Nanoparticles

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Mannose-6-Phosphate Receptor: A Target for Theranostics of Prostate Cancer**

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Abstract: *The development of personalized and non-invasive cancer therapies based on new targets combined with nano-devices is a major challenge in nanomedicine. In this work, the over-expression of a membrane lectin, the cation-independent mannose 6-phosphate receptor (M6PR), was specifically demonstrated in prostate cancer cell lines and tissues. To efficiently target this lectin a mannose-6-phosphate analogue was synthesized in six steps and grafted onto the surface of functionalized mesoporous silica nanoparticles (MSNs). These MSNs were used for in vitro and ex vivo photodynamic therapy to treat prostate cancer cell lines and primary cell cultures prepared from patient biopsies. The results demonstrated the efficiency of M6PR targeting for prostate cancer theranostic.*

Prostate cancer is the most commonly diagnosed malignancy and the second cause of cancer death among men.[1–4] Current detection and treatment of prostate cancer relies on the use of prostate specific antigen (PSA) expressing cells. Serum PSA is the most used method of detecting localized prostatic cancer and of monitoring response to treatment. However, this test presents a low specificity because PSA is also increased in most patients with benign prostatic hyperplasia. This low specificity could induce an over treatment of early and less aggressive cancers.[5] Therefore, new personalized treatments are urgently needed. The development of new systems combining theranostics and targeting represents an important challenge in nanomedicine for prostate cancer. Currently the specific targeting of prostate tumors with nanosystems relies on the well-known prostate specific membrane antigen (PSMA), which is the most prevalent prostate cancer cell biomarker, and is targeted with either antibodies, aptamers, or peptides.[6] In this work, we have identified the cation-independent mannose-6-phosphate membrane receptor (M6PR) as a new and very efficient target, and an alternative biomarker to PSMA for nanomedicine applications. M6PR is an ubiquitous receptor involved in several biological functions, and mainly in addressing enzymes to the lysosomes.[7–9] In the literature, M6PR is sometimes described as a tumor suppressor because of the decrease of its expression in liver, lung, or ovarian cancers and adenocortical tumors.[10–12] However, in a screening study, Huang et al.[13] identified circulating auto-antibodies against M6PR in sera from 30% of the patients with prostate cancer, thus suggesting the presence of this receptor in association with prostate cancer. We show here that this receptor was indeed over-expressed in prostate cancer cell lines and in tissue samples obtained from patient biopsies, compared to normal tissues. A new carbohydrate derivative, an analogue of mannose-6-phosphate, was prepared and

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specifically targeted this receptor. As photodynamic therapy is emerging as an alternative and less invasive treatment for prostate cancer,[14] its combination with new targeted biomarkers will be particularly useful for personalized nanomedicine of this cancer. Therefore, this carbohydrate was anchored on the surface of functionalized mesoporous silica nanoparticles (MSNs), and one- or two-photon photodynamic therapy of prostate cancer cells and tissues was achieved through an active endocytosis pathway involving M6PR.

The expression profile of M6PR was first studied in both normal and cancer cell lines by Western blot using anti-M6PR antibody. The first blot, made from with whole-cell protein extracts of three prostate cancer cell lines, showed that M6PR was strongly over-expressed in LNCaP cells, and to a lesser extent in DU-145 cells (Figure 1a). Another blot comparing M6PR expression in different normal (HUVEC, fibroblasts, ARPE-19) and cancer cell lines (LNCaP, MCF-7, MDA-MB-231, MDA-MB-435, HCT-116, Y-79) demonstrated that only MCF-7 breast cancer and LNCaP prostate cancer cells over-expressed the receptor, whereas normal cells did not show any detectable expression of M6PR (Figure 1b). Furthermore, cancer prostate tissues from 126 patients were collected during prostate surgery and paraffin-embedded for immunohistochemical analysis. They were compared with 39 normal prostate tissues and two adenomas. Each sample was analyzed by a pathologist to define the Gleason scores corresponding to the severity of the cancer (see Table S1 in the Supporting Information). Immunostaining of prostate cancer tissues using anti-M6PR antibody indicated two different expression patterns. The first type of staining observed in 23% of the samples is granular and perinuclear. This staining is the most conventional (Figure 1c) and generally observed in normal M6PR-rich tissues.[15–17] The second type, observed in 61% of samples, is also granular but more diffused in the cytoplasm (Figure 1d). By contrast, none of the 39 normal tissues and two adenomas were stained, thus suggesting that the over-expression of M6PR is specific of malignant cells (Figure 1e,f). The diffused staining of cancer cells was totally prevented by incubation of the M6PR antibody with an excess of free M6PR (Figure 1g,h), and thus validated the specificity of M6PR staining. In normal prostate samples, the expression of M6PR, revealed by immunohistochemistry is low, that is, less than 10% of stained cells (Figure 1i). By contrast, 84% of the prostate cancer samples presented an over-expression of M6PR (Figure 1j). These results provide the first evidence of M6PR-specific overexpression in prostate cancer cells as compared to normal prostate epithelial cells, and suggest its potential use for nanomedicine applications.

Therefore, we investigated targeting M6PR with carbohydrates and we synthesized an analogue of M6P, **M6C** (Scheme 1). After the trimethylsilylation of 4-nitrophenyl α-D-mannopyranoside (**1**), the selective deprotection at position 6 of the persilylated compound **2** was carried out to give the primary alcohol **3**. The alcohol **3** was next subjected to a Swern oxidation to afford the aldehyde intermediate which was then treated with the anion of triethyl phosphonoacetate to lead to the carboxylate ester **4**. The carboxylate ester **5** was readily obtained after removal of the TMS groups. The reduction of the double bond and the nitro function under

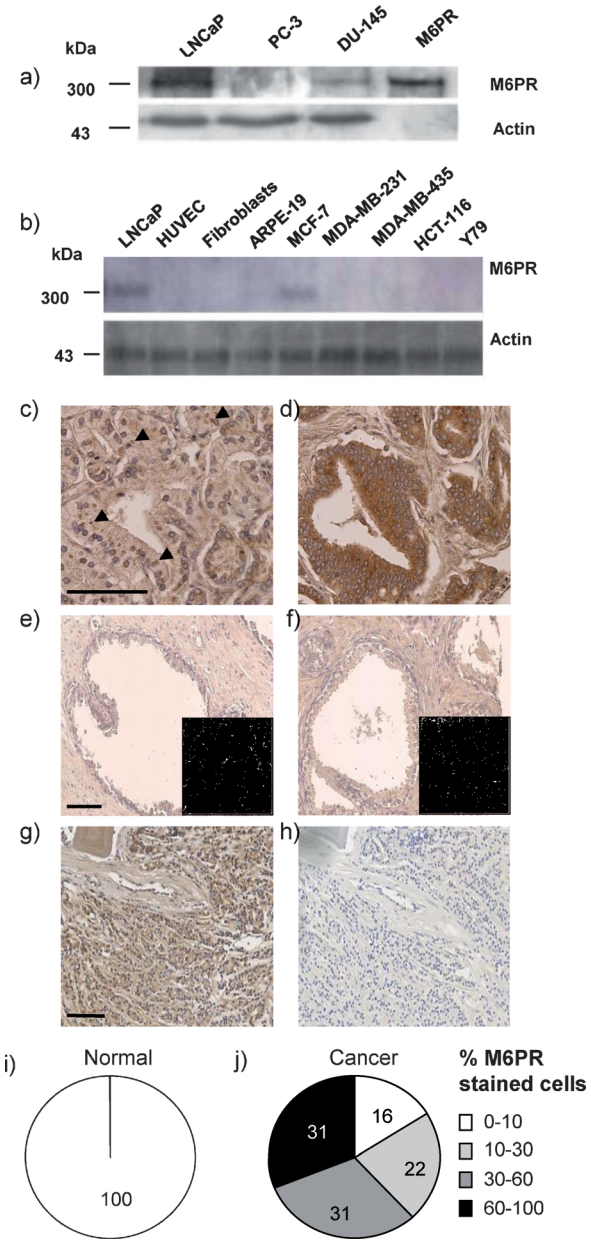


Figure 1. M6PR expression patterns in either normal or cancer cell lines and normal or cancerous prostate tissues.

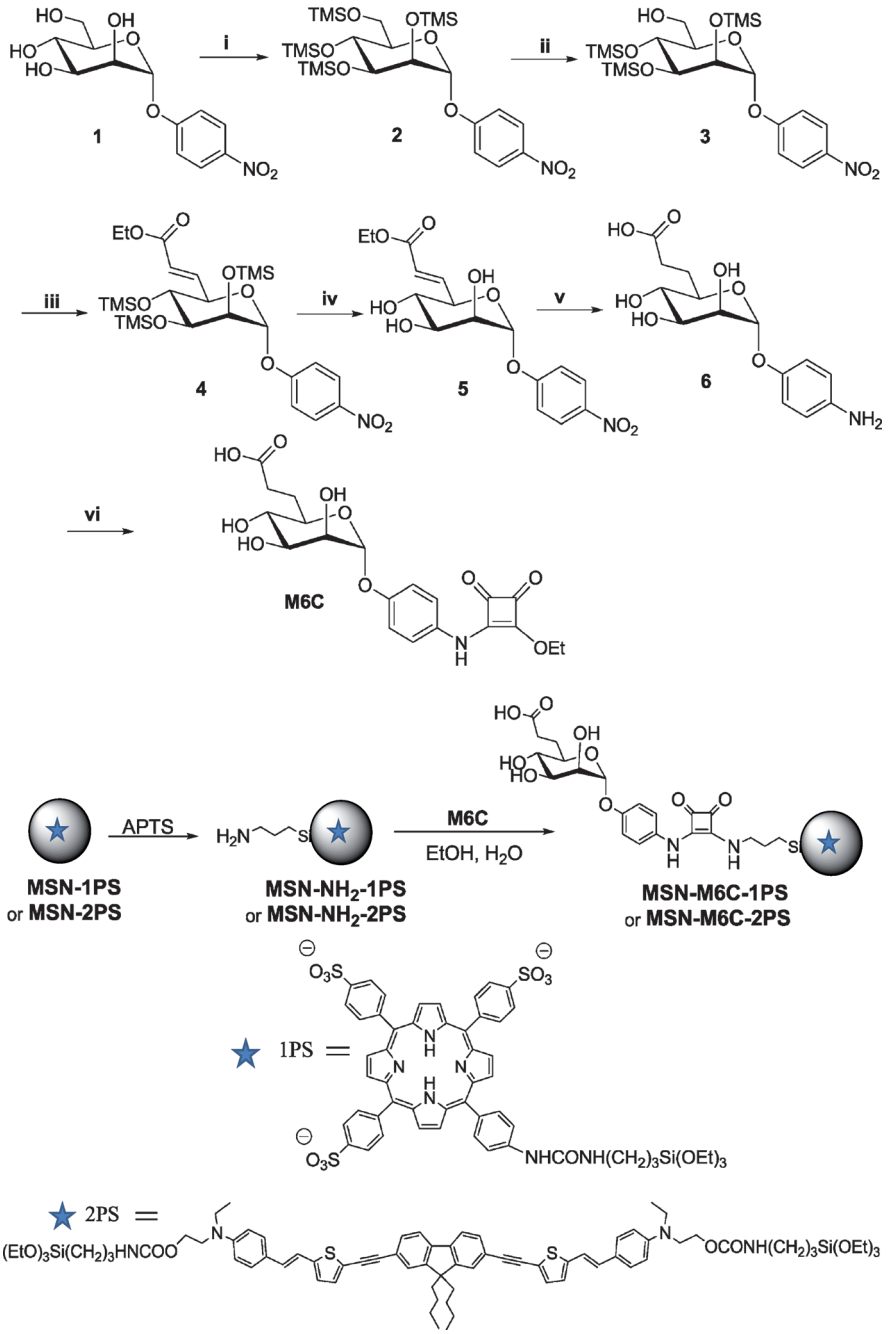
catalytic hydrogenation, followed by the saponification of ester **5** provided the carboxylate **6**. The carboxylate **6** was then treated with diethyl squarate to afford the carboxylate phenylsquarate **M6C. M6C** thus possesses a lateral chain with a carboxylate function bioisostere of the phosphate moiety, which is insensitive to serum phosphatase.[18,19] The affinity of **6** for the M6PR was evaluated and was high (see Table S2 in the Supporting Information). Mesoporous silica nanoparticles covalently incorporating one- and two-photon photosensitizers, MSN-NH_2-1PS[20] and MSN-NH_2-2PS,[21] respectively, were prepared as previously described. **M6C** was then anchored on the surface of these particles using the procedure we developed with mannose,[22] thus leading to MSN-M6C-1PS and MSN-M6C-2PS.

The potential of M6PR for theranostic applications was first evaluated on the LNCaP prostate cancer cell line. Two-photon fluorescence imaging of living LNCaP cells was performed at a $\lambda = 900$ nm excitation wavelength and a low laser power (Figure 2). MSN-M6C-2PS was incubated for 24 hours at a concentration of 100 $\mu g\,mL^{-1}$ and were highly endocytosed in LNCaP cancer cells, and were colocalized with the lysosomes (see Figure S1 in the Supporting Information). Moreover, M6PR-mediated endocytosis of MSN-M6C-2PS was shown by the competitive inhibition of the cell internalization of MSN-M6C-2PS when incubated for 2 hours with an excess of M6P (10 mM; Figure 2). Two-photon excited photodynamic therapy[21] (TPE-PDT) on LNCaP cancer cell lines was then investigated (Figure 3).

LNCaP cancer cells were incubated for 24 hours with either MSN-2PS or MSN-M6C-2PS, and then irradiated with a multiphotonic laser or not (Figure 3a). Irradiation was performed at $\lambda = 760$ nm for a very short time (3×1.57 s), with a focused laser beam and at maximum laser power. The photodynamic therapeutic potential of MSN-M6C-2PS was higher than that of MSN-2PS (55% cell death versus 30% cell deaths, respectively; Figure 3b). The addition of an excess of M6P before TPE-PDT with MSN-M6C-2PS in the culture medium totally inhibited cancer cell death (2 h incubation; see Figure S2), thus showing the active endocytosis of MSN-M6C-2PS through M6PR.

One-photon excited PDT (OPE-PDT) at $\lambda = 650$ nm (6.5 $J\,cm^{-2}$) using either MSN-M6C-1PS or MSN-1PS was also performed on LNCaP, DU-145, and PC-3 prostate cancer cell lines (see Figure S3) and was very efficient on LNCaP cells (96% cancer cell death with MSN-M6C-1PS). The involvement of M6PR in the active endocytosis of MSN-M6C-1PS was shown as well. Interestingly, in normal fibroblasts, the same treatment did not induce any cell death, thus suggesting an absence of internalization of MSN-M6C in normal cells (Figure S3).

To further demonstrate the efficiency of the M6PR targeting, OPE-PDT experiments using MSN-M6C-1PS were performed ex vivo on prostatic cancer cells from patients. After verification by immunohistochemistry of M6PR over-expression in prostate cancer tissues (Figure 4a), primary cultures were treated with 20 $\mu g\,mL^{-1}$ of either MSN-M6C-1PS or MSN-1PS and submitted to monophotonic irradiation at $\lambda = 650$ nm (6.5 $J\,cm^{-2}$). The laser alone or MSNs without irradiation did not induce damage on primary culture cells (Figure 4b). The quantification of PDT activity on primary culture cells with MSN-6C 1PS induced 75% cell death, while untargeted MSN-1PS induced only 28% cancer



Scheme 1. Synthesis of an M6C analogue of M6P and grafting on MSNs. i) TMSCl, CH_2Cl_2, Et_3N, RT, 21 h, 95%; ii) K_2CO_3, MeOH, 0°C, 15 min, 50%; iii) 1. DMSO, $(COCl)_2$, Et_3N, THF, −78°C, 2. triethyl phosphonoacetate, NaH, THF, RT, 90 min, 73% over 2 steps; iv) HCl 0.5 N, THF, RT, 10 min, 91%; v) 1. H_2, Pd/C, ethanol/H_2O (4:1) RT, overnight, 98% 2. NaOH 0.1 N, THF, RT, 6 h, 91%; vi) diethyl squarate, EtOH/H_2O (2:1.5), RT, 4 h, 56%. DMSO = dimethylsulfoxide, THF = tetrahydrofuran, TMS = trimethylsilyl.

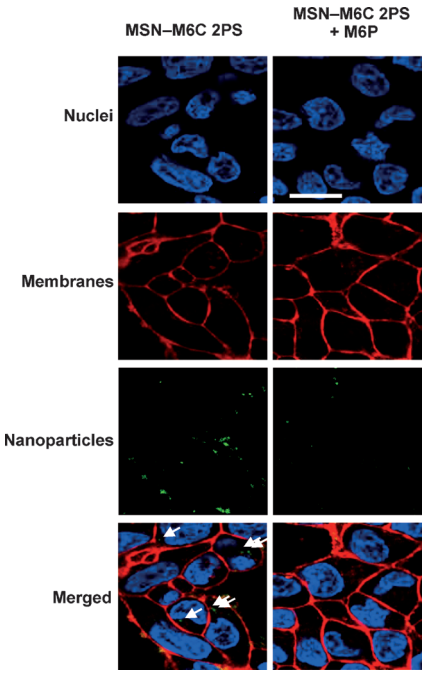


Figure 2. Two-photon fluorescence imaging of LNCaP living cells with MSN-M6C-2PS.

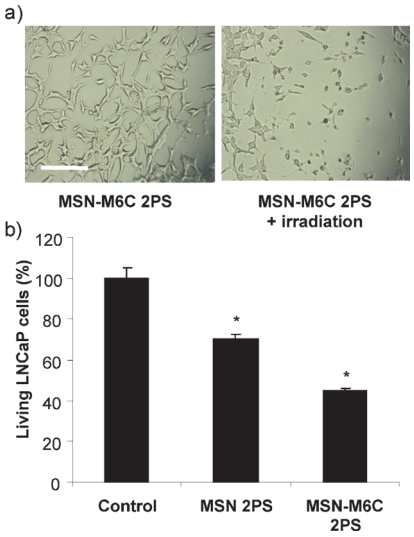


Figure 3. TPE-PDT on LNCaP cancer cells with MSN-M6C-2PS.

cell death (Figure 4c). These data demonstrate that the therapeutic strategy using M6PR as a target for prostate cancer is strongly effective on primary cultures directly isolated from tissues.

The search of new biomarkers remains of major importance for theranostic applications in nanomedicine. Our study presents M6PR as a new target because it fulfills important and necessary properties: 1) over-expression with a very large percentage (84%) in prostate cancer tissues, 2) no expression in normal tissues or non-cancerous hypertrophy of prostate, and 3) over-expression in low-grade cancers. Furthermore, the membrane location of M6PR is an important feature for

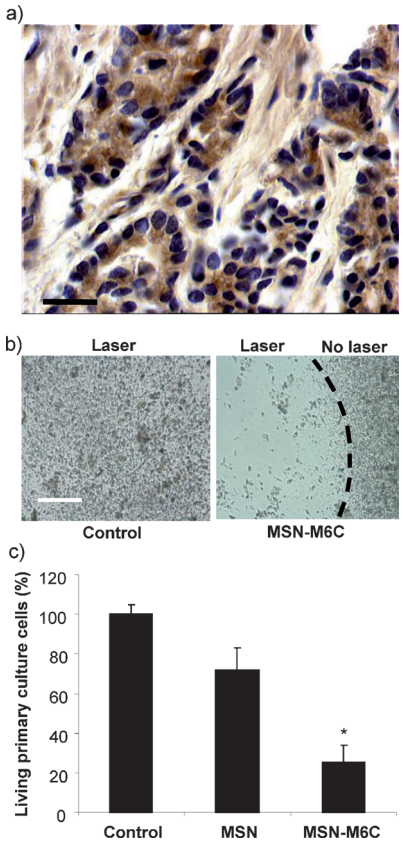


Figure 4. The ex vivo PDT efficacy on primary cultures. a) Cancer prostate tissues, from patients, were immunostained with anti-M6PR antibody. Scale bar: 25 μm. b) Microscopy pictures of primary cultures established from tissue submitted to or not submitted to PDT (18 h incubation with 20 $\mu g\,mL^{-1}$ MSN-M6C, irradiation at 650 nm). c) Comparison of PDT efficiency on primary cultures with MSNs and MSN-M6C. Values represent the percent of living cells as compared to control. Means ± s.d. ($n=3$). *$p<0.01$ versus control.

nanomedicine applications. An analogue of mannose-6-phosphate (M6C), which is stable in culture medium was therefore synthesized to target M6PR, and it was demonstrated that M6C-functionalized MSNs were very efficient for M6PR targeting, imaging, and photodynamic therapy. Therefore M6PR appears as a new promising target for nanomedicine applications in prostate cancer, particularly for a non-invasive and personalized therapy of small-sized prostate tumors.

Keywords: cancer · carbohydrates · nanoparticles · receptors · synthesis design

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