Multifunctional ultrasmall nanoplatforms for vascular-targeted interstitial photodynamic therapy of brain tumors guided by real-time MRI

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Abstract

Photodynamic therapy (PDT) for brain tumors appears to be complementary to conventional treatments. A number of studies show the major role of the vascular effect in the tumor eradication by PDT. For interstitial PDT (iPDT) of brain tumors guided by real-time imaging, multifunctional nanoparticles consisting of a surface-localized tumor vasculature targeting neuropilin-1 (NRP-1) peptide and encapsulated photosensitizer and magnetic resonance imaging (MRI) contrast agents, have been designed. Nanoplatforms confer photosensitivity to cells and demonstrate a molecular affinity to NRP-1. Intravenous injection into rats bearing intracranial glioma exhibited a dynamic contrast-enhanced MRI for angiogenic endothelial cells lining the new vessels mainly located in the peripheral tumor. By using MRI completed by NRP-1 protein expression of the tumor and brain adjacent to tumor tissues, we checked the selectivity of the nanoparticles. This study represents the first in vivo proof of concept of closed-head iPDT guided by real-time MRI using targeted ultrasmall nanoplatforms.

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Key words: Multifunctional nanoplatforms; Targeting; Brain tumor; iPDT; Real-time MRI

Abbreviations: a.i., arbitrary intensity; a.u., arbitrary unit; ASL, arterial spin labeling; ATWLPPR, H-Ala-Thr-Trp-Leu-Pro-Pro-Arg-OH; BAT, brain adjacent tumor; Ct, creatine; CT, Computed tomography; DTPA, diethylene triamine pentaacetic acid; DTPADA, diethylenetriaminepentaacetic dianhydride; EPI, echo planar imaging; FAIR, flow-sensitive alternating inversion recovery; FDG, fluoro-2-deoxyglucose; FGR, fluorescence-guided resection; FLASH, fast low-angle shot; FOV, field of view; HBSS, Hank’s buffered salt solution; HER2, human epidermal growth factor receptor 2; HMGN2, high-mobility-group nucleosomal binding protein 2; ICP-MS, inductively coupled plasma mass spectroscopy; iPDT, interstitial photodynamic therapy; LWRPTPA, H-Leu-Trp-Arg-Pro-Thr-Pro-Ala-OH; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NP, nanoparticle; NRP-1, neuropilin-1 receptor; PDD, photodynamic diagnosis; PAS, periodic-acid-Schiff; PET, positron emission tomography; PRESS, point-resolved spectroscopy sequence; RGD, H-Arg-Gly-Asp-OH; ROI, region of interest; ROS, reactive oxygen species; TE, echo time; TPC, 5-(4-carboxyphenyl)-10,15,20-triphenylchlorin; TPC-NHS, 5,10,15,trip-(p-tolyl)-20-(p-carboxylphenyl)chlorinsuccinidyl ester; VEGF, vascular endothelial growth factor; VTP, vascular targeted photodynamic therapy; \( \Phi_f \), fluorescence quantum yield; \( \Phi_{\Delta} \), \( 1O_2 \) quantum yield.

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Background

The poor outcome of primary malignant brain tumors is due to local invasion and local recurrence. Standard treatment of high-grade astrocytic tumors usually consists of cytoreductive surgery followed by radiation techniques and chemotherapy; however, these tumor types usually recur despite treatments. Once progression of a tumor occurs, treatment options include repeat surgical resection, radiosurgery, chemotherapy with standard agents, novel therapies, or a combination of the above. Surgical resection is the mainstay of treatment removing tumor material with the aim of reducing intracranial pressure without worsening neurological function. However, in most cases curative resection is not possible due to infiltrating growth of the tumor into normal brain parenchyma.

The wide majority of glioblastoma multiforme (GBM) recur locally and patients often succumb to and die from local recurrence, indicating that a more aggressive local therapy is required to eradicate it. However, complete radical surgical excision is hindered by the elusive nature of these tumors: a significant number of cells are not visible and require the aid of the surgical microscope. Moreover, side effects of radiotherapy can have considerable influence on health and quality of life. In this unfavorable context, photodynamic therapy (PDT) appears as an innovative technology being investigated to fulfill the need for a targeted cancer treatment that may reduce recurrence and extend survival with few side effects. PDT aims at selectively killing neoplastic lesions by the combined action of a photosensitizer and visible light whose combined action mainly results in the formation of ROS and singlet oxygen (1O2), which is thought to be the main mediator of cellular death induced by PDT.

A number of clinical studies, including phase-III randomized prospective clinical trials of PDT, have been reported, using different technologies such as photodynamic diagnosis (PDD), fluorescence-guided resection (FGR), interstitial PDT (iPDT) and intraoperative PDT.1–12 Interstitial PDT offers a localized treatment approach in which improvements in local control of GBM may result in significant improved survival.1,3,11 FGR promotes resection of the tumor and infiltrating areas which are not visible during conventional surgery, by taking into account the safety margins determined by the delineation of gross tumor volume and by planning the anatomical volume including the adjacent brain to tumor (BAT).

Death of the vasculature may indirectly lead to tumor eradication, following deprivation of life-sustaining nutrients and oxygen,13,14 and this effect is thought to play a major part in the destruction of some tumors by PDT.15–23 Hence, tumor vasculature is a potential target of PDT damage. Receptors specifically located on angiogenic endothelial cells, such as receptors to vascular endothelial growth factor (VEGF), can be used as molecular targets. We have previously described the conjugation of a chlorin (TPC) to a heptapeptide (ATWLPPR), specific for the VEGF receptor, neuropilin-1 (NRP-1).22,23 We evidenced a significant decrease in the conjugated photosensitizer cellular uptake after RNA interference-mediated silencing of NRP-1.24,25 This new targeted photosensitizer proved to be very efficient in vitro in human umbilical vein endothelial cells compared to its non-conjugated form.23 In vivo, we demonstrated the interest of using this active-targeting strategy, allowing efficient tumor tissue uptake of the conjugated photosensitizer. In mice ectopically xenografted with U87 human malignant glioma cells, we evidenced that only the conjugated photosensitizer allowed a selective accumulation in endothelial cells of tumor vessels.26 Thanks to an experimental design, an optimal vascular targeted PDT (VTP) condition was selected to show the effects and inter-dependence of photosensitizer dose, fluence and fluence rate on the growth of U87 cells ectopically xenografted in nude mice.27 Using the peptide-conjugated photosensitizer, induction of tissue factor expression immediately post-treatment, led to the establishment of thrombogenic effects within the vessel lumen.28

As we previously described, non-biodegradable nanoparticles seem to be very promising careers satisfying all the requirements for an ideal targeted PDT.29,30 We recently described the design and photophysical characteristics of multifunctional nanoparticles consisting of a surface-localized tumor vasculature targeting peptide and encapsulated PDT and imaging agents. The elaboration of these multifunctional silica-based nanoparticles was previously reported.31 Nanoparticles functionalized with four peptides specifically bound to NRP-1 recombinant protein. Nanoparticles conferred photosensitivity to cells over-expressing NRP-1 receptor and provided evidence that the photosensitizer grafted within the nanoparticle matrix can be photo activated to induce cytotoxic effects in vitro.31

For the first time in this study, we challenged to validate the interest of multifunctional ultrasmall nanoplatforms, consisting of a surface-localized tumor vasculature targeting NRP-1 peptide and encapsulated PDT and imaging agents, for iPDT of brain tumors guided by interventional MRI. We developed and optimized ATWLPPR-targeted silica-based nanoparticles encapsulated gadolinium oxide as MRI contrast agent and a chlorin as a photosensitizer. More precisely, these hybrid non-biodegradable nanoparticles consisted of a gadolinium oxide core, a silica shell containing the covalently grafted chlorin molecules, diethylene triamine penta-acetic acids (DTPA, an active chelator substance) as surfactant and ATWLPPR (or LWRPTPA, a scramble peptide, as Q6 targeting units. Multifunctional nanoparticles were evaluated in a series of in vitro experiments for their ability to produce 1O2, to target NRP-1 recombinant protein, and to confer photosensitivity. Photodynamic activity of these nanoparticles resulted in the loss of cell viability related to chlorin concentration and light dose. In vivo studies revealed that nanoparticles could be visualized into rats bearing an orthotopic U87 using MRI analysis, leading to the optimization of the optical fiber implantation just before iPDT. Several clinical studies demonstrated that PET (Positron Emission Tomography) and CT (Computed Tomography), when used together, increased the diagnostic accuracy.32–34 MRI, MRS (Magnetic Resonance Spectroscopy) and PET–CT allowed us to monitor post-iPDT effects, validating this concept of iPDT guided by MRI. We checked the functionalized nanoplatforms selectivity by determining NRP-1 protein expression into the tumor tissue related to MRI perfusion profile. After intravenous injection of ATWLPPR-targeted nanoplatforms, the positive contrast enhancement of the tumor by MRI allowed us to visualize the proliferating part of the tumor tissue compared to un-conjugated or LWRPTPA-conjugated nanoparticles.
Methods

Binding test

The binding of functionalized nanoparticles to recombinant NRP-1 protein has been widely described previously by our group.31 Reported values are the average of triplicate measurements.

Cell line, dark cytotoxicity and photodynamic activity

To study the involvement of NRP-1, MDA-MB-231 breast cancer cells were used, strongly over-expressing NRP-1 receptor. Cell line and culture conditions have been described previously by our group.31,24,25 Cell survival and photodynamic activity after incubation with the different batches of nanoparticles in the dark were measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously.31,35

Animals and tumor model

All experiments were performed in accordance with animal care guidelines (Directive 2010/63/EU) and carried out by competent and authorized persons (personal authorization number 54-89 issued by the Department of Veterinary Services) in a registered establishment (establishment number C-54-547-03 issued by the Department of Veterinary Services).

Male athymic nude rats (rnu−/−) were used for this study (Harlan, Gannat, France). The rats were used for tumor implantation at age of 8 weeks (150-180 g). During microsurgery (implantation or treatment protocol) and all acquisitions with microimaging, rats were anesthetized with a mixture of air and isoflurane concentrate (1.5%-2% depending on the procedure) and all acquisitions were performed under sterile conditions. The rat was placed into a Kopf stereotactic frame (900 M Kopf Instruments, Tujunga, CA). A midline incision was done and a burr hole was drilled 0.5 mm anterior and 2.7 mm lateral to the bregma. A skull anchor was fixed. 5.10⁴ U87 cells were suspended in 5 μL Hank’s Buffered Salt Solution (HBSS, 1×) and were injected in 4.4 mm into the anterior and 2.7 mm lateral to the bregma. A skull anchor was fixed. 5.10⁴ U87 cells were suspended in 5 μL Hank’s Buffered Salt Solution (HBSS, 1×) and were injected in 4.4 mm into the brain parenchyma with a flow of 0.2 μL/min using a 10 μL Hamilton syringe. After injection, the scalp incision was sutured (Suture 6.0 filament) and the surface was antiseptically cleaned.

Nanoparticles preparation for in vivo studies

Nanoparticles were suspended in ultrapure water and NaCl 9% (50:50) to obtain an equivalent concentration of 2.5 mM TPC or 200 mM Gd. Each batch of nanoparticles was buffered in order to obtain an iso-osmolar solution and pH 7.4 and conserved at 5 °C. Injected TPC amounted to 1.75 μmol/kg as previously described.27,28 The injection solution was prepared by dissolution in 9% NaCl to obtain an injection volume of 600 μL (e.g. 0.437 μmol of TPC or 84.2 μmol of Gd for a body weight of 250 g) and injected, followed by 600 μL of 9% NaCl injected during 1 min.

Inductively coupled plasma-mass spectroscopy

A Varian 820 MS instrument (Varian, Les Ulis, France) was used. All samples were completely dissolved with 70% HNO₃ and heated at 90 °C until total mineralization. Each mineralized sample was solubilized in 25 mL of ultrapure water (resistivity >18.2 MΩ) and analyzed by ICP-MS (Laboratoire Environnement-Hygienè of ASCAL, Forbach, France). All removed samples were stored at −80 °C prior to elemental analysis.

Nanoparticles biodistribution

MRI experiments were performed at 7 T in a horizontal bore magnet (Bruker, Biospec, Ettingen, Germany). Reference images (“Scout views”) were first realized to obtain the brain position or abdominal position inside the magnet. 15 slices were obtained, 5 in each plan. For cerebral imaging, a volume coil (internal diameter 72 mm) was used for radio frequency emission, and a surface coil was placed on the animal skull for the reception of the signal.

T2 weighted images

For the abdominal imaging, a quadrature volume coil (inner diameter of 72 mm) was used for radio frequency emission and reception. Acquisitions were synchronized to the breath to prevent the kinetics blurring. The T1 weighted turboRARE36 spin echo sequence was performed in coronal plan to characterize the clearance of the nanoparticles after injection.

MRI-guided iPDT and light delivery

Light delivery fiber was inserted through the skull anchor (Patent WO2012176050 A1) into the tumor tissue. The fiber tip (272 nm diameter, ULS 272, OFS, Norcross, U.S.A.) delivered light (652 nm, 50 mW, 8 min 40 s, 26 J). A RARE T1 and T2 weighted imaging was performed before iPDT to control the positioning of the optical fiber inside the brain.

Perfusion MRI

Arterial Spin Labeling (ASL)37 techniques were able to provide quantitative information about local tissue blood flow by observing the inflow of magnetically tagged arterial blood into imaging slice.

Magnetic resonance spectroscopy (MRS) analysis

A 1.7 mm cubic voxel was positioned in the glioma and in the striatum (contralateral side). Before the spectroscopic PRESS (Point-Resolved Spectroscopy Sequence) sequence acquisition a FastMAP38 was performed in order to homogenize the magnetic field in the voxel.

PET–CT acquisition procedures

Metabolic brain imaging was performed by using a small animal PET–CT (MicroPET/CT INVEON, Siemens Preclinical Medical Solutions). The animal was deprived only of food 6 h...
prior to intravenous injection of $[^{18}F]$FDG. At 40 min after, the
anesthetized animal was positioned on the scanner bed, which
automatically moves inside the gantry of the CT scanner then
further into the PET field of view; the acquisition protocol
started. The CT scanner provided information with regards to
tissue attenuation that is necessary for PET imaging accurate
reconstruction. PET images were reconstructed with iterative
algorithms of OSEM2D and corrected for attenuation and scatter.
Imaging data analyses were performed on all frames by use of
IRW (version 3.0) software.

**Immunohistological analysis**

Brain tissue was fixed during 10 days at room temperature in
formal. Macro-samples of each brain (5 mm) were realized with
a large rat coronal blocker (DKI-PA-001, David Kopf Instru-
ments, Pymep, Paris, France) and fixed still during 24 h.
Samples were dehydrated in ethanol (96° followed by 100°).
Histopathology was performed on 5 μm paraffined tissue
sections. Hematoxylin, eosin and safran (HES) staining and
Periodic-Acid-Schiff (PAS) were performed. Each section was
pre-treated by EDTA (10 mM, pH: 7.8) at 121 °C during 3 h. To
detect tumor cellular proliferation, sections were incubated for 1
night at room temperature with the primary antibody (rabbit
monoclonal antibody anti-Ki67, 1:200 dilution buffer; SP6,
RM-9106-S0, S1, NeoMarkers, Labvision). Glial fibrillary
acidic protein (GFAP) was analyzed using a mouse polyclonal
antibody anti-GFAP (1:2000 dilution buffer, MS-280-P0,
Thermo Fischer Scientific) and NRP-1 expression with a rabbit
polyclonal antibody anti-NRP-1 (1:400 dilution buffer, Invitro-
gen Corporation, Camarillo, CA). VEGF165 expression was
detected with a rabbit polyclonal antibody anti-human VEGF
(1:100 dilution buffer, AB-2, PC-37, Oncogene Science, Inc.,
Cambridge, MA, USA). After washing, the slides were incubated
for 1 h with the secondary goat polyclonal antibody anti-rat
biotinylated IgG (1:400 dilution in PBS-Tween E0432, Dako-
cytomation, Denmark). The revelation of secondary biotinylated
antibodies was performed with a streptavidin–horseradish
peroxidase complex (1 h at room temperature, diluted 1:400 in
PBS-Tween, Dakocytomation, Denmark) and the peroxidase
substrate (5 min, Vector® NovoRedTM Substrate Kit for
peroxidase, HistoGreen, Vector Laboratories, Paris). A hema-
toxylin counterstaining was performed to visualize the section by
optical microscopy (AX-70 Provis, Olympus, Rungis, France).

**Statistical analysis**

Mann–Whitney U test was used to assess the significant level
between independent variables. The level of significance was set
10 to $P < 0.05$.

**Results**

**Nanoparticles characterizations: photophysical and chemical
properties, size distribution and zeta potential**

The synthesis pathway has been previously described in
Couleaud et al, 2011. Supplementary Figure 1 shows the
photophysical and chemical characterization of nanoparticles.
Absorption spectra (Supplementary Figure 1, A), fluorescence
spectra (Supplementary Figure 1, B) and $^{18}O_2$ luminescence spectra
(Supplementary Figure 1, C) in ethanol of free TPC and TPC grafted
to nanoparticles. No significant changes in the quantum yields of
fluorescence and $^{18}O_2$ production have been observed between free
TPC and TPC grafted onto nanoparticles (Supplementary
Figure 1, E). Fluorescent of TPC (excitation and emission
wavelengths at 420 and 600-800 nm, respectively) and fluorescence
of tryptophan residues of ATWLPPR (excitation and emission
wavelengths at 280 and 350 nm, respectively) have been used to
type TPC and ATWLPPR grafted onto the nanoparticles. We
found an average of 2 TPC molecules per nanoparticle and 4, 9 or 15
ATWLPPR peptides per nanoparticle depending on the amount we
needed. By measuring the partition coefficients of free TPC,
NP-TPC, and NP-TPC-ATWLPPR, we find that the formulation we
developed has a higher hydrophilic character than the free TPC
(Supplementary Figure 1, E). DLS and HR-TEM measurements have
permit to find a consistent diameter of 2.9 ± 0.7 nm and 2.8 ±
0.2 nm, respectively (Supplementary Figure 1, D). The presence of
ATWLPPR peptide on the surface of the NPs induces a significant
increase in the surface charge, as measured by zeta potential at pH
7.4 (Supplementary Figure 1, E). As expected, the derivatization
of the nanoparticles by DTPA rendered them water soluble in a wide
pH range, including pH of biological fluid whereas the colloidal
stability of uncoated nanoparticles was not sufficient.

**Molecular affinity**

As previously described, the endothelium-homing peptide
ATWLPPR selectively targets NRP-1 receptor overexpressed by
neo-angiogenic vasculature. ATWLPPR grafted onto nanoparticles was measured as previously described. We tested different ATWLPPR grafted ratios onto nanoparticles: 4 peptides per nanoparticles (NP-TPC-ATWLPR), 9 peptides per nanoparticle (NP-TPC-(ATWLPPR)$_9$) or 15 peptides per nanoparticle (NP-TPC-(ATWLPPR)$_{15}$). Molecular affinity of these functionalized nanoparticles to recombinant NRP-1 protein has been estimated using binding tests. As VEGF165 binding to its receptors is heat-independant, the competitive binding experiments were always carried out in the presence of heparin. Nanoparticles conjugated to ATWLPPR indeed bound to recombinant NRP-1 chimeric protein (Figure 1, A). Binding of biotinylated VEGF165 to NRP-1 was displaced by NP-TPC-ATWLPPR in a peptide concentration-dependent manner (IC$_{50}$ = 27 μM, Figure 1, B).

**In vitro dark cytotoxicity without light exposure**

We used MDA-MB-231 breast cancer cells that strongly
over-express NRP-1 receptor, as previously demonstrated. MTT
test was used to evaluate the dark cytotoxicity of the different
batches of nanoparticles, control nanoparticles without TPC (NP),
nanoparticles with TPC but without peptides (NP-TPC), and
nanoparticles with 4 peptides (NP-TPC-ATWLPR) for TPC
concentrations ranging from 0.10 to 20.00 μM. A 24 h-incubation
of MDA-MB-231 with nanoparticles in the absence of light
which are involved in renal excretion, showed a positive contrast due to the ATWLPPR-targeting moiety. It appears that only the kidneys and the bladder, which are involved in renal excretion, showed a positive contrast enhancement for the peripheral tumor tissue. As illustrated in the kinetic profiles between MRI signal intensity percentage of the ROIs of the total tumor area and the peripheral tumor tissue areas. However, for NP-TPC-ATWLPPR peptide without affinity for NRP-1 used as negative control. Just after intravenous injection, whatever the batches of nanoparticles delivered to the tumor site and that the presence of the peptide units onto the nanoparticle surface yielded a mean surviving cell fraction of more than 70% for concentrations up to 1.00 μM of TPC (Figure 1, C). All subsequent in vitro experiments were carried out at concentrations equal or inferior to 1.00 μM of TPC. At 10.00 μM of TPC, we can noticed that the presence of peptide units onto the nanoparticle increased significantly its cytotoxic effect probably due to an improved uptake but this effect was not verified for 20.00 μM concentration (Figure 1, C).

In vivo biodistribution and tumor tissue selectivity

We followed the in vivo biodistribution of NP-TPC-ATWLPPR and NP-TPC nanoparticles 2 and 24 h post-intravenous injection. It appears that only the kidneys and the bladder, which are involved in renal excretion, showed a positive contrast enhancement of the MRI signal intensity (Figure 2, A and B).

Similar biodistribution results were obtained for both un-conjugated and peptides-conjugated nanoparticles. Gadolinium concentration of each sample was measured by ICP-MS also demonstrating high levels in the kidneys 2 h after intravenous injection (Figure 2, C).

To investigate tumor tissue selectivity, we used U87 orthotopic model about 10 days after stereotactic implantation in nude rats. MRI analysis of the tumor tissue was investigated for un-conjugated and ATWLPPR- or LWRPTPA-targeted nanoparticles. LWRPTPA is a mix of amino acids of ATWLPPR for un-conjugated and conjugation with ATWLPPR significantly enhanced photodynamic efficiency (Figure 1, D). A statistically significant influence was also evaluated on the photodynamic activity for un-conjugated nanoparticles and peptides-functionalized nanoparticles with 1.00 μM of TPC using light doses from 5 to 20 J/cm² (Figure 1, E).

Figure 1. Molecular affinity of nanoparticles with peptides, in vitro dark cytotoxicity and photodynamic activity. Binding of NP-TPC-ATWLPPR (black), NP-TPC-(ATWLPPR)₉ (dark grey) and NP-TPC-(ATWLPPR)₁₅ (clear gray) to recombinant NRP-1 protein compared to nanoparticles without ATWLPPR (A). Binding of biotinylated VEGF₁₆₅ (5 ng/mL; 110 pM) to NRP-1 in the presence of 2 μg/mL heparin was evaluated when increasing concentrations of nanoparticles were added (data points show the mean ± SD, n = 3). Binding curve of nanoparticles with 4 peptides (NP-TPC-ATWLPPR to recombinant NRP-1 protein (EC₅₀ = 27 μM) (data points show the mean ± SD, n = 3) (B). Dark cytotoxicity and photodynamic therapy sensitivity to different formulations: NP-TPC-ATWLPPR (black), NP-TPC (dark gray) and control NP (without TPC or peptide in white) in MDA-MB-231 cells depending on nanoparticle concentration, as determined by MTT test (data points show the mean ± SD, n = 6) (C). Measurements of photosensitivity of MDA-MB-231 cells to NP-TPC (black) and NP-TPC-ATWLPPR (gray) (corrected by respective nanoparticles in dark cytotoxicity) (D-E). Survival was obtained for cells incubated with different concentrations of nanoparticles for 24 h before exposure to doses of light from 1 to 20 J/cm² at 0.1 μM of TPC (D) and at 1 μM of TPC (E) by MTT test (data points show the mean ± SD, n = 6).
respective inserts of the Figure 3, D, the tumor periphery was not delimited by a margin of connective tissue; conversely MRI images from NP-TPC and NP-TPC-(LWRPTPA)_4 appeared well demarcated.

In order to complete these investigations and to understand the tropism of NP-TPC-ATWLPPR for the tumor tissue periphery, we performed dynamic contrast-enhanced perfusion MRI and as expected, we clearly visualized that the margins of the tumor volume were more vascularized than its center (Figure 4, A). Vascular phenotype in angiogenic vessels was characterized by an over expression of NRP-1 protein mainly in this peripheral interest area (Figure 4, B); the margins of the tumor tissue were more vascularized and the neoangiogenic vessels from the peripheral interest area between the tumor tissue and the brain adjacent to tumor expressed NRP-1 protein. It appears that ATWLPPR-conjugated nanoparticles target vessels mainly located in the peripheral tumor tissue with an angiogenic phenotype.

**In vivo interstitial stereotactic PDT by interventional MRI**

Tumors tissue, visualized by T1-weighted imaging after injection of nanoparticles, was illuminated via an optical fiber placed stereotactically into the brain of each animal. The fiber position was confirmed by another coronal T1-weighted MRI acquisition (Figure 5, A) and visualized by a colocalization between MRI combined with PET–CT images (Figure 5, A, left pictures) (Patent WO2012176050 A1). Brain tumor tissue was illuminated about 1 h post-injection with nanoparticles, taking into account the drug–light interval according to the MRI signal intensity.

Following iPDT using these ultrasmall nanoplatforms, advanced imaging complementary techniques (perfusion MRI, proton MRS, PET–CT) were applied as a proof of concept study. Before and immediately after iPDT, cerebral perfusion MRI was realized for each treated animal (Figure 5, B). Top and bottom of Figure 5, B show the tumor perfusion images.
tumor perfusion images have been obtained for two animals and they illustrate clearly that the intratumoral blood perfusion significantly decreased for tumors treated with both batches of nanoparticles. The most interesting point is that blood perfusion declined for more than 80% mean of the initial values only for tumors treated with NP-TPC-ATWLPPR.

Magnetic resonance spectroscopy acquisition in tumor was performed to quantify intratumoral metabolites, using creatine as reference. Proton MRS provides a noninvasive method for evaluating some metabolic components. Because this technique measures the presence of specific metabolites, it is independent of anatomic information and may be used to characterize lesions.
Metabolites over-expressed 24 h after iPDT were listed in the Figure 5, as metabolites/creatine ratios. After iPDT for tumors treated by NP-TPC-ATWLPPR, CH₃/creatine and CH₂/creatine lipid ratios related to the tumor cell necrosis, increased by a factor of 3.3 and 3.0, respectively. Changes in the concentrations of choline-containing metabolites have been implicated in both cell proliferation and death processes. An increase of 1.9- and 2.1-fold of choline/creatine ratios was measured after treatment for NP-TPC-ATWLPPR and NP-TPC, respectively.

In order to estimate tumor tissue metabolism, sample images were performed 4 and 6 days after treatment using a combination of PET-CT imaging technology. This modality allowed intra-tumor metabolism detection after incorporation of [¹⁸F]FDG by cells. The uptake of [¹⁸F]FDG in tumor tissue and in surrounding healthy tissue is time-dependent. [¹⁸F]FDG uptake reflects tumor physiology, tumor cell density, and blood supply (Figure 6). Using non-conjugated nanoparticles, the percentage of injected [¹⁸F]FDG dose increased from 1.40 (just before iPDT) to 1.80 (four days after iPDT) and to 1.70 (six days after treatment). At the same times, NP-TPC-ATWLPPR-treated tumor also described an increase related to time post-treatment in percentage of injected dose with 1.40 before treatment followed by 1.53 and 1.92, four and six days after treatment, respectively. The calculated values of the tumor metabolism after non-conjugated nanoparticles treatment were 0.040 ± 0.002 and 0.023 ± 0.002 for 1 mm³ of tumor tissue, two days and six days post-iPDT, respectively. For conjugated nanoparticles treatment, the calculated values were 0.028 ± 0.001 and 0.021 ± 0.001 for 1 mm³ of tumor tissue, 2 days and 6 days post-iPDT, respectively.

Histological examination of tissue sections taken from brain tissue immediately after iPDT indicated a vascular disruption and edema into both tumor and BAT areas (Figure 7, A). The alteration of the extra cellular matrix after treatment was suggested by a decrease in PAS protein expression.
<table>
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<th>Ratio</th>
<th>Controlateral brain before iPDT*</th>
<th>Controlateral brain 24 h after iPDT*</th>
<th>Intratumoral before iPDT*</th>
<th>Intratumoral 24 h after iPDT**</th>
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<td>NP-TPC-ATWLPPR</td>
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<td>Myo-inositol/crea</td>
<td>0.25±0.07</td>
<td>0.37±0.10</td>
<td>1.04±0.54</td>
<td>1.71</td>
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<td>Glutamate/crea</td>
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<td>0.88±0.17</td>
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<td>Taurine/crea</td>
<td>0.37±0.09</td>
<td>0.68±0.32</td>
<td>0.38±0.2</td>
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* Average ± S.D., n=3; ** one sample analyzed
studies have been performed to actively target tumor vascular peptide was also grafted onto polyacrilamide nanoparticles cells, including MDA-MB-435 tumor cells. It is expressed in the nuclei of tumor and endothelial vasculature. This peptide is an N-terminal fragment (amino acid peptide has also been coupled to nanoparticles to target tumor selective targeting in cells overexpressing HER2 and a photodynamic effect related to the expression of HER2. F3 nanoparticle increased significantly its cytotoxic effect probably due to an improved cellular uptake. Moreover, we demonstrated we also observed that the presence of peptide units onto the nanoparticle increased significantly its cytotoxic effect probably due to an improved cellular uptake. Moreover, we demonstrated that nanoparticles conferred photosensitivity to cells, providing evidence that the chlorin molecules grafted within the nanoparticle can be photoactivated to yield photocytotoxic effects. Our strategy aims to favor the vascular effect of PDT by targeting tumor-associated vascularization. Our preliminary approach consisted of the conjugation of a chlorin to a heptapeptide ATWLPPR targeting NRP-1, over-expressed by tumor angiogenic vessels. This conjugated-chlorin proved to be very efficient in vitro in human umbilical vein endothelial cells compared to its non-conjugated form. In this study, in order to check the absence of dark cytotoxicity of the different batches of nanoparticles and to assess the impact of the uptake improvement on the photodynamic efficiency according to the nanoparticles grafting level, MDA-MB-231 cells were selected. As previously demonstrated, this cell line strongly over-expresses NRP-1 receptor, leading us to evidence a statistically significant decrease of the conjugated photosensitizer cellular uptake after RNA interference-mediated silencing of NRP-1. Here, we designed ultrasmall ATWLPPR-targeted silica-based nanoparticles encapsulated gadolinium oxide as MRI contrast agent and a chlorin as photosensitizer. We previously described the in vitro photodynamic efficiency of multifunctional silica-based nanoparticles for PDT. In this study, we demonstrated that nanoparticles conjugated to ATWLPPR bound to recombinant NRP-1 chimeric protein and interestingly, the best binding value was evidenced with four peptides per nanoparticle with a decrease of the affinity related to the number of peptides. Binding of biotinylated VEGF to NRP-1 was displaced by NP-TPC-ATWLPPR in a peptide concentration-dependent manner. This decrease of affinity related to the number of grafted peptides maybe related to the steric hindrance due to the number of peptide units on comparison with the ultra-small size of the nano-object. By in vitro experiments with MDA-MB-231 cells over-expressing NRP-1, we also observed that the presence of peptide units onto the nanoparticle increased significantly its cytotoxicity effect probably due to an improved cellular uptake. Moreover, we demonstrated that nanoparticles conferred photosensitivity to cells, providing evidence that the chlorin molecules grafted within the nanoparticle matrix can be photoactivated to yield photocytotoxic effects in vitro but also in vivo.

Discussion

Kopelman et al were the first to describe targeted nanoplatforms combining both MRI and PDT agents. They described a nanoplatform based on PAA (polyacrylamide acid)-modified core of iron oxide, coupled to the RGD peptide. Several potent small-molecule αβ antagonist-based RGD compounds have been studied under clinical trials for anti-angiogenesis, drug delivery, and cancer imaging. The promising results have highly suggested that integrin receptors are important targets for molecular imaging, drug delivery and therapy. Recently, another team grafted an anti-HER2 antibody onto gold nanoparticles. Human Epidermal Growth Factor Receptor-2 (HER2) belongs to the HER family involved in intracellular signaling mechanisms including cell proliferation. By coupling the anti-HER2 to nanoparticles, Suchinskaya et al demonstrated in vitro a selective targeting in cells overexpressing HER2 and a photodynamic effect related to the expression of HER2. F3 peptide has also been coupled to nanoparticles to target tumor vasculature. This peptide is an N-terminal fragment (amino acid sequence 17-48) of the high human protein 2 (HMGN2) mobility group. It is expressed in the nuclei of tumor and endothelial cells, including MDA-MB-435 tumor cells. In vivo, in a model of orthotopic glioma, polyacrilamide F3-conjugated nanoparticles containing Photofrin led to a photodynamic efficiency. F3 peptide was also grafted onto polyacrilamide nanoparticles encapsulated methylene blue. Only a very limited number of studies have been performed to actively target tumor vascular endothelial cells.
Figure 7. Histological images of U87 tumor and BAT before (left) and immediately after iPDT (right) using NP-TPC-ATWLPPR. After treatment, representative edema images of hematoxylin–eosin–safran staining obtained from brain section in glioma were compared to before treatment (A). Representative images of PAS staining (B) and Ki67 staining counterstained with hematoxylin (C). VEGF staining counterstained with hematoxylin (D) was highly expressed in BAT before iPDT and unexpressed immediately after iPDT (BAT: brain adjacent to tumor; O: oedema; T: tumor; V: vessels).
For the first time, we evidenced the in vivo tropism of ATWLPPR-conjugated nanoparticles targeting NRP-1 receptor for the peripheral tumor tissue. Indeed, after intravenous injection of non-conjugated nanoparticles when we selected an ROI corresponding to the total tumor tissue area, we only observed the Enhanced Permeability and Retention (EPR) effect.\textsuperscript{58,49} Leaky fenestration caused extravasations of non-conjugated nanoparticles out of the vasculature. Due to an inefficient lymphatic drainage, there was a poor clearance of the nanoparticles into the interstitial space of the tumor tissue. In contrast after intravenous injection of ATWLPPR-conjugated nanoparticles, they provided a more selective contrast enhancement for angiogenic endothelial cells that line the neo-vessels mainly located in the peripheral tumor and over expressing NRP-1. Results from perfusion MRI argue that the margins of the tumors were more vascularized. Using ATWLPPR-targeted nanoplatforms, the positive contrast enhancement of the tumor by MRI, allowed us to visualize the proliferating part of the tumor tissue, which was not the case with unconjugated nanoparticles.

The average size of these nanoparticles makes them amenable to renal clearance and to avoid retention. Three hours after intravenous injection less than 0.2% of the injected nanoparticles are in organs other than kidneys and bladder\textsuperscript{50} and the uptake into different brain tumor models (U87 and 9 L) was demonstrated to be sufficient to perform MRI imaging.\textsuperscript{51} After intravenous injection of the nanoparticles (with or without peptide), the positive contrast enhancement of the tumor tissue by MRI allowed us to optimize the optical fiber implantation. With \textsuperscript{1}H-MRS, we applied the quantitative spectral analysis, allowing us to measure and to compare metabolite expression before and after iPDT for tumor and contralateral hemisphere. Lehtimaki et al used BT4C tumors undergoing (ganciclovir-HSV-tk) gene therapy as a model of programmed cell death.\textsuperscript{52} They characterized metabolic changes associated with programmed cell death, most notably a large increase in polyunsaturated and saturated fatty acids. As explained by Hakumaki et al, saturated and polyunsaturated lipid concentration extensively increase during programmed cell death despite severe cell loss.\textsuperscript{53} In our study, water-suppressed \textsuperscript{1}H NMR spectra from U87 in vivo are dominated by strong lipid signals arising from a $-\text{CH}_2\text{CH}_2\text{CH}_2-$ of saturated lipids and a $-\text{CH}_2\text{CH}_3-$ of saturated lipids. Moreover, these peak intensities increase $\approx 2$ folds after treatment. Choline-containing metabolites (choline, phosphocholine, glycerophosphocholine, taurine, myo-inositol) decreased at an advanced stage of apoptotic cell death.\textsuperscript{54} Choline-containing metabolite level increased after treatment, suggesting a presence of an acute tumor inflammatory response. In patients at the very early stage of multiple sclerosis with acute inflammatory processes, choline-containing metabolites increased with the decrease in N-acetyl aspartate levels.\textsuperscript{55} It is also well known that PDT induced inflammatory response.\textsuperscript{57} Moreover, localized edema was observed just after treatment by histological analysis. This inflammatory reaction may be secondary to an ischemic-related cell death and cytokines production. We previously demonstrated that tumors treated with the peptide-conjugated photosensitizer showed an increase in TNF-\(\alpha\) and IL-6 protein levels.\textsuperscript{28} PDT-induced inflammatory changes were widely characterized by enhanced expression of a number of pro-inflammatory cytokines, including IL-1\(\beta\), TNF-\(\alpha\) and IL-6.\textsuperscript{56,57}

Using the peptide-conjugated photosensitizer, we demonstrated an induction of tissue factor expression immediately post-treatment, leading to the establishment of thrombogenic effects within the vessel lumen.\textsuperscript{28} Tissue factor pathway can also influence inflammatory signaling by activation of protease-activated receptor-1 and -2 or expression of TNF-\(\alpha\) and IL-6.\textsuperscript{58} Szotowski et al explained that aHTF (alternatively spliced human tissue factor) released from endothelial cells contributes to the creation of an imbalance in hemostasis. This soluble tissue isoform released from endothelial cells in response to inflammatory cytokines becomes pro-coagulant in presence of phospholipids.\textsuperscript{28,58}

After iPDT, non-invasive imaging approaches and histological examination indicated a vascular disruption and edema into both tumor and BAT areas using NP-TPC-ATWLPPR. Even if it is well known that inflammatory response contributes to in vivo PDT efficiency, these effects may increase the risk for a compression syndrome. Our finality is currently to follow the tumor response to the iPDT by non-invasive imaging monitoring that could give warning signs before the tumor regrowth and thus, could provide a basis for a rational approach to determine a schedule of irradiation. A judicious choice of iPDT regimens could minimize inflammatory responses. Specific dosimetry for PDT is challenging owing to the nonlinear interaction between light dose, irradiation time, and concentration of both the photosensitizer and molecular oxygen.\textsuperscript{59,60} The effect of PDT on any tumor is dependent on a number of factors. These include the light energy absorbed by the target tumor tissue, the concentration of the photosensitizer in the tumor tissue, and the inherent sensitivity of the tissue to the photodynamic effect. The dose delivered during iPDT is determined by the amount of reactive oxygen species (ROS) that are generated, itself dependent on the photosensitizer, its concentration, the local fluence and the availability of oxygen. Tissue hypoxia resulting from vascular damage is also a continual source of ROS production. Further, pro-inflammatory cytokines and growth factors greatly increase intracellular ROS generation. A combination of both explicit and implicit parameters, monitored during iPDT would be valuable tools.

These nanoparticles provide interesting possibilities for new avenues to significantly improve iPDT. For example, the traditional delay between photosensitizer administration and light exposure needed to allow for enough clearance from normal adjacent tissue to occur along with prolonged cutaneous photosensitization is a well-known disadvantage of PDT.\textsuperscript{55} However, this disadvantage was not observed in the application in this study involving a covalently grafted photosensitizer in nanoparticles. These nanoparticles were produced to contain both a magnetic resonance contrast agent along with a therapeutic agent. The ability of vascular targeting along with imaging capability while carrying a payload of a drug by these nanoparticles provides proof for a multifunctional nanoparticle technology that can be adapted for other therapeutic purposes in future studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnano.2014.12.007.

References


Multifunctional ultrasmall nanoplatforms for vascular-targeted interstitial photodynamic therapy of brain tumors guided by real-time MRI

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This study is the first in vivo proof of concept of closed-head interstitial photodynamic therapy guided by real-time MRI using targeted ultrasmall nanoplatforms. After treatment, non-invasive imaging approaches and histological examination indicated a vascular disruption and edema into the tumor tissue areas using the conjugated nanoparticles.