles is associated with a reduction of silencing efficiency. The parental peptide MPG ^{Δ NLS} also forms stable particles with siRNA with an optimal cyclin B1 silencing response (50%) for a molar ratio of 20/1 and particle size of 260 ± 50 nm diameter (Figure 1B). However, the efficiency of MPG ^{Δ NLS} is 2-fold lower than that of MPG-8 at a 20/1 ratio. Therefore, to ensure optimal biological conditions for siRNA delivery, throughout the rest of our study, MPG-8/siRNA particles were systematically prepared at 20/1 ratio. In addition, the toxicity of MPG-8-based formulations was investigated using either MTT assay or by monitoring cyclophilin mRNA levels. As reported in Figure 1C, no toxicity was detected up to a concentration of 20 μ M of MPG-8/siRNA particles and only 10–15% of cell death was observed with 100 μ M of MPG-8/siRNA particles.

MPG-8-mediated delivery of siRNA targeting cyclin B1 induces G2 arrest

Dose-response experiments performed on cultured cells revealed that MPG-8-mediated delivery of siRNA (Cyc-B1) induced a robust biological response associated with downregulation of both cyclin B1 protein and mRNA levels (Figure 2A and B). An siRNA concentration of 5 nM was sufficient to reduce cyclin B1 levels by more than 85% in HeLa cells (Figure 2A) and IC₅₀ of 1.1 ± 0.3 nM and 0.9 ± 0.2 nM were estimated for downregulation of protein levels, and of 0.6 ± 0.1 nM and 0.4 ± 0.1 nM for mRNA levels, for non-transformed HS68 fibroblasts (Figure 2B) and HeLa cells (data not shown), respectively. In comparison, when siRNA were delivered with MPG ^{Δ NLS}, IC₅₀ values of 24 ± 5 nM and 35 ± 7 nM were obtained for downregulation of protein and mRNA levels, respectively (Figure 2C). That MPG is 30- to 60-fold less efficient than MPG-8 can be directly correlated to differences in stability, solubility and size of the siRNA/MPG complexes. Reduction of cyclin B1 protein levels was directly associated with accumulation of cells with a 4N content, consistent with downregulation of Cdk1-Cyclin B1 activity, and was optimally obtained with 2 nM siRNA and IC₅₀ values estimated to 0.8 ± 0.2 nM and 1.2 ± 0.4 nM for HeLa and HS68 cells, respectively (Figure 2D). In contrast, no effect on cyclin B1 levels and cell cycle progression was observed with 200 nM of an unrelated siRNA (si-GAPDH), or of a mismatch siRNA harbouring two mutations (Cyc-B3) complexed with MPG-8 at a 20/1 ratio, or with MPG-8 carrier alone (100 μ M).

MPG-8-mediated delivery of siRNA targeting cyclin B1 blocks cancer cell proliferation

The potency of MPG-8/siRNA particles to block cancer cell proliferation was evaluated using MCF-7, PC3, SKBr3-HER2 cell lines and compared to the response observed for HS68 fibroblasts. Cells were treated on Day 1 with different concentrations (0.125–20 nM) of siRNA, MPG-8/siRNA and MPG-8/m-siRNA complexes, and inhibition of cell proliferation was determined

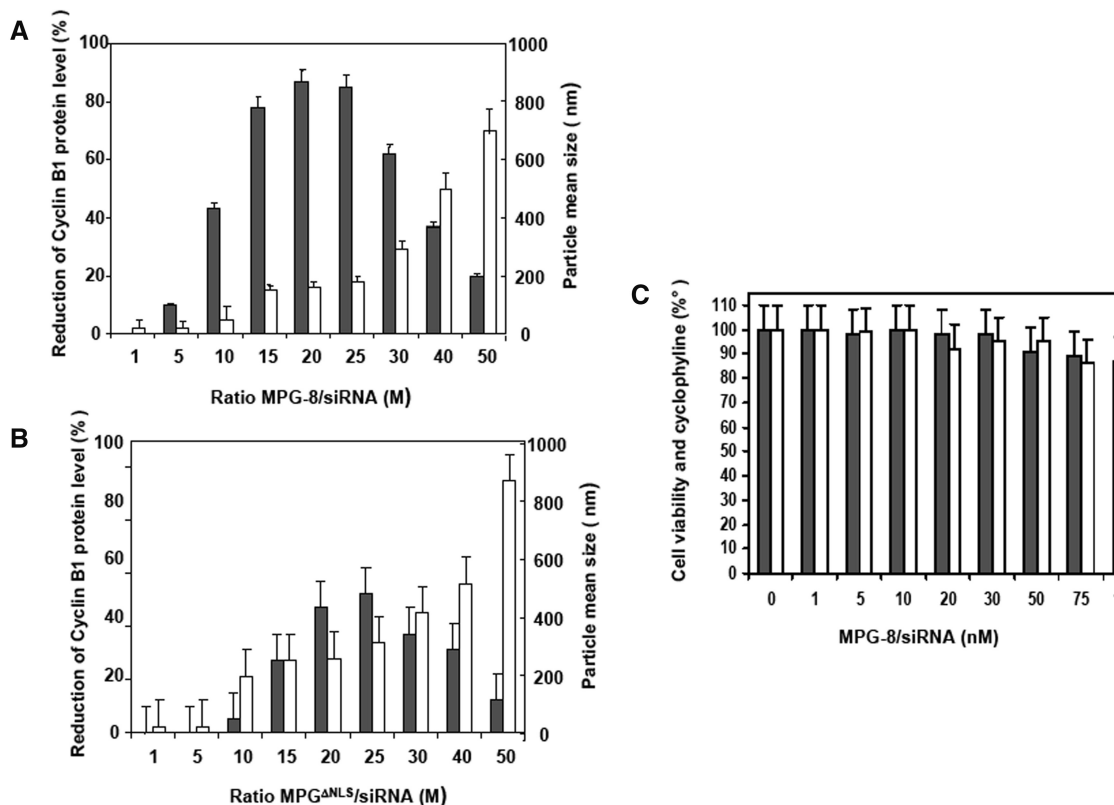


Figure 1. MPG-8 nanoparticle-mediated delivery of siRNA targeting cyclin B1. Impact of MPG-8 particle size on silencing efficiency (**A** and **B**): A fixed concentration of 20 nM of siRNA (Cyc-B1) was associated with different molar ratios of MPG-8 (**A**) or MPG (**B**) ranging from 1/1 to 50/1. The size of the MPG-8/siRNA or MPG/siRNA particles were measured by light scattering (white bars) and the biological response associated with siRNA internalization was evaluated in cultured cells by measuring reduction of cyclin B1 protein levels 24 h after transfection (grey bars). Toxicity of MPG-8 particles (**C**): The toxicity of MPG-8 particles was investigated by MTT assay (grey bars) and by monitoring the level of cyclophilin mRNA (white bars). HeLa cells were treated with increasing concentrations of MPG-8/siRNA particles ranging from 1 to 100 μ M and toxicity was then evaluated 12 h (Cyclophilin mRNA) or 24 h (MTT) after treatment. Reported data are the average of three separate experiments.

after 7 days. As reported in Figure 3, subnanomolar concentrations of Cyc-B1 siRNA complexed to MPG-8 significantly blocked proliferation of the three cancer cell lines, with IC_{50} values of 0.6 ± 0.2 nM, 0.9 ± 0.3 nM and 1.1 ± 0.3 nM, for PC3, MCF7 and SKBr3-HER2 cells, respectively, whereas no effect was observed with control siRNA or carrier alone. In comparison, the effect of this treatment on cell proliferation of HS68 fibroblasts was quite limited, despite efficient downregulation of cyclin B1 levels (Figure 3D). Although MPG-8/cyclin B1 siRNA compromised proliferation of cancer cells, it only had a moderate effect on non-cancer cells. These results are in agreement with previous reports showing that downregulation of proteins required for the G2/M transition including Plk1 kinase or cyclin B1 have a limited effect on non-cancer cells with an active G2/M checkpoint machinery and do not induce mitotic catastrophes (22,29).

***In vivo* MPG8-mediated Cyclin B1 siRNA delivery blocks tumour growth upon topical injection**

The potential of MPG-8 to deliver cyclin B1 siRNA *in vivo* was first evaluated on human prostate carcinoma cell PC3-xenografted mice (Figure 4A). The effect of local intra-tumoral administration of MPG-8/siRNA particles (molar ratio 20/1) on the growth of established

subcutaneous tumours was evaluated. At Day 50, tumour sizes in the control cohort, injected with PBS increased by about 3.5-fold. Reduction of tumour growth by 75% was observed using 1 μ g (0.05 mg/kg) of siRNA/MPG-8 and tumour growth was completely prevented with 5 μ g (0.25 mg/kg) siRNA/MPG-8 (Figure 4A). In contrast, intra-tumoral administration of 5 μ g (0.25 mg/kg) MPG^{ΔNLS}/siRNA particles did not significantly reduce tumour growth, as only 7–10% of growth curve deviation was observed in comparison to the saline control. The poor efficiency of MPG^{ΔNLS}/siRNA is associated with low stability of the particles after injection and the tendency of MPG^{ΔNLS}/siRNA complexes to precipitate at the concentration required for *in vivo* administration. At Day 48, we validated that the Cyc-B1 siRNA-mediated inhibition of tumour growth was directly associated with a decrease in the level of cyclin B1 mRNA. As reported in Figure 4B, the level of cyclin B1 mRNA was reduced by 58% and 82% with 1 and 5 μ M of siRNA formulated with MPG-8, respectively. In contrast, 5 μ M MPG^{ΔNLS}/Cyc-B1siRNA reduced cyclin B1 mRNA by only 7%, in perfect agreement with the observed variation in tumour growth (Figure 4B). As a control, we showed that administration of 50 μ g (intra-tumoral) or 100 μ g (intravenous) naked siRNA or MPG-8 carrier alone had

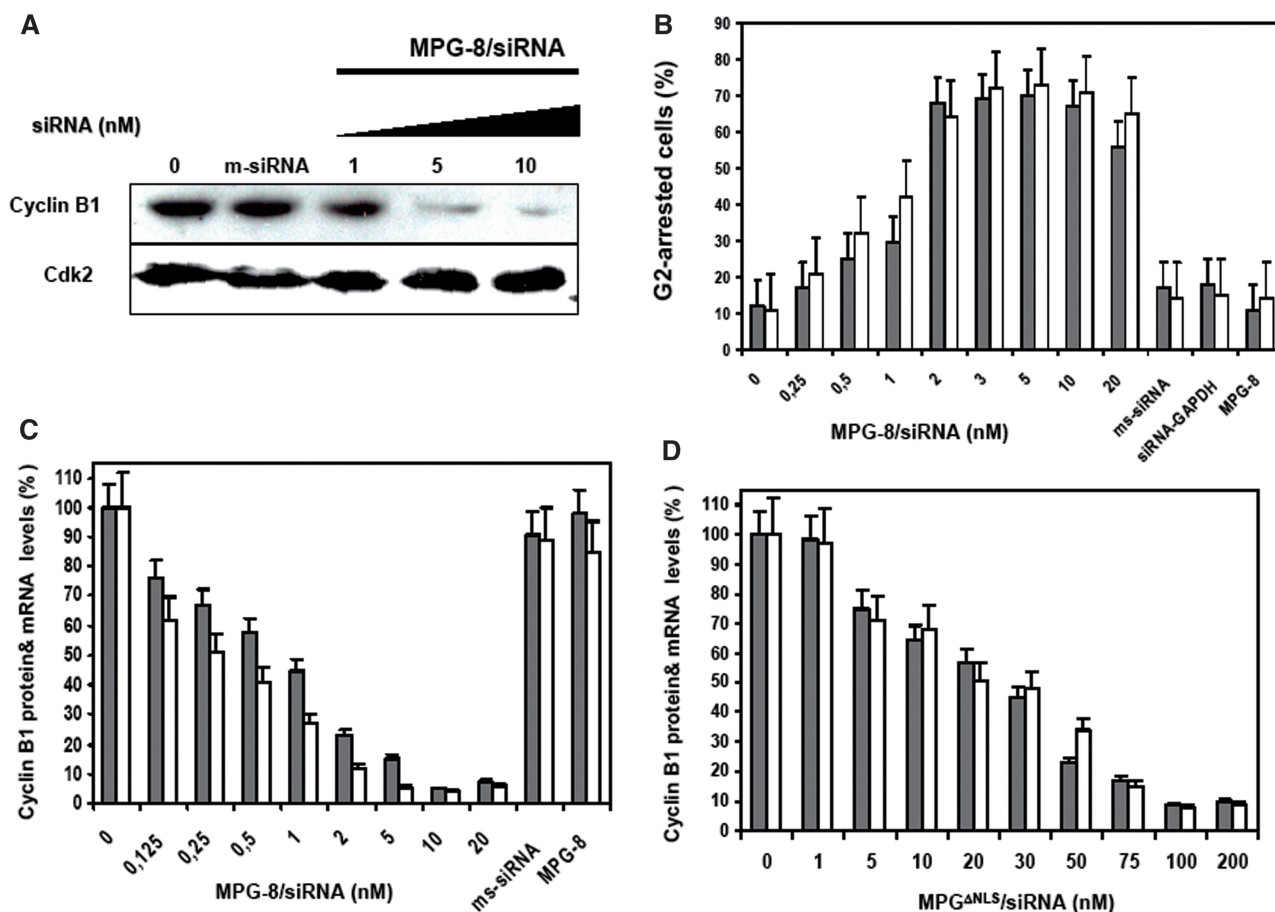


Figure 2. MPG-8-mediated delivery of siRNA targeting cyclin B1 induces G2-arrest. MPG-8 (A and B) and MPG (C) dose-response of Cyclin B1 silencing at the protein and mRNA levels. Stock solutions of MPG-8/siRNA (100 nM) or MPG/siRNA (500 nM) particles were prepared at a molar ratio of 1/20, and lower concentrations (from 200 nM to 0.125 nM) were obtained by serial dilution of the stock solution in PBS. HeLa (A) and HS-68 (B and C) cells (60% confluency) were overlaid with preformed complexes for 30 min, then fresh DMEM supplemented with 10% FCS was added directly to the cells, which were then returned to the incubator for 24 h. Cyclin B1 protein levels were determined by western blotting using Cdk2 as a control for quantification (grey bars). Cyclin B1 mRNA levels were measured 12 h after transfection using Quantigen technology (white bars). Mismatched Cyc-B3 siRNA associated with MPG-8 (200 nM) and empty MPG-8 particles (20 μ M) were used as a control. Dose-response of G2-arrest associated with Cyclin B1 silencing (D). HeLa (grey bars) and HS68 (white bars) cells were treated with increasing concentrations of MPG-8/siRNA-Cyc-B1 from 0.25 to 20 nM. The cell cycle status was evaluated by FACS analysis. Mismatched Cyc-B3 siRNA (100 nM) and GAPDH siRNA (100 nM) associated to MPG-8 as well as to MPG-8 carrier alone (20 μ M) were used as controls. Results are the means \pm of four separate experiments.

no significant effect on tumour growth. Moreover, inhibition of tumour growth was siRNA sequence-specific as a cyclin B1 siRNA harbouring two mutations (Cyc-B3) complexed with MPG-8 and injected into mice at 0.5 mg/kg was unable to inhibit tumour growth (Figure 4A).

Functionalization of MPG-8 improves *in vivo* stability and distribution of MPG-8/siRNA complexes

The stability of drug-carrier formulations *in vivo* and in the blood circulation is a major issue for systemic administration of therapeutics. Despite its demonstrated potency *in cellulo*, intravenous injection of MPG-8/siRNA formulations (0.5 mg/kg) only produces an anti-tumoural response of about 12% (Figures 4A and 7A). Cholesterol modification of siRNA has been reported to enhance their potency and stability *in vivo* by maintaining the siRNA in the circulation for longer periods of time (30,31).

Hence, in order to improve the bioavailability and stability of the MPG-8/siRNA particles, thereby rendering them more suitable for systemic administration, the surface layer of MPG-8/siRNA particles was functionalized with a cholesterol-moiety at the N-terminus of MPG-8 (Chol-MPG-8), through activation of the N-terminal beta alanine amino group. Cholesterol-functionalized MPG-8/siRNA particles were obtained stepwise by complexing siRNA molecules with MPG-8 at a molar ratio of 20/1, followed by coating of particles with a second layer of Chol-MPG-8. The optimal ratio of Chol-MPG-8 required was determined experimentally, by assessing the ability of the different particles containing between 5 and 50% Chol-MPG-8 to deliver Cyc-B1 siRNA. Below a ratio of 15% Chol-MPG-8/MPG-8, no significant difference in the efficiency of cyclin B1 silencing, with 85–92% knockdown of protein or mRNA levels (Figure 5A), nor of the associated G2 cell arrest (Figure 5B) were observed. In these

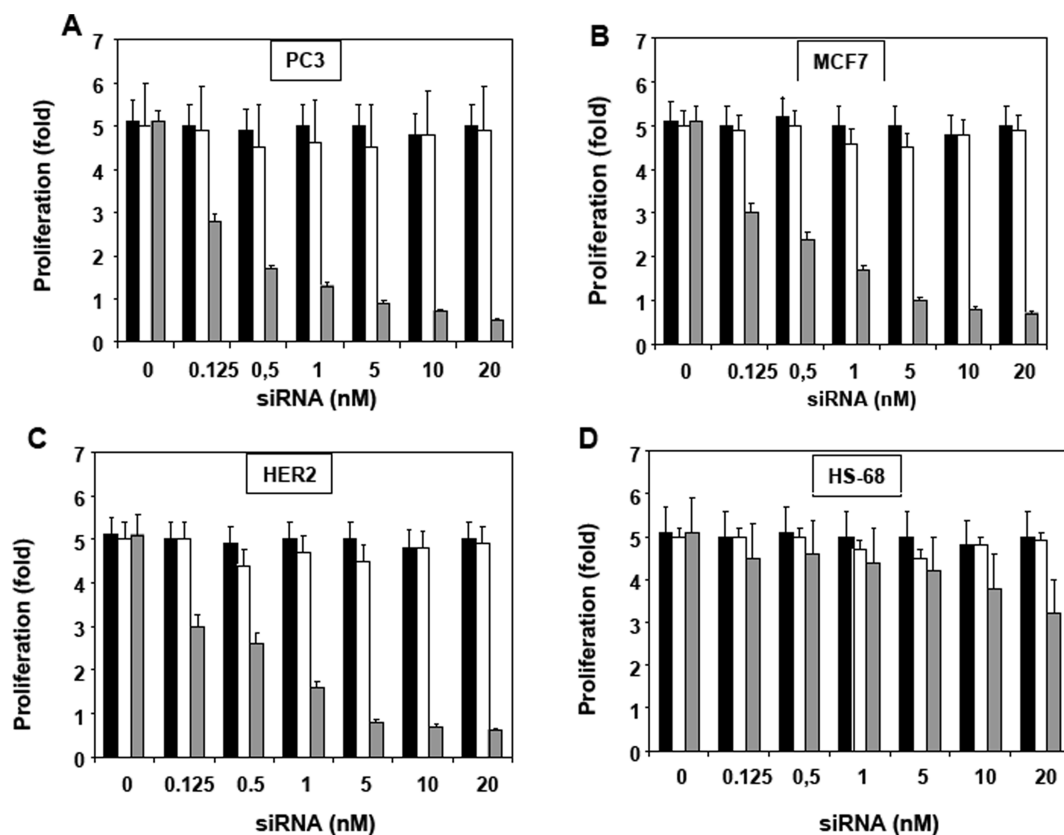


Figure 3. Cyclin B1 siRNA-MPG-mediated delivery reduces cancer cell proliferation. PC3 (A), MCF7 (B) SKBR3-HER2 (C) tumour cell lines and HS-68 (D) were treated on Day 1, with increasing concentrations of MPG-8/siRNA-Cyc-B1 (from 0.125 to 20 nM). The concentrations of formulated siRNA (from 20 to 0.125 nM) were obtained by serial dilution as described in Figure 2. The proliferation of cells treated with MPG-8/siRNA (grey bars) was evaluated on Day 7 and compared to controls including a MPG-8/Cyc-B3 mismatch siRNA (black bars) and MPG-8 carrier alone (white bars). Results are the average of four independent experiments.

conditions, an IC_{50} of 1.1 ± 0.2 nM was obtained for HS68 cells, similar to the value obtained for non-functionalized MPG-8 particles. Functionalized-MPG-8/siRNA particles with 15% of Chol-MPG-8 measure 180 ± 45 nm in diameter and are characterized by a zeta potential of 14 ± 2 v. These results indicate that functionalization of the MPG-8/siRNA particles with 15% of cholesterol alters neither their physicochemical parameters nor their efficiency to deliver siRNA. We next investigated to what extent cholesterol functionalization of MPG-8 influenced the *in vivo* bio-distribution of MPG-8/siRNA particles. Mice were injected intravenously with $10 \mu\text{g}$ of Alexa⁷⁰⁰ labelled-siRNA either naked or complexed with MPG-8 or MPG-8Chol/MPG-8. Kinetics of siRNA biodistribution were measured during the first 15 min (Figure 6A), then every hour for 5 h (Figure 6B) and fluorescence was quantified in the different organs 24 h after injection (Figure 6C). Naked siRNA was reported to be rapidly degraded, with a half-life of a few hours *in vivo*. Therefore, as expected, the control experiment performed with naked siRNA revealed that it rapidly accumulated in the bladder and in the liver over the first hours and was barely distributed throughout the rest of the body (Figure 6A, panel 1). In contrast, MPG-8/siRNA (Figure 6A, panel 2) and MPG-8/MPG-8-Chol/siRNA

(Figure 6A, panel 3) formulations favoured the rapid distribution of siRNA throughout the body within the first 15 min following injection, more prominently for the cholesterol functionalized-particles (Figure 6A, panel 2). In both cases, MPG-8/siRNA (Figure 6B, top panel) and MPG-8/MPG-8-Chol/siRNA (Figure 6B, bottom panel) were found to access all tissues and siRNA distribution was optimal at 5 h, accumulating mainly in the lung, liver, plasma, skin and kidney, adrenal gland and spleen. Although we cannot exclude that fluorescence is due to degradation of the siRNA, the fact that siRNA remains in the plasma and in most of the tissues 24 h after injection (Figure 6C), and also to a certain extent in the brain, ovary and uterus, confirms the high stability of MPG-8/siRNA and MPG-8/MPG-8chol/siRNA particles. No major differences were obtained between MPG-8/siRNA and MPG-8/MPG-8chol/siRNA particles in terms of tissue targeting. However, cholesterol-functionalization of the particles significantly increased the distribution kinetics of siRNA within the first 15 min and maintained a higher level of siRNA in the plasma even after 24 h, in comparison to MPG-8/siRNA particles, suggesting that it may limit siRNA clearance, thereby further favouring delivery of siRNA into the tumour.

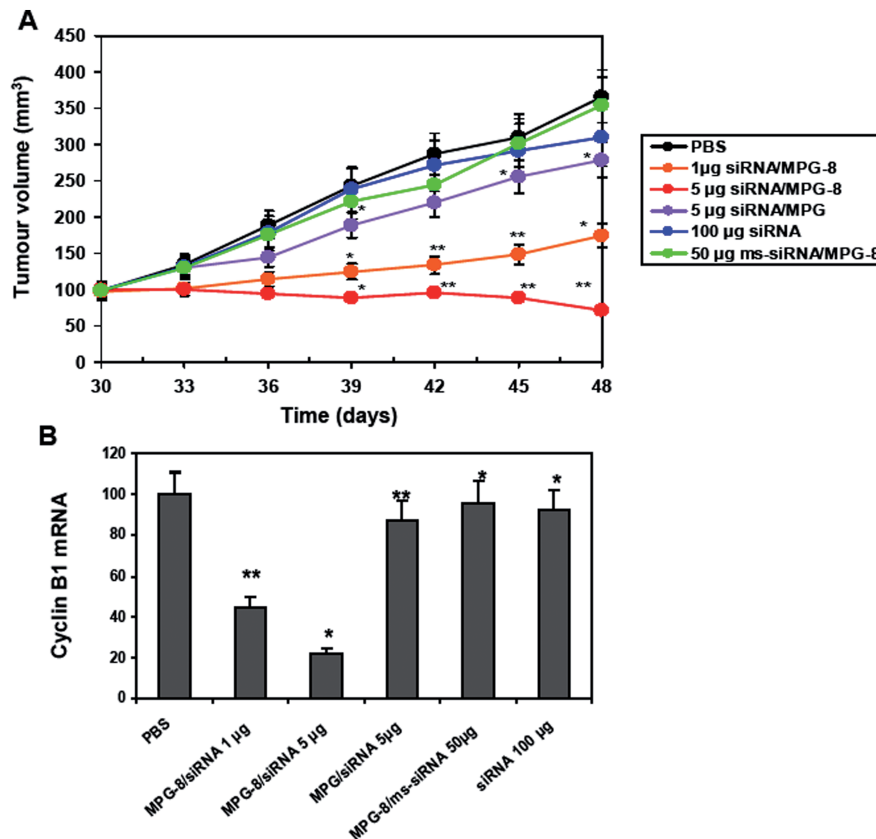


Figure 4. MPG-8-mediated Cyclin B1 siRNA delivery inhibits tumour growth upon intra-tumoural injection. (A) Swiss nude mice (a cohort of $N = 6$ animals) were injected subcutaneously with 106 PC3 cells. Thirty days after tumour implant, when tumour size reached 100 mm³, animals were treated by intratumoural injection, every 3 days, with a solution of 0.1 ml of either free Cyc-B1 siRNA (100 µg) (in blue), control siRNA Cyc-B3 (50 µg, in green), Cyc-B1 siRNA (1 µg in orange and 5 µg in red) complexed with MPG-8 at a 1/20 molar ratio, or Cyc-B1 siRNA (5 µg, in purple) associated with MPG at a 1/20 molar ratio. Curves show the mean value of tumour size. (B) After 48 days, PC3 tumours were removed, and Cyclin B1 mRNA levels were evaluated by Quantigen and normalized to GAPDH levels. * $P < 0.05$ versus saline control and ** $P < 0.01$ versus saline control.

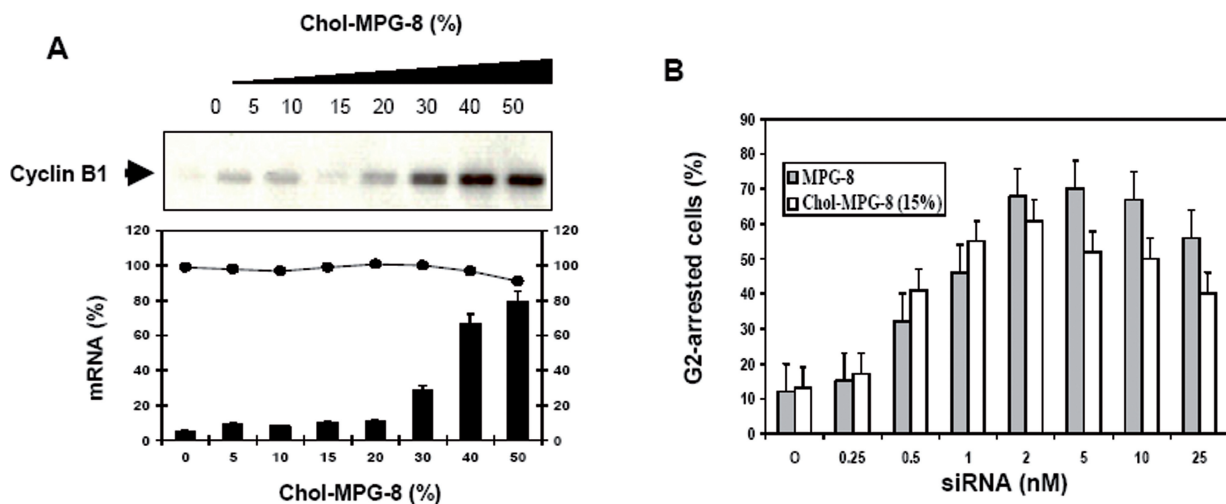


Figure 5. Cholesterol functionalization of MPG-8 does not affect *ex-vivo* efficiency. (A) Cholesterol functionalization of MPG-8-based particles. Formulations containing variable concentrations of Chol-MPG-8 were obtained by forming a precomplex of MPG-8/siRNA at a molar ratio of 1/20 and then increasing the ratio of siRNA/carrier up to 1/25 with Chol-MPG-8. The impact of the Chol-MPG-8 concentration on particle efficiency was evaluated using 5 nM Cyc-B1 siRNA and increasing concentrations of cholesterol-functionalized MPG-8, by measuring Cyclin B1 protein levels by western blot analysis 24h after transfection (A, top) and mRNA levels by Quantigen assay (A, bottom). mRNA levels were corrected using cyclophilin level as control (closed circles). (B) Dose-response of G2-arrest associated with Cyclin B1 silencing. HeLa cells were treated with increasing concentrations of MPG-8/siRNA-Cyc-B1 (grey bars) and of MPG-8/siRNA-Cyc-B1/cholesterol-functionalized MPG-8 (white bars) as in Figure 2. The concentrations of formulated siRNA (from 20 to 0.25 nM) were obtained by serial dilution of the stock solution in PBS. The cell cycle status was evaluated by FACS analysis. Results are the means \pm of four separate experiments.

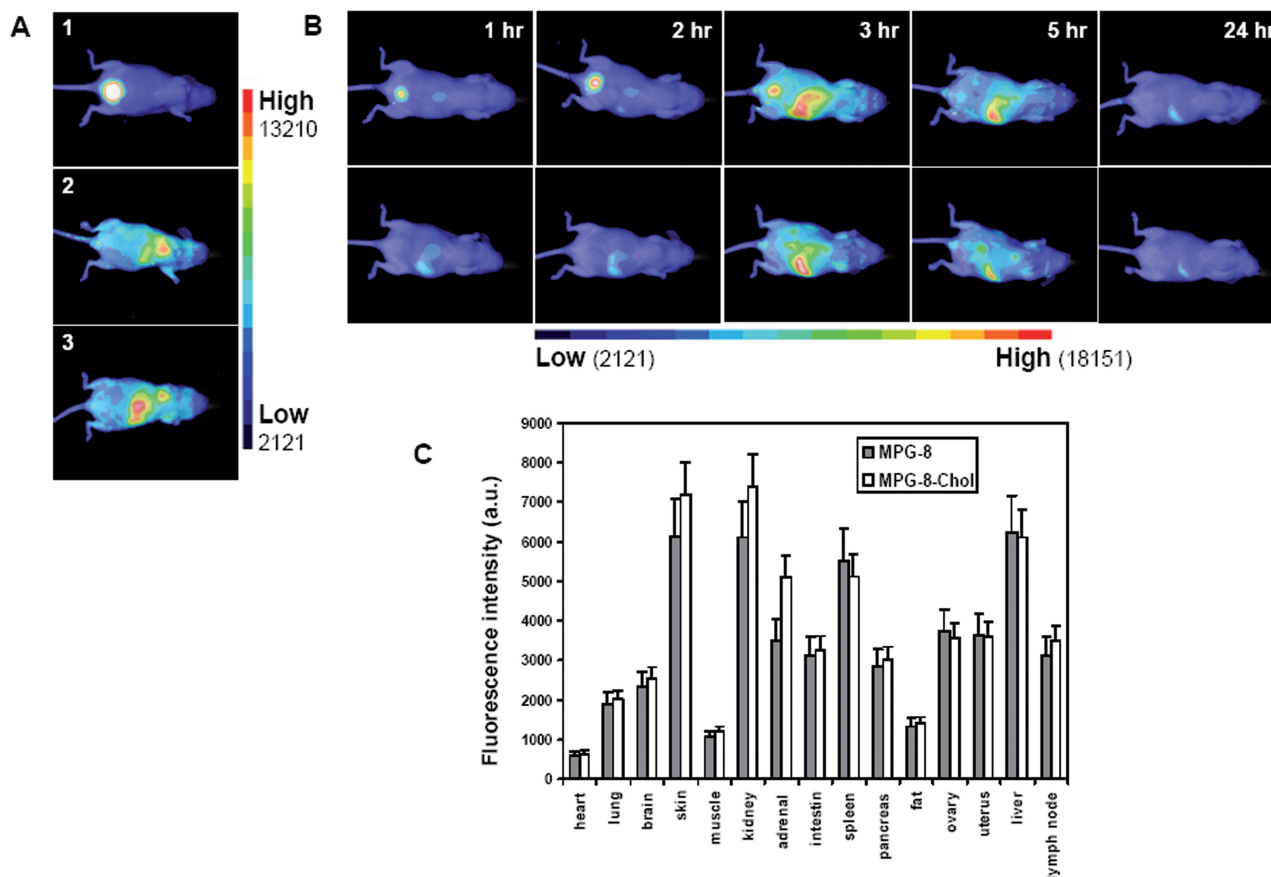


Figure 6. *In vivo* biodistribution of MPG-8 and cholesterol functionalization of MPG-8. Mice were injected intravenously with 10 μ g (200 μ l) of Alexa⁷⁰⁰ fluorescently labelled siRNA either naked (A, 1) or complexed with MPG-8 (A, 2) or MPG-8Chol/MPG-8 (A, 3). Anaesthetized mice were illuminated by 663 nm light emitting diodes equipped with interference filters and fluorescence images and real-time kinetics of biodistribution were acquired during the first 15 min with time of exposures ranging from 100 to 200 ms/image (A), then every hour for 5 h and after 24 h (B). The level of fluorescently labelled siRNA in the different organs was quantified 24 h after administration (C). Results are the average of three animals per group.

Functionalized MPG8-mediated Cyclin B1 siRNA delivery blocks tumour growth upon systemic injection

In order to analyse if increase in the distribution of siRNA associated to functionalized-MPG-8 particles directly affects its potency to inhibit tumour growth, the particles were used for systemic intravenous administration into two xenografted tumour mouse models; human prostate carcinoma (PC3) and human lung cancer (SCK3-HER-2) injected subcutaneously into the flanks of NCR nude mice. Five micrograms (0.25 mg/kg) and 10 μ g (0.5 mg/kg) of Cyc-B1 siRNA complexed with MPG/Chol-MPG-8 at a 20/1 ratio were injected intravenously every 3 days into mice bearing PC3 xenografted tumour and a significant reduction in PC3 tumour size was observed at Day 50, with 60% and 92% inhibition with 5 μ g and 10 μ g of siRNA, respectively (Figure 7A). The reduction in tumour size was directly correlated to reduction of cyclin B1 protein levels, as evaluated by western blotting, by 60% and 80% in animals treated with 5 μ g and 10 μ g of siRNA complexed to MPG-8/Chol-MPG-8, respectively (Figure 7A, insert). These results together with the lack of anti-tumoural activity of the mismatch siRNA/MPG/

Chol-MPG-8 (10 μ g) or of MPG-8/Chol-MPG-8 carrier alone, underscores the robustness and specificity of the biological response associated with systemic delivery of cyclin B1 siRNA. MPG/Cyc-B1-siRNA/Chol-MPG8 particles were next evaluated on human lung cancer cells (SCK3-HER-2) xenografted mice. After 20 days, reduction of tumour growth of 70% was achieved with 10 μ g (0.5 mg/kg) of Cyc-B1-siRNA/MPG-8/Chol-MPG-8 (data not shown). Further analysis of the mice that received (10 μ g) of Cyc-B1-siRNA/MPG-8/Chol-MPG-8 in comparison to control (PBS) or mice injected with Cyc-B1-siRNA/MPG-8 or Cyc-B3 siRNA/MPG-8/Chol-MPG-8, demonstrated a significant increase in survival at Day 40, with 70% of survival, instead of 20% with cholesterol free MPG-8 formulation (Figure 7C). Moreover, 50% of survival was observed after 100 days through injection of cholesterol-functionalized particles every 3 days for 20 days, then every 10 days (10 μ g) (Figure 7B), demonstrating the long-term efficiency of this approach. In contrast, no significant effect was observed with non-functionalized MPG-8-particles, suggesting that cholesterol increases the biodistribution

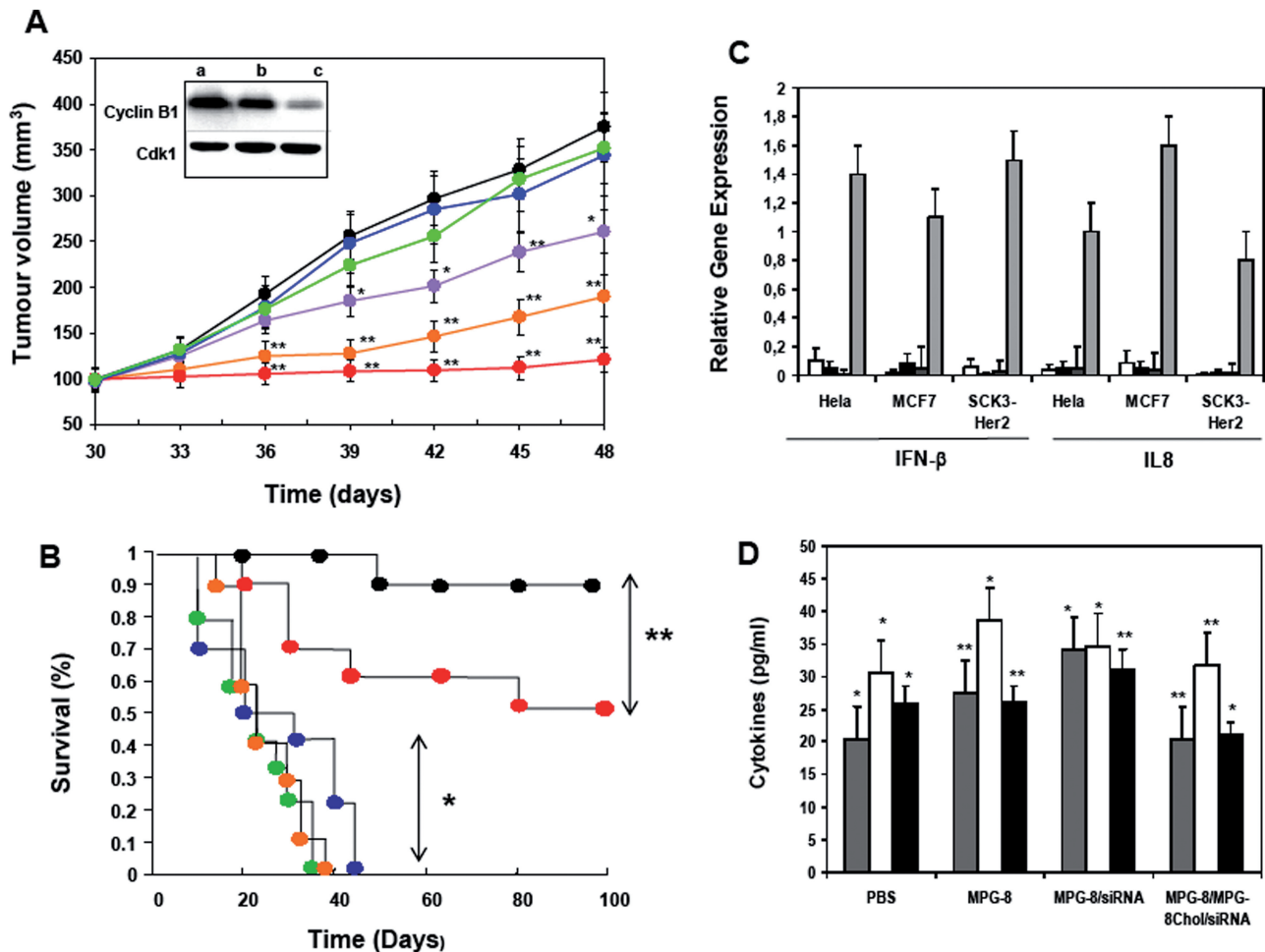


Figure 7. Systemic administration of MPG-8/MPG-8chol/cyclin B1 siRNA blocks tumour growth in vivo. (A) Inhibition of PC3 tumour growth upon intravenous injection. Swiss nude mice (a cohort of $N = 6$ animals) were injected subcutaneously with 106 PC3 cells and tumour analysis was performed as described in Figure 4A. Animals were treated by intravenous tail vein injection, every 3 days, with a solution of 0.1 ml of either PBS (black), free Cyc-B1 siRNA (100 μ g; blue), Cyc-B1 siRNA (10 μ g) complexed with MPG-8 (purple), control siRNA Cyc-B3 (100 μ g; green) or Cyc-B1 siRNA (5 μ g; orange and 10 μ g; red) complexed with MPG-8/Chol-MPG-8 at a 1/20 molar ratio. Curves show the mean value of tumour size in a group of six animals. After 48 days, PC3 tumours were removed, and Cyclin B1 protein levels were evaluated by western blotting (insert) in control (lane a), 5 μ g siRNA (lane b) and 10 μ g siRNA (lane c) complexed with MPG-8/Chol-MPG-8 at a 1/20 molar ratio. * $P < 0.05$ versus saline control and ** $P < 0.01$ versus saline control. (B) Inhibition of SK-BR3 HER2 tumour growth upon intravenous injection. Swiss nude mice (a cohort of $N = 10$ animals) were injected subcutaneously with 106 SK-BR3 HER2 cells. Ten days after tumour implant, when tumour size reached 100 mm³, animals were treated by intravenous tail vein injection, every 3 days from D10 to D30, then every 10 days, with a solution of 0.1 ml of either PBS (green), Cyc-B1 siRNA (10 μ g) complexed with MPG-8 (blue) and Cyc-B3 (100 μ g; orange) Cyc-B1 siRNA (10 μ g; red) complexed with MPG-8/Chol-MPG-8. Control mice treated by intravenous tail vein injection of (10 μ g) Cyc-B1 siRNA complexed with MPG-8/Chol-MPG-8 (black) * $P < 0.05$ versus saline control and ** $P < 0.01$ versus saline control. (C) Expression of IFN response genes: expression of INF- β and IL8 relative to GAPDH was analysed by quantitative RT-PCR. HeLa, MCF7 and SCK3-Her2 cells were treated with MPG-8 carrier alone (white) (20 μ M), 20 nM of Cyc-B1 siRNA associated with MPG-8 (black) or MPG-8/Chol-MPG8 (light grey) particles. Poly(I:C) was used as a positive control to induce interferon response (grey). (D) MPG-8 formulation does not induce interferon response in vivo. MPG-8/siRNA, MPG-8/MPG-8-Chol/siRNA (0.5 mg/kg), and MPG-8 carrier were intravenously injected into mice and IL-6 (grey), TNF- α (white) and IFN- γ (black) induction were measured in the serum 6 h after injection by sandwich ELISA and expressed as pg/ml. (P versus saline controls * <0.05 , ** <0.01) each value is the mean of three separate experiments.

of siRNA in the tumour by maintaining siRNA in the plasma.

The potential induction of inflammatory cytokine and interferon responses by siRNA has been reported as a major limitation to their therapeutic application (32–34). As interferon response has been recently reported for covalent CPP-siRNA formulations (34), we investigated the ability of MPG-8 and MPG-8/Chol formulations to induce innate immune response in cultured HeLa, MCF7 and SCK3-HER-2 cells, by monitoring expression of INF-

β and of IL8 by quantitative RT-PCR (Figure 7C), and *in vivo* by quantifying the level of IFN- α , TNF- α and IL6 in the plasma (Figure 7D). As reported in Figure 7C, none of the formulations triggered activation of interferon response, the level of expression of INF- β and IL8 being similar to those of the negative control, in contrast to poly(I:C) (positive control) which induced 1.0–1.7-fold gene expression depending on the gene and the cell line. Similarly no increase in cytokine level, in comparison to saline solution, was observed in the plasma, 6 h after

injection of any formulation (Figure 7D). Interferon response of siRNA and formulations has been associated to the binding to Toll Like Receptors within the endosome (33). As such, the lack of activation of interferon response with MPG-8 is in perfect agreement with an endosomal-independent uptake pathway (10).

CONCLUSIONS

Despite the potential of siRNA *in vitro* and *ex vivo*, their systemic delivery remains a major obstacle to therapeutic administration (4). Several reports using polycationic formulations (35), protamine-antibodies (36,37) and peptides (12,17) have largely proven that non-covalent strategies are very suitable approaches for siRNA delivery, as they improve the stability of siRNA in biological fluids, thereby increasing their overall potency. In the present work, we have developed a potent strategy to deliver siRNA *in vivo* based on an improved variant of the primary amphipathic peptide carrier MPG, MPG-8, which forms stable, yet non-covalent nanoparticles with siRNA, ensuring its efficient delivery *in vivo*. We provide a proof of concept that MPG-8 mediates efficient delivery of low effective concentrations of siRNA (0.5 mg/kg) *in vivo*. Moreover, we demonstrate that siRNA-targeting of cyclin B1 compromises tumour cell proliferation and tumour growth in a xenografted tumour mouse model.

The high stability of MPG-8-based particles, together with the slow release of the siRNA within cells, allows the use of low concentrations of siRNA, thereby limiting side effects whilst ensuring long-term response associated with siRNA delivery. MPG-8-based nanoparticles can be functionalized, and we have shown that an additional layer of cholesterol-functionalized-MPG-8 at the surface of MPG-8/siRNA particles is sufficient to stabilize these formulations in biological fluids, thereby enhancing the biological response associated with the siRNA delivered upon systemic injection. Likewise, functionalization of MPG-8 with other chemical groups or biological moieties could be applied to generate formulations to target specific cell types or tissues will be of a major interest for future development. Taken together, these findings provide new perspectives for the specific targeting and treatment of tumours, and the marked anti-tumoural effect achieved with the MPG-8-based nanoparticle system, with very low concentrations of siRNA and the lack of induction of innate immunoresponse, infers that it constitutes a promising technology for systemic administration of siRNA in a therapeutic context.

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