

Targeting the Oncofetal Thomsen–Friedenreich Disaccharide Using Jacalin-PEG Phthalocyanine Gold Nanoparticles for Photodynamic Cancer Therapy**

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Photodynamic therapy (PDT) is an emerging therapeutic modality for cancer treatment during which a photoactive drug, termed a photosensitizer, is used to destroy tumors upon activation with red or near-infrared light through the production of cytotoxic singlet oxygen.^[1] PDT has shown significant efficacy with photosensitizers, such as Photofrin and Foscan, which are in current clinical use for the treatment of a number of cancers.^[2]

The “ideal” photosensitizer has been shown to possess hydrophobic characteristics^[1] and consequently suffers from limited aqueous solubility, thus impairing its systemic biodistribution.^[3] An additional drawback is that some photosensitizers exhibit limited selectivity towards cancerous tissue, thus resulting in nonspecific photodamage of peripheral tissue. To overcome these limitations there have been considerable efforts to develop nanoparticle-based systems for use as photosensitizer drug delivery vehicles.^[2] Such systems maintain the stability and activity of hydrophobic photosensitizers in aqueous environments, while the nanoparticle itself provides a platform for further functionality with targeting moieties for cancer-specific PDT.^[2] The targeting of tumors using nanoparticle systems can be achieved either passively or actively. Passive targeting with nanoparticles involves the enhanced permeability and retention (EPR) effect that is invariably observed in tumors.^[4] Active targeting of nanoparticles can be achieved through the conjugation of biological ligands, such as antibodies,^[5] peptides,^[6] carbohydrates,^[7] and folic acid,^[8] each with an affinity for a specific surface receptor expressed by cancer cells.^[9]

During the transformation of healthy to malignant cells aberrations in the glycosylation of cell-surface glycoproteins occur. It has been suggested that these glycosylation patterns play roles in cancer cell metastasis, survival, and evasion of immune surveillance.^[10] One of the most therapeutically attractive cancer-associated carbohydrates is the Thomsen–Friedenreich disaccharide antigen (T antigen).^[11] The T anti-

gen is a truncated O-glycan (Gal β 1-3GalNAc-*O*-serine/threonine) that is expressed in more than 90% of primary human carcinomas, but is usually cryptic in normal cells.^[11] This antigen is often referred to as being oncofetal, as its exposure at the cell surface is only observed in developing embryonic cells or cancer cells.^[11,12] Since the T antigen is expressed in such a large number of cancers and its exposure on non-developmental cells is restricted to malignant tissue, it is clearly an appealing molecular target for cancer therapies, such as PDT.

Herein we present, for the first time, the synthesis and application of gold nanoparticles that are stabilized by a mixed monolayer of a hydrophobic zinc phthalocyanine photosensitizer and a water-soluble thiol-functionalized poly(ethylene glycol) (PEG) to which jacalin (a lectin) is covalently conjugated to specifically target the T antigen disaccharide. Previously, lectin-functionalized nanoparticles have been used for the targeted delivery of drugs to the brain,^[13] lungs,^[14] and colon,^[15] while other lectin–nanoparticle conjugates, based on gold nanoparticles^[16] and quantum dots,^[17] have been used for bioimaging.^[16–18] Lectins (specifically, wheat germ agglutinin loaded on polymeric nanoparticles and fluorescently labeled nanospheres functionalized with peanut agglutinin) have been used to enhance drug delivery to colon cancer cells^[19] and to detect implanted colorectal tumors expressing the T antigen,^[20] respectively. Within PDT, wheat germ agglutinin has been used with liposomes carrying the photosensitizer temoporfin for antimicrobial studies,^[21] and Poiroux et al.^[22] directly coupled the Morniga G lectin to a water-soluble porphyrin photosensitizer to target the T antigen and the Tn antigen (another cancer-associated carbohydrate) expressed on Jurkat leukemia cells.

We have now demonstrated that the jacalin-PEG phthalocyanine gold nanoparticles described herein target the T antigen expressed on HT-29 colon adenocarcinoma cells. The jacalin is shown to promote cellular adhesion and uptake of the nanoparticle conjugates. As a result of jacalin targeting, the specificity of which was confirmed through inhibition studies, enhanced cellular uptake of the nanoparticles was achieved. Exceptional PDT efficacy with the light-activated jacalin–nanoparticle conjugates for the destruction of the colon cancer cells has been demonstrated. Finally, it has been established that necrosis was the mechanism of jacalin-targeted photodynamic toxicity. The results highlight that jacalin-PEG phthalocyanine nanoparticles can selectively target the important T antigen on the surface of colon

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cancer cells and elicit exceptional photodynamic efficacy of cell death.

To achieve these results, gold nanoparticles were first synthesized by the reduction of gold(III) chloride trihydrate using sodium borohydride in the presence of two stabilizing ligands; the octaalkyl-substituted zinc(II) phthalocyanine (C11Pc) photosensitizer^[5a] and a water-soluble thiolated poly(ethylene glycol).^[23] Experimental methods are provided in the Supporting Information. The as-synthesized gold nanoparticles have a mean diameter of 4.3 nm as determined by TEM (Figure S1 in the Supporting Information). The thiolated PEG used herein together with the disulfide C11Pc photosensitizer simultaneously self-assembled onto the nanoparticle surface through gold–sulfur interactions to form a mixed monolayer that preserved the active monomeric form of the C11Pc and provided water solubility for the nanoparticle system; these nanoparticles are referred to as C11Pc-PEG gold nanoparticles throughout. By determining the ratio of Au/Zn using inductively coupled plasma optical emission spectroscopy (ICP-OES; see the Supporting Information) the number of zinc phthalocyanine photosensitizer molecules on each nanoparticle was estimated to be 155. The UV/visible absorption spectrum of the C11Pc-PEG gold nanoparticles in aqueous buffer (Figure S2 in the Supporting Information) confirmed that the C11Pc on the gold nanoparticle surface was predominantly monomeric.

For active targeting, the C11Pc-PEG gold nanoparticles were conjugated to jacalin, which is a tetrameric lectin extracted from Jackfruit (*Artocarpus integrifolia*) that has a high specificity towards the T antigen.^[24] Jacalin has been shown to bind specifically to the T antigen expressed on the surface of HT-29 cells with a dissociation constant (K_d) of (500 ± 50) nM.^[25] Conjugation was achieved using *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as activation reagents to form stable amide bonds between the lysine residues of the jacalin and the terminal carboxyl groups of the PEG on the gold nanoparticle surface (Figure 1). To ensure that the jacalin-conjugated C11Pc-PEG gold nanoparticles (hereafter referred to as jacalin-conjugated nanoparticles) were able to produce singlet oxygen, a buffered solution of the conjugates was irradiated at 633 nm in the presence of the singlet-oxygen probe, anthracene-9,10-dipropionic acid (ADPA). Progressive photobleaching of the ADPA (Figure S3 in the Supporting Information) confirmed that the jacalin-conjugated nanoparticles produce singlet oxygen.

HT-29 colon adenocarcinoma cells that were incubated with either jacalin-conjugated or nonconjugated nanoparticles were imaged with combined DIC and confocal fluorescence microscopy. The intracellular localization and accumulation of the jacalin-conjugated and nonconjugated nanoparticles were visualized by exploiting the intrinsic red fluorescence of the C11Pc photosensitizer on the nanoparticles. The internalization of the nanoparticle conjugates is visible in Figure 2 where it is clear that the extent of endocytosis of the jacalin-conjugated nanoparticles (Figure 2A) is significantly greater than that observed for the nonconjugated nanoparticles (Figure 2B). The jacalin-conjugated nanoparticles appear to be distributed throughout the

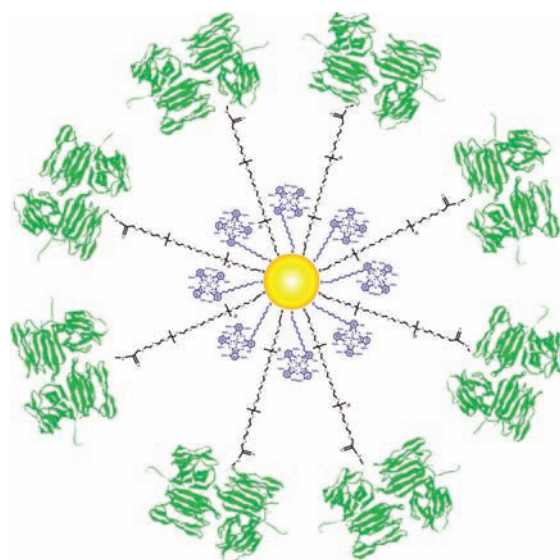


Figure 1. Schematic of the T antigen-specific lectin, jacalin (green), conjugated to the mixed monolayer of the phthalocyanine C11Pc photosensitizer (blue) and the thiol-functionalized PEG (black) on the gold nanoparticle surface. The structure of jacalin was obtained from the Protein Data Bank (PDB ID 1JAC).^[26]

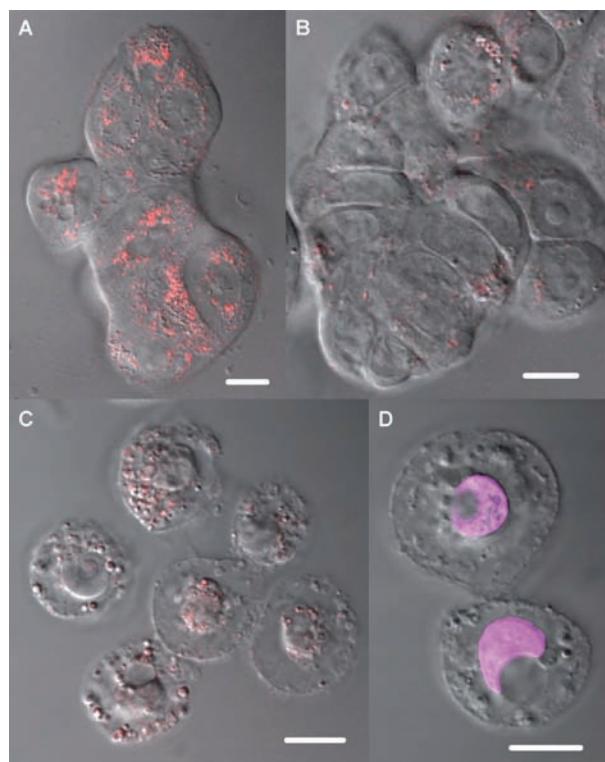


Figure 2. Merged differential interference contrast (DIC) and confocal fluorescence microscopy images of HT-29 colon adenocarcinoma cells incubated for three hours with A) jacalin-conjugated nanoparticles ($2 \mu\text{M}$ C11Pc equivalent) and B) nonconjugated nanoparticles ($2 \mu\text{M}$ C11Pc equivalent). C) After PDT treatment, the cell morphology of HT-29 cells incubated with jacalin-conjugated nanoparticles dramatically changed, and D) the HT-29 cells after PDT with the jacalin-conjugated nanoparticles stained positive for propidium iodide ($5 \mu\text{g mL}^{-1}$). Scale bars = $10 \mu\text{m}$.

HT-29 cells, thus suggesting that they have been internalized through receptor-mediated endocytosis.

After irradiation using a 633 nm HeNe laser, the morphology of the HT-29 cells incubated with the jacalin-conjugated nanoparticles dramatically changed (Figure 2C). The attachment of the cells to the coverslip weakened, and the cells became spheroid in nature with a poorly defined periphery, features typical of dead cells. Cell membrane perforation and degradation is a key indicator of cell death through the necrotic pathway. Perforation of the cell membrane was assessed using the fluorescent marker propidium iodide, which is a membrane-impermeable nucleotide interchelator that specifically binds to DNA once the cell membrane is damaged.^[27] The HT-29 cells that were incubated with the jacalin-conjugated nanoparticles stained positive for propidium iodide after PDT treatment (Figure 2D), thereby indicating a significant loss in membrane integrity post PDT and possibly necrosis.

To establish the PDT efficacy of the jacalin-conjugated nanoparticles, a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell viability assay was used. Metabolically active cells reduce MTT to formazan, which is soluble in organic solvents, such as dimethylsulfoxide (DMSO), and can be quantified by measuring the absorbance intensity at 550 nm.^[28] HT-29 cells were incubated with varying concentrations of the jacalin-conjugated or nonconjugated nanoparticles for three hours. The cells were subjected to PDT using 633 nm light from a HeNe laser, fitted with a biconvex diverging lens that was positioned 50 cm above the cells; this configuration gave an increased beam diameter of 5 mm. Cell viability was assessed 48 h after PDT treatment, and cells that were kept in the dark were used as a control. The viability of the HT-29 cells after PDT treatment is shown in Figure 3. HT-29 cells incubated with the nonconjugated nanoparticles exhibited minimal phototoxicity after PDT treatment. At the maximum concentration of 1.15 μM C11Pc equivalent of the nonconjugated nanoparticles only approximately 8% (8.32 ± 1.22 %) of the cells were killed (Figure 3A). However, when jacalin was conjugated to the nanoparticles an exceptional proportion of cells were killed, approximately 95% (95.56 ± 2.34 %), after one cycle of targeted PDT treatment (Figure 3B). In both instances, with or without jacalin conjugation, there was only minimal dark toxicity of the C11Pc-PEG gold nanoparticles as shown by the limited decrease in cell viability after incubation of

the nanoparticles without light irradiation (Figure 3A and B). It is important to note that the free C11Pc photosensitizer is not water-soluble. Consequently, it is not possible to directly compare the PDT efficacy of the free photosensitizer with the jacalin-conjugated (or the nonconjugated) nanoparticles. A sigmoidal dose dependency for the phototoxicity of the jacalin-conjugated nanoparticles was observed (Figure 3C). Figure 3C clearly shows the enhanced photodynamic efficacy when the jacalin is conjugated to the nanoparticles for targeting the HT-29 colon cancer cells. The enhanced PDT efficacy highlights the effectiveness of jacalin for specifically targeting cancerous cells for efficient cancer therapy.

To confirm that the enhanced phototoxicity of the HT-29 cells was due to specific interactions between the jacalin that was conjugated to the nanoparticles and the T antigen that was exposed at the cell surface, competitive inhibition studies were performed using methyl- α -D-galactopyranoside and asialofetuin (a glycoprotein expressing T antigens on its surface^[29]). Both bind to jacalin, thus competitively inhibiting jacalin-targeted phototoxicity of HT-29 cells. The inhibition of the photodynamic toxicity, as assessed by the MTT viability assay, is shown in Figure 3D. The photodynamic toxicity is expressed as a difference of the percentage viability of the HT-29 cells between dark toxicity and photodynamic toxicity after PDT treatment. It is clear from Figure 3D that the presence of either methyl- α -galactopyranoside or the asialofetuin inhibited the photodynamic efficacy of the jacalin-conjugated nanoparticles towards the HT-29 cells. The results

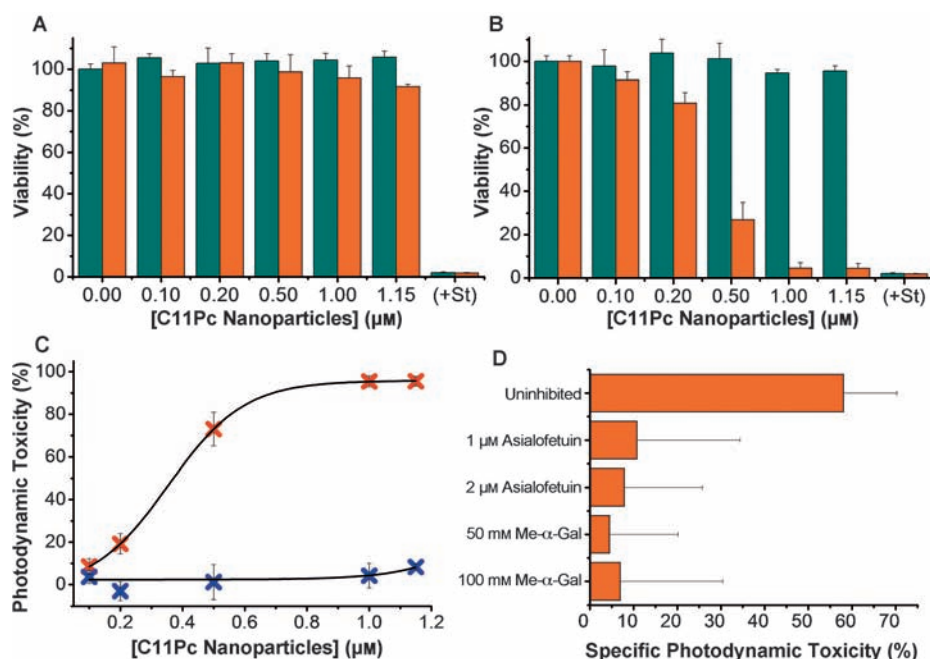


Figure 3. MTT viability assay of HT-29 cells that were incubated for three hours with varying concentrations of A) nonconjugated and B) jacalin-conjugated nanoparticles, with (orange) and without (dark cyan) irradiation. (+ St) refers to cells treated with staurosporine (20 μM), a positive control for cytotoxicity. Viability is expressed as a percentage of untreated, non-irradiated cells. The concentration [C11Pc Nanoparticles] in (A–C) refers to the C11Pc equivalents of the used nanoparticles. C) Photodynamic toxicity of jacalin-conjugated (red X) and nonconjugated (blue X) nanoparticles after PDT treatment of HT-29 cells. D) Inhibition of photodynamic toxicity of jacalin-conjugated nanoparticles (0.5 μM C11Pc equivalent) using asialofetuin (1 μM or 2 μM) and methyl- α -D-galactopyranoside (50 mM or 100 mM, Me- α -Gal). (Error bars are $\pm$ 95% confidence intervals, $n = 3$).

suggest that the enhanced photodynamic efficacy observed with the jacalin-conjugated nanoparticles was dependent on the targeted binding of the jacalin to the T antigen on the HT-29 cell surface.

After photodynamic treatment with the jacalin-conjugated nanoparticles the HT-29 cells stained positive for propidium iodide (Figure 2D), thus indicating that necrosis could be the mechanism of cell death. To determine the exact mechanism of cell death the ApoTox-Glo™ triplex assay was performed. These three assays assess cell viability, cytotoxicity, and apoptosis. The HT-29 cells incubated with jacalin-conjugated nanoparticles, with and without irradiation, were first assessed for viability and cytotoxicity using the GF-AFC viability reagent and the bis-AAF-118 cytotoxicity reagent, respectively. After irradiation at 633 nm the HT-29 cells that were incubated with the jacalin-conjugated nanoparticles showed a remarkably low viability of (2.67 ± 0.67) % (Figure 4A), thereby further highlighting the PDT efficacy of the nanoparticle conjugates. Without irradiation the cell viability remained at (78.27 ± 6.1) %. Since the rate of cleavage of the GF-AFC viability substrate depends on the rate of cell proliferation, it is possible that the slight loss in viability that was observed when the jacalin–nanoparticle conjugates had not been irradiated is due to the noncytotoxic, reversible inhibition of proliferation that jacalin induces with HT-29 cells upon binding.^[25] However, both of the cell viability assays used in this study confirm the excellent photodynamic efficacy of the jacalin-conjugated nanoparticles towards the HT-29 colon cancer cells.

After irradiation the HT-29 cells that were incubated with the jacalin-conjugated nanoparticles exhibited a sharp increase in cytotoxicity (Figure 4B). The cytotoxicity levels were approximately double that of the cytotoxicity levels of HT-29 cells incubated with staurosporine, which induces cell death mainly through the apoptotic pathway.^[30] This result supports the observation that the HT-29 cells have died through the necrotic pathway as indicated by positive staining of the cell nucleus with propidium iodide (Figure 2D). After PDT treatment with the jacalin-conjugated nanoparticles, the HT-29 cells were assessed for apoptosis using the Caspase-Glo 3/7 bioluminescent component of the triplex assay (Figure 4C); the presence of caspase-3 or caspase-7 is indicative of cell death through the apoptosis pathway. The HT-29 cells treated with staurosporine appeared to have a 14-fold higher activity of caspase-3 or caspase-7 as compared to the untreated, non-irradiated control. However, when the HT-29 cells were incubated with jacalin-conjugated nanoparticles, there was no caspase-3 or caspase-7 activity with or without irradiation at 633 nm (Figure 4C). These combined results confirm that PDT with the jacalin-conjugated nanoparticles, under the defined conditions used in this study, induces cytotoxicity through the necrotic pathway with no indication of apoptosis.

In summary, the lectin jacalin has been conjugated to C11Pc-PEG gold nanoparticles to target the T antigen on the surface of HT-29 human colorectal adenocarcinoma cells. The jacalin–nanoparticle conjugates were able to specifically recognize and bind to the cancer-associated T antigen disaccharide to deliver the phthalocyanine photosensitizer for

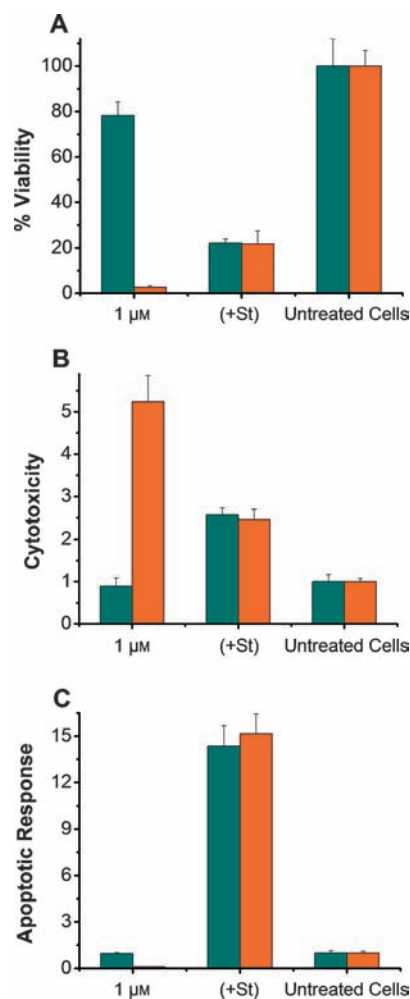


Figure 4. ApoTox-Glo™ triplex assay results of HT-29 cells incubated with jacalin-conjugated nanoparticles (1 μ M C11Pc equivalent) for three hours with (orange) and without (dark cyan) irradiation at 633 nm measuring A) cell viability, B) cell cytotoxicity, and C) apoptosis. Cell viability is expressed as a percentage of fluorescence emission (492 nm) of untreated, non-irradiated cells. Cell cytotoxicity is given as normalized fluorescence emission (530 nm) of untreated, non-irradiated cells. Apoptosis is given as a normalized bioluminescence emission (576 nm) of untreated, non-irradiated cells. Staurosporine (20 μ M; +St) was used as a positive control for apoptosis. (Error bars are $\pm$ 95% confidence intervals, $n=3$.)

photodynamic cancer therapy. After irradiation with 633 nm light, the lectin-targeted nanoparticle conjugates induced cell death of a remarkable proportion of cancer cells (ca. 95–98%) compared to the nonconjugated nanoparticles or the conjugated nanoparticles without irradiation. The mechanism of photodynamic action was established to be predominantly through the necrotic pathway. Since the T antigen is expressed by more than 90% of primary human carcinomas,^[11] the ability to target this cell-surface antigen with the jacalin-conjugated nanoparticles and then to efficiently destroy the cells with PDT is a significant first and potentially an important development in cancer therapy.

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