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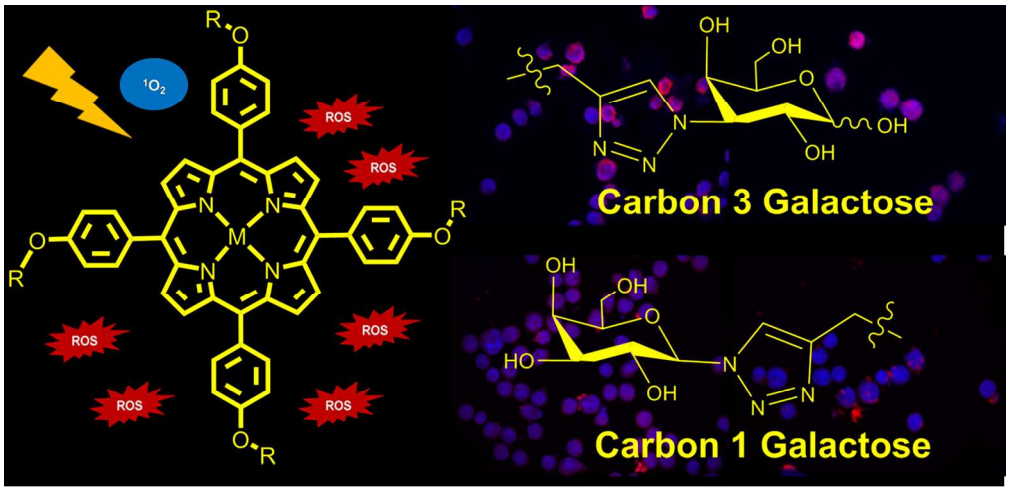
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Carbon-1 versus carbon-3 linkage of *D*-galactose to porphyrins: Synthesis, uptake, and photodynamic efficiency

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ABSTRACT: The use of glycosylated compounds is actively pursued as a therapeutic strategy for cancer due to the overexpression of various types of sugar receptors and transporters on most cancer cells. Conjugation of saccharides to photosensitizers such as porphyrins provides a promising strategy to improve the selectivity and cell uptake of the photosensitizers, enhancing the overall photosensitizing efficacy. Most porphyrin-carbohydrate conjugates are linked *via* the carbon-1 position of the carbohydrate because this is the most synthetically accessible approach. Previous studies suggest that carbon-1 galactose derivatives diminish binding since the hydroxyl group in the carbon-1 position of the sugar is a hydrogen bond acceptor in the galectin-1 sugar binding site. We therefore synthesized two isomeric porphyrin-galactose conjugates using click chemistry: one linked *via* the carbon-1 of the galactose, and one linked *via* carbon-3. Free base and zinc analogs of both conjugates were synthesized. We assessed the uptake and photodynamic therapeutic (PDT) activity of the two conjugates in both monolayer and spheroidal cell cultures of four different cell lines. For both the monolayer and spheroid models, we observe that the uptake of both conjugates is proportional to the protein levels of galectin-1 and the uptake is suppressed after pre-incubation with an excess of thiogalactose, as measured by fluorescence spectroscopy. Compared to the carbon-1 conjugate, the uptake of the carbon-3 conjugate was greater in cell lines containing high expression levels of galectin-1. After photodynamic activation, MTT and lactate dehydrogenase assays demonstrated that the conjugates induce phototoxicity in both monolayers and spheroids of cancer cells.

INTRODUCTION

Galectins are animal lectins with high affinity for β-galactose-containing oligosaccharides.[1] Galectin-1, a member of this family, has a key role in different events associated with cancer biology including tumor transformation. Galectin-1 has been exploited as a target for anticancer drugs, since its expression is higher in tumors compared to normal tissues.[2]

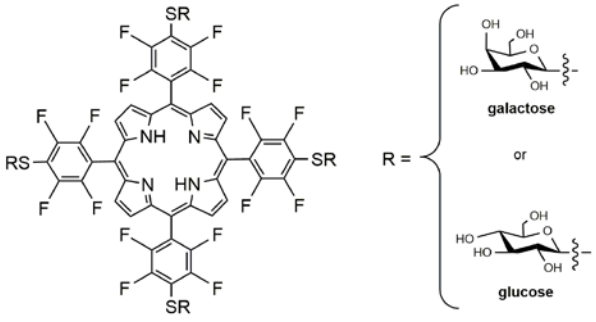


Figure 1. Tetrathioglycosylated porphryins.

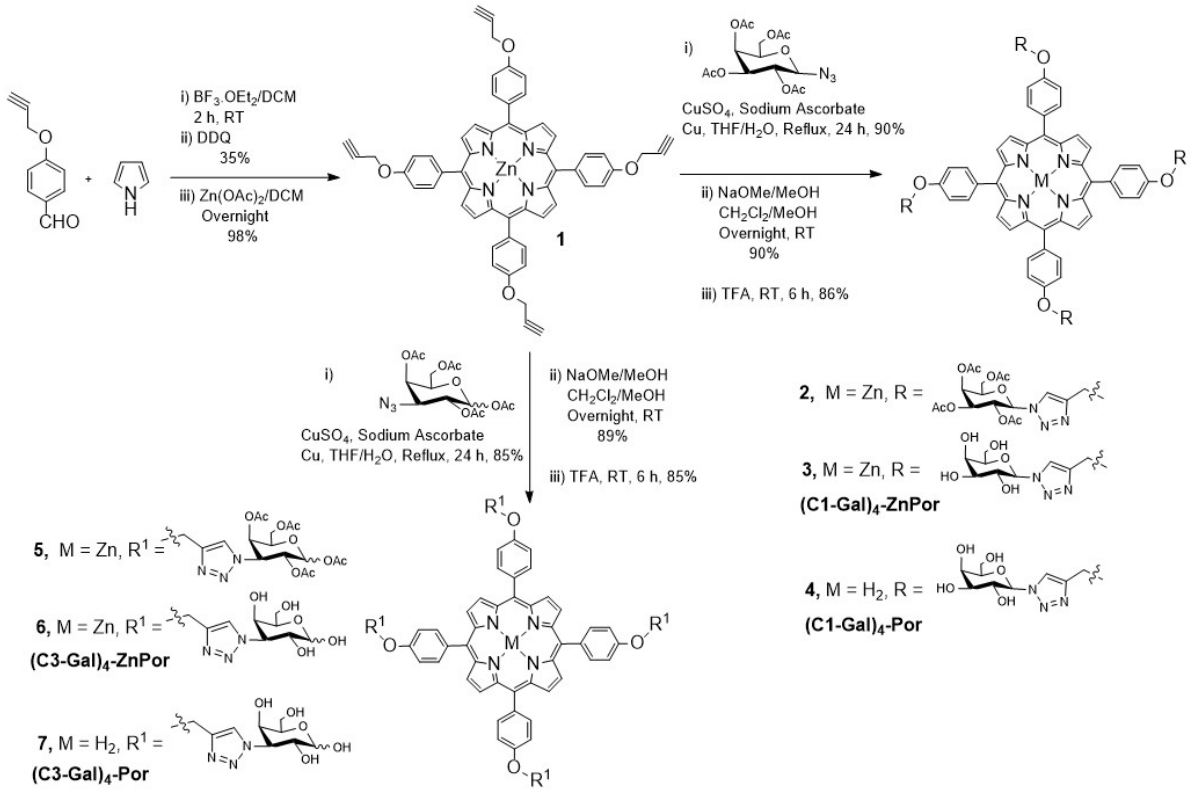
Drugs and other molecules can be taken up by cells *via* active or passive transport mechanisms, or combination of both.[3] Passive mechanisms involve simple diffusion of the molecule through the cell membrane and depends on the molecular hydrophobicity,[4] *e.g.* the octanol/water partition coefficient, while active uptake requires the target molecules to be recognized by specific receptors and other intermolecular interactions. Porphyrinoids and other photosensitizers (PS) can be targeted toward cell receptors by appending appropriate targeting moieties. For example, chromophores appended with carbohydrates are directed to specific cell surface carbohydrate receptors or carbohydrate-binding proteins overexpressed in cancer cells. After light activation, glyco-PS can sensitize the formation of

reactive oxygen species and induce cytotoxicity.[5-16] Simple click-type reactions were reported to append polyglycerol dendrimers, glucose, polyethylene glycols, polyamines and polysaccharides to porphyrinoids.[5, 17-20] Since most glycosylated porphyrinoids have *O*- linkages where hydrolysis diminishes *in vivo* effectiveness, non-hydrolysable linkages can be more effective.[5]

PS such as porphyrins are commonly used in photodynamic therapy (PDT).[6-9, 11, 16, 21] PDT is a rapidly growing modality to treat age-related macular degeneration, cancer, and various skin disorders using visible light.[4, 21, 22] Previously, our group appended PEGs, polyamines, lysines, alkanes, fluorous alkanes, sugars, and nucleotides with *S*-, *O*- and *N*- linkages to porphyrins.[5, 23] The PDT activity of many of these derivatives was studied with a variety of cell lines, and the glycosylated compounds affect both necrosis and apoptosis depending on PS concentration and light flux.[23] We reported the synthesis of nonhydrolyzable tetrathioglucose and tetrathiogalactose porphyrins[21] connected through carbon-1 of the sugar (Figure 1). The glucose derivative proved to be selective towards cancer cells overexpressing glucose transporters and an effective PDT agent *in vitro* using cell lines such as MDA-MB-231 human breast cancer, whereas the galactose derivative was much less effective.[21] In addition to upregulation of glucose transporters expression, such as Glut-1, many cancer cells exhibit overexpression of galectin-1. Galactose derivatives linked at the carbon-1 position bind to galectins 200 fold less than corresponding carbon-3 derivatives.[24]

Galactosylated photosensitizers have demonstrated an excellent potential in PDT due to their ability to target galactose-binding proteins such as galectin-1.[25] Photosensitizers linked to galactose *via N*-, *S*-, *C*- and *O*- bonds at carbon-1 (anomeric) or at carbon-6 of the sugar to the porphyrinoids are reported.[6] To improve affinity for galactose-binding proteins, galactosylated dendritic motifs appended to porphyrinoids were developed to enable multivalent interactions.[26, 27] Monovalent galactose derivatives attached at carbon-3 yielded compounds with greater affinity to galectin-1 than the corresponding carbon-1 derivatives.[24] Therefore, we hypothesized that attachment of a porphyrin through the carbon-3 of galactose will result in improved uptake to cancer cells overexpressing galectin-1 protein.[28] This report is the first direct comparison of the uptake and PDT efficiency of carbon-1 versus carbon-3 linked porphyrin-galactose conjugates.

We attached galactose units through carbon-3 or carbon-1 to a Zn(II) porphyrin by click chemistry to yield (C1-Gal)$_4$-ZnPor and (C3-Gal)$_4$-ZnPor, respectively. Removing the metal ion yields the (C3-Gal)$_4$-Por and (C1-Gal)$_4$-Por derivatives (Scheme 1). Porphyrins conjugated with galactose through the carbon-3 of the sugar have not been previously explored in PDT. The main focus of the present research was to study the photophysical properties, *in vitro* cell uptake, and photodynamic efficiency of the carbon-1 versus the carbon-3 conjugates. *In vitro* cell studies were performed using HCT-116 colon cancer cells, MCF-7 breast cancer cells, UM-UC-3 bladder cancer cells and HeLa cervical cancer cells, which exhibit different levels of the galectin-1 protein (HeLa > UM-UC-3 > HCT-116>MCF-7 cells). In addition to the monolayer cancer cell culture studies, we performed studies using 0.05 mm^3 three-dimensional spheroids because they more closely mimic the tumor microenvironment and diffusion processes that take place in solid tumors; this 3D-spheroid culture model enables more accurate predictions of *in vivo* of photosensitizer efficacy.[29-31] Recently, we have observed that monolayer cultures and tumor spheroids differ in glucose metabolism, endogenous reactive



Scheme 1. Synthesis of porphyrin-galactose conjugates.

Table 1. Photophysical properties of C-1 and C-3 porphyrin derivatives.

Compound	Solvent	UV-visible[a] λ_{max} (nm)	Emission[b] λ_{max} (nm) 424 nm excitation	Φ_F[c]	DLS (PDI) mean diameter & poly dispersity index[d] (nm)
$(C1\text{-}Gal)_4$-ZnPor (**3**)	DMSO	431, 568, 608	609, 656	0.035	-
	Ethanol	425, 564, 604	604, 651	0.03	74(0.39)
	PBS buffer	410, 425, 446, 571, 613	655, 718	0.002	16, 38 (0.39)
$(C3\text{-}Gal)_4$-ZnPor (**6**)	DMSO	430, 568, 608	610, 650	0.03	-
	Ethanol	425, 564, 604	602, 651	0.025	79, 164 (0.23)
	PBS buffer	424, 440, 453, 454, 573, 617	655, 723	0.003	24 (0.33)
$(C1\text{-}Gal)_4$-Por (**4**)	DMSO	424, 523, 559, 588, 614	610, 654, 715	0.05	-
	Ethanol	419, 523, 562, 599, 651	605, 654	0.05	80 (0.30)
	PBS buffer	402, 429, 530, 556, 603, 655	658, 734	0.01	24 (0.34)
$(C3\text{-}Gal)_4$-Por (**7**)	DMSO	424, 524, 560, 588, 613	608, 654, 714	0.06	-
	Ethanol	419, 522, 563, 602, 657	604, 654	0.06	67 (0.23)
	PBS buffer	410, 427, 530, 567, 602, 659	657, 718	0.008	28 (0.33)

[ab] Spectra in supporting information, [c]Φ_F are ± 10%, [d]DLS histograms in supporting information.

oxygen species (ROS), and galectin-1 and GLUT-1 protein levels.[28] Here, we report that even though the cell uptake for carbon-3 conjugates is significantly greater than the carbon-1 derivatives and proportional to the protein levels of galectin-1, the PDT activities are about the same.

RESULTS AND DISCUSSION

Synthesis and Characterization of the conjugates

The synthesis and characterization of porphyrin **1** is typical for tetraaryl porphyrins, and proceeds in a ca. 33% overall yield. The zinc complex was made to avoid copper metalation of the macrocycle during the next step wherein copper salts were used for the click reaction, because copper (II) porphyrins are difficult to demetalate and have complex photophysics.[32] Appending the commercially available or synthesized azido sugars to compound **1** were carried out using standard click chemistry conditions in 85-90% yields of compounds **2** and **5** without transmetalation of the macrocycle. Deprotection by removal of the acetate groups on the sugars was efficiently accomplished with sodium methoxide in methanol. Herein, a large excess of the base was avoided to prevent hydrolysis of the sugars, and Sephadex columns were used for purification. The zinc was removed using 1:1 methanol:TFA in about 6 hr without hydrolysis of the sugars.

The ^{1}H NMR characterization of porphyrins **2** and **3** showed characteristic peaks for β pyrrolic porphyrin at ~ 8.5 ppm, 2,6- and 3,5- phenyl protons at ~ 8.1 and 7.2 ppm, galactopyranose peaks around 4.0-5.6 ppm, and the acetate peaks were found between 1.8-2.2 ppm. Due to the anomeric galactopyranoside (1,2,4,6-tetra-O-acetyl-3-azido-3-deoxy-D-galactopyranose), porphyrin **5** has a distinct multiplicity compared to porphyrin **2**. ^{13}C NMR confirmed the formation of the products where the carbonyl carbon peaks of acetates were found around 170 ppm and porphyrin aromatic peaks were found between 110-158 ppm. HRMS is consistent with the reported structures.

After deprotection of the sugar hydroxyl groups on porphyrins **2** and **5**, no peaks between 1.8-2.2 ppm in the ^{1}H NMR and no carbonyl peaks in the ^{13}C NMR were observed, indicating that the acetate groups were effectively removed. The free base porphyrins are characterized by the UV-visible spectra, the observation of the pyrrole N-H at -2.7 ppm, and HRMS.

Photophysics

The photophysical properties of chromophores such as porphyrins with polar substituents depend on the solvent. The amphipathic nature of sugar substituted porphyrin allows it to partition into lipophilic, hydrophilic, or amphipathic cellular structures.[5] Therefore, we have studied the photophysical properties in DMSO, ethanol and DPBS (pH = 7.4). The electronic spectra of the free base and metalated derivatives are significantly different. Zn analogues show red-shifted Soret bands and the typical two Q-bands between the 500-700 nm, while freebase analogues show the expected four Q bands. The absorbance and fluorescence emission spectra for the four compounds are correspondingly different and have a strong solvent dependence (Table 1).

Compared to the fluorescence in DMSO, the fluorescence emission of both the free bases and the zinc complexes are ca. 80-90% quenched in PBS. There are negligible Stokes shifts for all compounds in DMSO and ethanol solvents, indicating minimal specific solvent-solute interactions with the macrocycle effecting vibrational processes. Excitation spectra of all compounds indicate the presence of only one species (data not shown). The 0.05 and 0.06 fluorescence quantum yields for **(C1-Gal)$_4$-Por (4)** and **(C3-Gal)$_4$-Por** (**7**) is similar to other free base glycosylated porphyrins, and almost twice the zinc complexes.[5, 33] Because of the orthogonal phenyl groups, the 1,2,3-triazole and hydrophilic sugar molecules has minimal effect on photophysical properties, but their presence modulates the amphipathicity to promote solvent dependence. The split Soret bands in the UV-visible spectra in PBS at ca. 0.1 μM of all four conjugates (Supporting Information) indicate some degree of aggregation even at this low concentration. Thus, some of the quenching observed in PBS is due to the internal shading and the branching deactivation of aggregates.

All deprotected compounds show normal UV-visible spectra in DMSO as expected for typical solvated porphyrins (Supporting Information). In ethanol, the peaks are somewhat broadened indicating some level of aggregation. In PBS buffer, **(C1-Gal)$_4$-ZnPor** (**3**), **(C1-Gal)$_4$-Por** (**4**) and **(C3-Gal)$_4$-Por** (**7**) are broadened and have shoulders to the blue and red of the main absorption in the UV-visible spectra indicating both J and H aggregates. The exception is **(C3-Gal)$_4$-ZnPor** (**6**), which the electronic spectra indicates only J aggregates in PBS. To investigate the aggregation, the DLS studies were done in PBS buffer and ethanol. All compounds in PBS buffer yield 16-30 nm diameter aggregates. Larger 44-80 nm aggregates are observed in ethanol (Supporting Information). Aggregates less than ca. 50 nm are known to be taken up by cancer cells.[6]

Upon irradiation with 550 ± 10 nm light, DMF solutions of galactose-conjugated porphyrins catalyze the formation of $^1O_2$ as indicated by the $^1O_2$ scavenger 1,3-diphenylisobenzofuran (DPBF) to a similar extent as the reference tetraphenyl-porphyrin (TPP) (Supporting Information).[34] Consistent with similar porphyrinic compounds in this solvent the Zn(II) complexes (**C1-Gal)$_4$-ZnPor** and **(C3-Gal)$_4$ZnPor** have about the same $^1O_2$ yields as the free base compounds **(C1-Gal)$_4$-Por** and **(C3-Gal)$_4$-Por** measured by this method even though the heavy atom effect increases intersystem crossing to the triplet state.[35] Importantly, there is no significant difference between the C1 and the C3 isomers. Though there are different values in the literature, both TPP and ZnTPP have $^1O_2$ quantum yield ($\phi\Delta$) of ca. 0.52 ± 0.12 in DMF.[34-36] Thus, both the free base and the Zn(II) C1- and C3 porphyrins have about the same $\phi\Delta$ as the TPP reference in DMF.

Uptake/binding

It is generally accepted that there is a correlation between the expression level of receptors on the cell membrane and the uptake and/or binding of drugs appended to the ligands for the receptor. However, nonspecific partition into the membrane is also possible with hydrophobic or amphipathic molecules. Targeted porphyrin conjugates increase the local concentration of the dye around cancer cells, and when the binding constant is not large, there is an equilibrium between the free and bound conjugate. For dyes weakly bound to receptors, such as the compounds reported herein, the high local concentration of unbound dye around the cells increases passive diffusion, so uptake also depends on the hydrophobicity of the molecules.[37]

Table 2. Partition coefficient (log *P* *n*-octanol/PBS) of the galactose-conjugates.

Compound	logP±S.D.
(C1Gal)$_4$-Por (4)	-1.36±0.05
(C3Gal)$_4$-Por (7)	-1.50±0.12
(C1Gal)$_4$-ZnPor (3)	-0.99±0.01
(C3Gal)$_4$-ZnPor (6)	-1.10±0.10

Octanol-PBS partition coefficient values

Octanol-PBS partition coefficient values were determined for **(C1-Gal)$_4$-ZnPor**, **(C1-Gal)$_4$-Por**, **(C3-Gal)$_4$-ZnPor** and **(C3-Gal)$_4$-Por** using the standard shake flask method.[5] Data show that the values of log *P* are somewhat greater for **(C3-Gal)$_4$-Por** compared to **(C1-Gal)$_4$-Por** (Table 2), indicating that **(C1-Gal)$_4$-Por** has somewhat less water solubility compared with the corresponding **(C3-Gal)$_4$-Por**. As observed for many other tetraarylporphyrins, the partition coefficients were greater for the free base porphyrins compared with Zn-porphyrin indicating the free bases are ca. 33% more soluble in PBS.[4] These differences in PS intrinsic lipophilicity can result in different subcellular localization and mechanisms of cell death after photoactivation.

In vitro studies with monolayer and spheroids

Despite the fact that the uptake of carbon-3 conjugates is greater relative to the carbon-1 conjugates in the four cell lines, compounds **6** and **7** are not significantly better PDT agents in both monolayer and spheroid cell cultures. Since the free base **(C1-Gal)$_4$-Por** and **(C3-Gal)$_4$-Por** and the zinc complexes (**C1-Gal)$_4$-ZnPor** and **(C3-Gal)$_4$-ZnPor** have similar $^1O_2$ quantum yields (vide supra),[38, 39] these observations suggest that there are important differences in uptake and intracellular transport between the carbon-1 and carbon-3 conjugates.

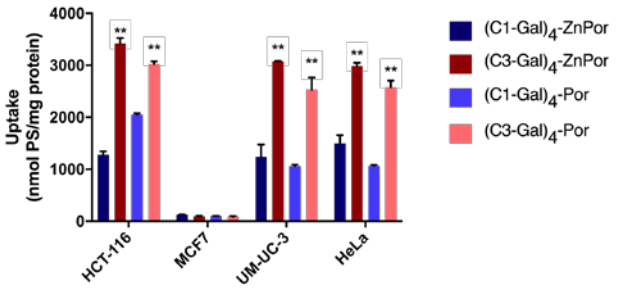


Figure 2. Intracellular uptake of galactose-porphyrins by monolayer cultures of HCT-116, MCF-7, UM-UC-3 or HeLa cancer cells. The concentration of porphyrins was determined by fluorescence spectroscopy after incubation of cancer cells with 9 μM of galactose-porphyrins for 4 h and the results were normalized to protein quantity. Data are the means ± S.D. of at least three independent experiments performed in triplicate. **($p<0.001$) significantly different from the corresponding C3-Gal$_4$-Por.

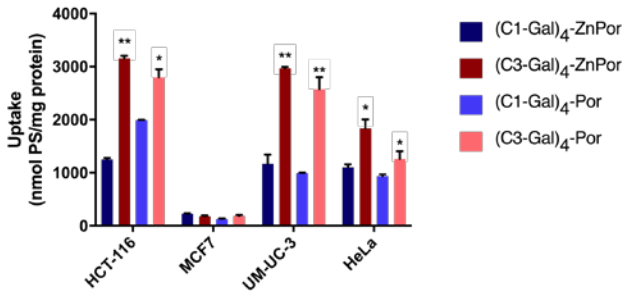


Figure 3. Intracellular uptake of galactose-porphyrins by spheroid cultures of HCT-116, MCF-7, UM-UC-3 or HeLa cancer cells. The concentration of porphyrins was determined by fluorescence spectroscopy after incubation of cancer cells with 9 μM of galactose-porphyrins for 4 h and the results were normalized to protein quantity. Data are the means ± S.D. of at least three independent experiments performed in triplicate. *($p<0.05$), **($p<0.001$) significantly different from the corresponding C3-Gal$_4$-Por.

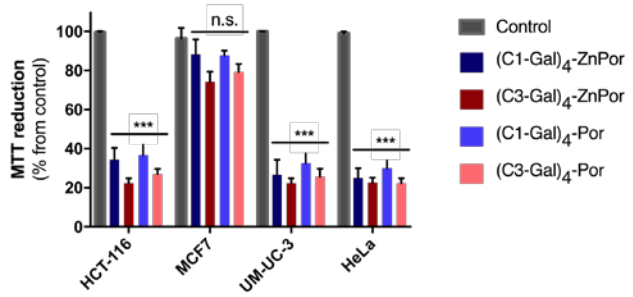


Figure 4. Cytotoxicity at 24 h after PDT with galactose-porphyrins in cancer cells growing in monolayers, determined using the MTT assay. Cells were incubated with 9 μM PS for 4 h and PDT was performed during 30 min at 0.44 mW/cm^2. Data are means ± S.D. of at least three independent experiments performed in triplicate. ***($p<0.0001$) significantly different from control cells.

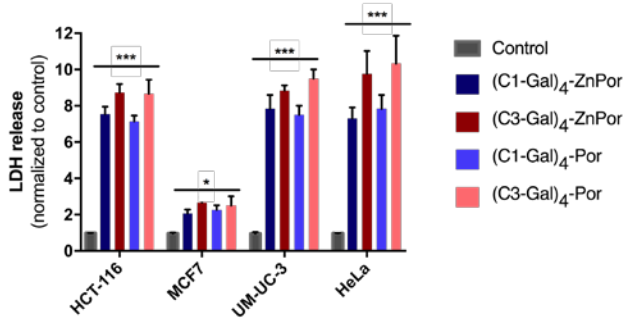


Figure 5. Cytotoxicity at 24 h after PDT with galactose-porphyrins in cancer cells growing in spheroids, determined using the LDH assay. Spheroids were incubated with 9 μM PS for 4 h and PDT was performed during 30 min at 0.44 mW/cm^2. Data are means ± S.D. of at least three independent experiments performed in triplicate. *($p<0.05$), ***($p<0.0001$) significantly different from control cells.

Previous studies reported this lack of association between uptake and phototoxicity of galactose-conjugated PS.[26] Factors such as different amounts of intracellular ROS and/or induction of a protective cellular mechanism after PDT can explain the reasons by which PSs with different uptake exhibit similar phototoxicity after PDT.[26] Additionally, differences in the intracellular localization of photosensitizers galactose-conjugated sugars can result in different cell death processes.[40]

Carbon-1 and carbon-3 galactose-porphyrins accumulate in cancer cells growing as monolayer and spheroid cultures and are non-toxic in the dark

To evaluate the photodynamic potential of the newly synthesized carbon-1 and carbon-3 galactose-porphyrins, we first evaluated their accumulation in monolayer and spheroid cultures by fluorescence spectroscopy and microscopy (Figures S32-S36) and dark cytotoxicity (Figures S37-S44). Monolayer cultures of cancer cells were incubated with increasing concentrations (2.25, 4.5 and 9 μM) of **(C1-Gal)$_4$-ZnPor**, **(C1-Gal)$_4$-Por**, **(C3-Gal)$_4$-ZnPor** and **(C3-Gal)$_4$-Por** in PBS containing a maximum of 0.5% v/v dimethyl sulfoxide, DMSO, for up to 4 h. Cancer cell-derived spheroids were incubated with the highest concentration (9 μM) of each photosensitizer.

No toxicity was observed in untreated cells (up to 4 h) in the presence of 0.5% (v/v) DMSO in the incubation medium.[28] Moreover, the galactose-porphyrins showed no significant dark cytotoxicity in monolayer and spheroid cultures at the 9 μM maximum concentration tested (Figures S37-S44).

The uptake by HCT-116, UM-UC-3 and HeLa cancer cells growing as monolayers or spheroids was greater for carbon-3 porphyrins than for carbon-1porphyrins (Figures 2,3). The uptake is proportional to the expression of the receptors, and competition experiments demonstrated that a pre-incubation with thiogalactose decreased the uptake of both the carbon-1 and carbon-3 galactose-porphyrins in UM-UC-3 cell cultures (Supporting Information).[41] Data showed that the reduction in uptake was higher for carbon-3 when compared with carbon-1 galactose-porphyrins. The uptake of galactose-porphyrins was greater in HCT-116, HeLa or UM-UC-3 cancer cells growing as monolayers than with monolayers of MCF-7 breast cancer cells. MCF-7 breast cancer cells have lower levels of galectin-1 protein compared to HCT-116, UMUC-3 and HeLa cancer cells.[28] When spheroids were used as cell culture model, a decrease in the uptake of galactose-porphyrins was observed in HeLa cancer cells.

Galactose-porphyrins induce cytotoxicity in monolayer and spheroid cultures after photodynamic activation

After performing uptake assays, we investigated the effect of galactose-porphyrins mediated PDT efficacy in monolayer and spheroid cultures. The cells were treated 48 h after plating, with 9 μM of galactosylated porphyrins for 4 h, and then exposed to light (420-700 nm) delivered at 0.44 mW/cm^2 during 30 min (0.792 J/cm^2). The 48 h time point following cell seeding was chosen based on the time needed for spheroids of the different cell lines to form and achieve reproducible and similar compactness (based on spheroid volume and shape) in each well.[28] In all monolayer cultures, the light activation of galactose-porphyrins induced reduction of cell viability.

As shown in Figure 4, the phototoxicity of carbon-3 porphyrins in cancer cells growing as monolayers was similar to carbon-1 porphyrins. As expected, the phototoxicity was greater in monolayer cultures of HCT-116, UM-UC-3 and HELA compared to MCF-7 cells because of the reduced galectin-1 expression level in the latter.[28] When PDT assays used spheroid cultures, the release of LDH into the culture medium was increased compared with control cells (Figure 5). After PDT, LDH released by HCT-166, UM-UC-3 and HeLa spheroids was greater than that by MCF-7 spheroid cultures. However, the amount of LDH leakage into the extracellular medium was lower for all cancer spheroid cultures when compared to monolayer countercultures. In spheroid cultures of HCT-166, UM-UC-3 and HELA the PDT efficacy is slightly greater for the carbon-3 derivative than for the carbon-1 derivatives, and again there is little effect on spheroids of MCF-7.

CONCLUSION

Carbon-1 and carbon-3 galactose-porphyrins demonstrate high accumulation and phototoxicity in cell lines (monolayers or spheroids) containing high expression of galectin-1 protein. The octanol/PBS partition coefficients indicate that the carbon-1 compounds are somewhat more lipophilic than the carbon-3 derivatives and indicates facilitated diffusion as a mode of uptake. Carbon-1 and carbon-3 galactose-porphyrins demonstrated low uptake in MCF-7 cancer cells, which contain low levels of galectin-1 protein. In cell lines containing high levels of galectin-1 (HCT-116, HeLa and UM-UC-3), carbon-3 compounds demonstrated greater uptake compared with carbon-1 galactose-porphyrins. The greater uptake of carbon-3 compared with carbon-1 galactose-porphyrin can be explained by its higher water solubility, as well as be the availability of the hydroxyl group at carbon-1 to bind the carbohydrate-recognition domain of galectin-1 protein.[24] The similar photodynamic efficiency of carbon-1 and carbon-3 galactose-porphyrins in cell lines containing different levels of galectin-1 protein can be a result of differences in intracellular localization, as well as differences in cellular oxygenation, cell death mechanism, and cellular antioxidant responses upon light activation.

EXPERIMENTAL PROCEDURES

Commercially available reagents such as trifluoroaceticacid (TFA), sodium methoxide, and solvents such as methanol, DMSO, and CH_2Cl_2, were purchased from Sigma-Aldrich, Fisher Scientific or Acros Organics and used without further purification. 1,2,4,6-tetra-O-acetyl-3-azido-3-deoxy-D-galactopyranose was prepared using the published procedure.[42] 5,10,15,20-tetrakis(4-propargyloxyphenyl)porphyrin was synthesized using a known procedure.[43] Analytical thin-layer chromatography (TLC) was performed on polyester-backed TLC plates 254 (precoated, 200 μm, Sorbent Technologies), and silica gel 60 (70−230 mesh, Merck) was used for column chromatography. ^1H and ^{13}C NMR spectra were obtained using a Brüker Avance III 400 MHz and Brüker Avance DRX 500MHz; chemical shifts are expressed in ppm relative to $CDCl_3$ (7.26 ppm, ^1H; 77.0 ppm,^{13}C), $(CD_3)_2CO$ (2.05 ppm, ^1H; 29.84 and 206.26 ppm, ^{13}C), CD_3OD (3.31 and 4.78 ppm, ^1H; 49.2 ppm, ^{13}C). Mass analyses were conducted at the CUNY Mass Spectrometry Facility at Hunter College on an Agilent iFunnel 6550 Q-ToF LC/MS System (for HRMS-ESI). The electrospray ionization was run in methanol, with 0.1% (v/v) formic acid. UV-visible spectra were recorded on a Lambda 35 PerkinElmer UV/Vis spectrophotometer. Steady-state fluorescence (emission) spectra were measured with a Fluorolog τ-3, Jobin-SPEX Instruments S.A., Inc.

5,10,15,20-tetrakis(4-propargyloxyphenyl)porphyrin was synthesized from commercially available 4-propargyloxy benzaldehyde and pyrrole.[43] Zn complexation was carried out using $Zn(OAc)_2$ in CH_2Cl_2 at room temperature overnight resulting in 98% overall yield of the metalloporphyrin **1**. 1,2,4,6-tetra-O-acetyl-3-azido-3-deoxy-D-galactopyranose was prepared using the published procedure,[42] and characterization was in agreement with the published spectra. The 1-azido-1-deoxy-β-D-galactopyranoside tetraacetate is commercially available. Either 1,2,4,6-tetra-O-acetyl-3-azido-3-deoxy-β-D-galactopyranose or 2,3,4,6-tetra-O-acetyl-1-azido-1-deoxy-β-D-galactopyranose (2.6 equivalents) were then refluxed overnight with one equivalent of porphyrin **1** using one equivalent Cu(0), 0.2 equivalents of $CuSO_4.5H_2O$, and 0.5 equivalents of Na-ascorbate dissolved in a mixture of THF/water resulting in porphyrins **2** and **5** in good yields after silica column purification using CH_2Cl_2/EtOAc (80:20) as eluent.

5,10,15,20-tetrakis(4-propargyloxyphenyl)porphyrinato Zn(II), compound 1

To 100 mg (0.12 mmol) of 5,10,15,20-tetrakis(4-propargyloxyphenyl)porphyrin in 20 mL CH_2Cl_2 was added 300 mg $Zn(OAc)_2$. The reaction mixture was stirred at room temperature overnight. The obtained mixture was evaporated using rotary evaporator under reduced pressure followed by a silica chromatography with CH_2Cl_2/EtOAc (95:5) as eluent resulting in compound **1** in 98% yield (105 mg, 0.117 mmol).^1H NMR (500 MHz, $CDCl_3$) δ 8.97 (s, 8H), 8.13 (d, *J*=8.35 Hz, 8H), 8.35 (d, J_1=8.35 Hz, 8H), 4.98 (s, 8H), 2.69 (s, 4H),); ^{13}C NMR (125 MHz, $CDCl_3$) δ 156.4, 134.5, 134.4, 118.5, 112.0, 77.6, 74.8, 55.1.

5,10,15,20-tetrakis[N-(2',3',4',6'-O-acetyl-1'-β-D-galactopyranosyl)-(4''-methylenoxytriazole)-phenyl]porphyrinato Zn(II), compound 2

Compound **1** (52.5 mg, 0.058 mmol), 1-azido-1-deoxy-β-D-galactopyranoside tetraacetate (56.24 mg, 0.15 mmol,) and (3.6 mg, 0.058 mmol) Cu(0) were dissolved in a mixture of THF/water (6 mL, 3:1), under inert gas atmosphere. To the above mixture, a solution of $CuSO4.5H_2O$ (2.9 mg, 0.0116 mmol) and Na-ascorbate (5.75 mg, 0.029 mmol) in THF/water (6 mL, 3:1) was added and the reaction mixture was refluxed overnight. Then the reaction mixture was cooled to room temperature and extracted using ethyl acetate and brine. The organic layer was washed twice with brine and the combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was then purified by silica chromatography. First using CH_2Cl_2 as eluent to remove any unreacted starting materials then followed by 80:20 (CH_2Cl_2:EtOAc) to obtain the desired product in 90% yield (125 mg, 0.052 mmol). ^1H NMR (500 MHz, $CDCl_3$) δ 8.95 (s, 8H), 8.13 (d, *J*=7.8 Hz, 8H), 7.77 (s, 4H), 7.24 (d, *J*= 7.8 Hz, 8H), 5.59 (t, *J*=9.2 Hz, 4H), 5.53 (t, *J*=2.9 Hz, 4H), 5.42-5.45 (m, 4H), 5.21 (dd, J_1= 2.95, J_2= 2.95 Hz, 4H), 4.67 (s, 8H), 4.16-4.20 (m, 8H), 4.09-4.13 (m, 4H), 2.20 (s, 12H), 2.05 (s, 12H), 2.00 (s, 12H), 1.80 (s, 12H). ^{13}C NMR (125 MHz, $CDCl_3$) δ170.3, 170.1, 169.9, 169.3, 157.7, 150.4, 144.5, 136.4, 135.7, 131.7, 120.9, 120.3, 112.9, 88.2, 86.1, 72.8, 70.7, 68.1, 66.9, 61.2, 61.2, 20.6, 20.6, 20.6, 20.9. HRMS (ESI) *m/z* calcd. for $C_{112}H_{112}N_{16}O_{40}Zn$ ($[M+2H]^{2+}$), 1194.3352, found 1194.3325.

5,10,15,20-tetrakis[N-(1'-β-D-galactosyl)-(4''-methylenoxytriazole)-phenyl]porphyrinato Zn(II), compound 3

Compound **2** (62.5 mg, 0.026 mmol) was dissolved in methanol/CH_2Cl_2 (3:1, 10 mL). To this mixture was added sodium methoxide (0.5 M solution in methanol, 3 mL). The reaction mixture was stirred at room temperature for 4 h and then the solvent was evaporated using rotary evaporator. The resulting crude product was passed through Sephadex LH-20 using MeOH as eluent to yield $(C1\text{-}Gal)_4$-ZnPor (**3)** in 84% yield (37.39 mg, 0.021 mmol). ^{1}H NMR (400 MHz, CD_3OD) δ 8.85 (s, 8H), 8.54 (s, 4H), 8.13 (d, *J*= 7.92 Hz, 8H), 7.51 (d, *J*= 8Hz, 8H), 5.62 (d, *J*= 9.04 Hz, 4H), 5.51 (s, 8H), 4.20 (t, *J*= 9.12 Hz, 4H), 3.81 (bs, 4H), 3.81-3.82 (m, 4H), 3.63-3.68 (m, 12H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.3, 150.11, 143.5, 136.0, 135.7, 131.9, 124.1, 120.3, 113.4, 88.8, 79.0, 74.4, 70.0, 69.1, 62.1, 61.1. HRMS (ESI) *m/z* calcd. for $C_{80}H_{80}N_{16}O_{24}Zn$ ($[M+H]^+$), 1715.4905, found 1715.4893.

5,10,15,20-tetrakis[N-(1'-β-D-galactosyl)-(4''-methylenoxytriazole)-phenyl]porphyrin, compound 4

$(C1\text{-}Gal)_4$-ZnPor (**3)** (37.39 mg, 0.021 mmol) was dissolved in methanol/TFA (1:1, 10 mL). The reaction mixture was stirred at room temperature overnight and then the solvent was evaporated using rotary evaporator. The resulting crude product was washed with CH_2Cl_2 and acetone several times to yield $(C1\text{-}Gal)_4$-Por (**4)** in 90% yield (31.18 mg, 0.018 mmol) as green solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.87 (bs, 8H), 8.60 (s, 4H), 8.18 (bs, 8H), 7.53 (bs. 8H), 5.72-5.76 (m, 8H), 5.49-5.62 (m, 8H), 4.47-4.59 (m, 4 H), 4.13-4.28 (m, 8H), 3.80-3.87 (m, 8H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.1, 156.5, 156.2, 142.7, 124.0, 123.9, 113.1, 88.2, 87.6, 78.5, 74.4, 73.7, 73.0, 69.3, 68.9, 68.6, 68.5, 68.0, 61.3, 60.5, 54.9. HRMS (ESI) *m/z* calcd. for $C_{80}H_{82}N_{16}O_{24}$ ($[M+H]^+$), 1652.5791, found 1652.5771.

5,10,15,20-tetrakis[N-(1',2',4',6'-O-acetyl-3'-β-D-galactopyranosyl)-(4''-methylenoxytriazole)-phenyl]porphyrinato Zn(II), compound 5

Compound **1** (52.5 mg, 0.058 mmol), 1,2,4,6-tetra-O-acetyl-3-azido-3-deoxy-D-galactopyranose (56.24 mg, 0.15 mmol) and Cu(0) (3.6 mg, 0.058 mmol) were dissolved in a mixture of THF/water (6 mL, 3:1), under inert gas atmosphere. To the above mixture, a solution of $CuSO_4.5H_2O$ (2.9 mg, 0.0116 mmol) and Na-ascorbate (5.75 mg, 0.029 mmol) in THF/water (6 mL, 3:1) was added and the reaction mixture was refluxed overnight. Then the reaction mixture was cooled to room temperature and extracted using ethyl acetate and brine. The organic layer was washed twice with brine and the combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was then purified by silica chromatography. First using CH_2Cl_2 as eluent to remove any unreacted starting materials then followed by CH_2Cl_2/EtOAc (80:20) to obtain the desired product in 84% yield (116 mg, 0.048 mmol).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.93 (bs, 8H), 8.11 (bs, 8H), 7.42-7.49 (m, 4H), 7.12-7.27 (m, 8H), 5.09-5.53 (m, 12H), 4.65-4.72 (m, 8H), 3.86-4.34 (m, 12H), 1.67-2.14 (m, 48H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.6, 169.5, 169.4, 168.6, 162.8, 143.7, 142.8,132.0, 132.0, 130.5, 126.0, 123.3, 115.1, 115.0, 90.1, 72.0, 71.5, 68.6, 66.0, 63.0, 62.0, 61.9, 60.4, 57.9, 20.98, 20.8, 20.4, 20.2, 20.2. HRMS (ESI) *m/z* calcd. for $C_{112}H_{112}N_{16}O_{40}Zn$ ($[M+2H]^{+2}$), 1216.3161, found 1216.4143.

5,10,15,20-tetrakis[N-(3'-β-D-galactosyl)-(4''-methylenoxytriazole)-phenyl]porphyrinato Zn(II), compound 6

Compound **5** (62.5 mg, 0.026 mmol) was dissolved in methanol/CH_2Cl_2 (3:1, 10 mL). To this mixture was added sodium methoxide (0.5 M solution in methanol, 3 mL). The reaction mixture was stirred at room temperature for 4 h and then the solvent was evaporated using rotary evaporator. The resulting crude product was passed through Sephadex LH-20 using MeOH as eluant to give $(C3\text{-}Gal)_4$-ZnPor (**6**)in 79% yield (35.16 mg, 0.020 mmol). ^{1}H NMR (500 MHz, CD_3OD) δ 8.81 (bs, 8H), 8.02 (bs, 8H), 7.65 (bs, 4H), 7.28 (bs, 8H), 5.45-5.47 (m, 4H), 5.31-5.33 (m, 8H), 4.90-5.12 (m, 24H); ^{13}C NMR (125 MHz, CD_3OD) δ 159.8, 151.7, 141.6, 137.3, 136.6, 132.5, 131.3, 121.5, 113.8, 63.4. HRMS (ESI) *m/z* calcd. for $C_{80}H_{80}N_{16}O_{24}Zn$ ($[M+H]^+$), 1715.4905, found 1715.4864; calcd. for $C_{80}H_{80}N_{16}O_{24}Zn$ ($[M+Na]^+$), 1736.4745, found 1736.4662.

5,10,15,20-tetrakis[N-(3'-β-D-galactosyl)-(4''-methylenoxytriazole)-phenyl]porphyrin, compound 7

$(C3\text{-}Gal)_4$-ZnPor (**6**) (35.16 mg, 0.020 mmol) was dissolved in methanol/TFA (1:1, 10 mL). The reaction mixture was stirred at room temperature overnight and then the solvent was evaporated using rotary evaporator. The resulting crude product was washed with CH_2Cl_2 and acetone several times resulting in $(C3\text{-}Gal)_4$-Por (**7**) in 87% yield (28.71 mg, 0.017 mmol) as a green solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.86 (s, 8H), 8.14 (s, 12H), 7.47 (bs, 8H), 5.54 (s, 8H), 3.76-4.79 (m, 28H), -2.82 (bs, 2H); ^{13}C NMR (500 MHz, DMSO-d_6) δ 158.5, 158.2, 158.1, 158.0, 157.7, 135.4, 133.8, 120.3, 119.6, 117.9, 115.6, 113.2, 76.5, 72.9; HRMS (ESI) *m/z* calcd. for $C_{80}H_{82}N_{16}O_{24}$ ($[M+H]^+$), 1652.5791, found 1652.5735; calcd. for $C_{80}H_{82}N_{16}O_{24}$ ($[M+Na]^+$), 1674.5610, found 1674.5545.

Photophysics

UV-visible and fluorescence measurements were performed on diluted solutions, typically about 0.1 μM, of compounds in ethanol, dimethylsulfoxide (DMSO) and Dulbecco's phosphate buffered saline (DPBS without $CaCl_2$ and $MgCl_2$ pH = 7.4 with 2% v/v DMSO). The UV-visible spectra were obtained from 350 nm to 750 nm using 1 cm quartz cuvettes. For steady-state fluorescence spectroscopy, samples were excited at 425 nm for all solvents at concentrations where absorbancies are less than 0.1. For emission spectra, both the excitation and detection monochromators had a band-pass of 1 nm. The corrected emission (for instrument response) and absorption spectra were used to calculate the quantum yield. Fluorescence quantum yields were determined for $(C3\text{-}Gal)_4$-Por (**7**), $(C3\text{-}Gal)_4$-ZnPor (**6**), $(C1\text{-}Gal)_4$-Por (**4**) and $(C1\text{-}Gal)_4$-ZnPor (**3**), solutions relative to a tetraphenylporphyrin standard in toluene, which has a fluorescence quantum yield of 0.11 in this and the other solvents.[5, 44] All experiments were carried out on the same day, using identical concentrations to minimize any experimental errors. (Supporting Information).

Dynamic light scattering (DLS) was used to determine aggregate size in the different solvents using a Malvern nano Zetasizer, modeled as a sphere and reported as the diameter. Samples were sonicated for about a minute and filtered with hydrophilic 200nm filters before measurements.

The partition coefficient (*P*) values were measured in an n-octanol/PBS (1:1, v/v) mixture at a concentration of 10 μM. After shaking the solution at room temperature, the two different

phases were obtained by centrifugation and the concentration of the PS into each phase was determined by UV-Vis using the molar absorptivity values.

For the singlet oxygen experiments, DMF solutions containing 50 μM of DPBF and 5 μM of porphyrins were irradiated at room temperature and under gentle magnetic stirring. DPBF depletion was monitored by measuring the decrease in absorbance (415 nm) at 2, 4, 6, 8 and 10 min after light irradiation. Irradiation with 0.49 mW/cm^2 light (measured at the front of the cuvette) from a xenon arc lamp in Horiba SPEX fluorometer with the monochonometer set at 550 nm and the band pass set at 10 nm to minimize absorption by DPBF.

Cell lines and culture

UM-UC-3 bladder cancer cell line, HeLa cervical cancer cell line, HCT-116 colon cancer cell line and MCF-7 breast cancer cell line were obtained from the American Type Culture Collection (ATCC®, Manassas, VA, USA).All batches of culture media were supplemented with 10% (v/v) of fetal bovine serum (Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma). HCT-116 colon cancer cells, MCF-7 and MDA-MB-231 breast cancer cells were cultured in Dulbecco´s Modified Eagle´s Medium (DMEM, Sigma). UM-UC-3 bladder cancer cells were cultured in Eagle´s Minimum Essential Medium (EMEM; Corning, NY, USA) with 1.5 g/L sodium bicarbonate, non-essential amino acids, L-glutamine and sodium pyruvate. HeLa cervical cancer cells were cultured in DMEM (Corning) with 4.5 g/L glucose, and L-glutamine without sodium pyruvate. All cells were maintained at 37 ºC in a 5% CO_2 humidified atmosphere. Agarose-coated 96-well plates were used for spheroid cultures as previously reported in theliterature.[28] Briefly, 5000 HCT-116 cells per well, 15000 cells MCF-7 per well, 20000 UM-UC-3 cells per well and 20000 HeLa cells per well were plated on agarose-coated microplates and left for 48 h undisturbed to obtain viable spheroids with volume ≈ 0.05 mm.[3]

Cellular uptake

After incubation of monolayers or spheroids of cancer cells with the photosensitizer in the dark, the cells were washed with PBS buffer (pH 7.60, 10 mM NaH_2PO_4, 70 mM Na_2HPO_4 and 145 mM NaCl) and scrapped in 1% (m/v) sodium dodecyl sulfate (SDS; Sigma) in PBS buffer at pH 7.0. The concentration of the porphyrins was determined by spectrofluorimetry using a microplate reader (Gemini EM Microplate Spectrofluorometer) with the excitation and emission filters set at 410 nm and 702 nm, respectively. The results were normalized for protein concentration using the bicinchoninic acid reagent (Pierce, Rockford, IL, USA). For the competition experiments, monolayers of UM-UC-3 cells were pre-incubated with the thiogalactose sugar (100 μM or 1.00 mM) for 1h. Next, those cells were incubated for 3h with Pors at a concentration of 9 μM.

For evaluation of cellular uptake by fluorescence microscopy, cells were grown on coated glass coverslips with poly-L-lysine (Sigma). The cells were incubated with 9 μM porphyrins for 4 h, at 37°C. After incubation, cells were washed twice with PBS and fixed with 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany) for 10 min at room temperature. The samples were then rinsed in PBS, and mounted in Vecta SHIELD mounting medium containing 4´,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA, Burlingame) for visualization under a confocal microscope (Nikon Eclipse Ti With Ultra High Speed Wavelength Source, Molecular Devices) equipped with Andor iXon EMCCD camera. For DAPI detection, the specimen was excited at 405 nm and light emitted was collected with a 460/50 nm band pass filter. Porphyrins fluorescence was obtained using 640 nm excitation and a 685/40 nm emission band pass filter.

PDT assays

After monolayers or spheroid cultures were incubated with 9 μM of porphyrins for 4 h, the cultures were gently rinsed with PBS, and then PDT assays were performed. The cells were exposed to light (420-700 nm) emitted by OLED Lumiblade Brite FL300-wm Level 4 (OLED Works, Rochester, NY, USA) delivered at 0.44 mW/cm^2 during 30 min (0.792 J/cm^2). The controls were performed by keeping monolayers or spheroids in the dark for the same durations and under the same conditions as the irradiated cells. Triplicate wells were established under each experimental condition and each experiment was repeated at least three times. Cytotoxicity was determined in monolayer or spheroid cultures 24 h after treatment using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) and lactate dehydrogenase (LDH) assays, respectively.[28]

MTT and lactate dehydrogenase assays

The MTT assay was used to determine metabolic activity of cells growing in monolayers by measuring their ability to reduce the yellow-colored MTT to a colored formazan using a microplate reader (PowerWave HT Microplate Spectrophotometer). The data were expressed in percentage of control (*i.e.* optical density of formazan from control cells). The CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) was used to determine cytotoxicity in spheroid cultures. The LDH activity was normalized to protein concentration and the data were normalized to the maximal LDH release.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Student's *t*-test was used to compare the treatment effects with that of control. P-value was considered at the 5% level of significance of the data. All graphs and statistics were prepared using the GraphPad Prism 5.0 software.

ASSOCIATED CONTENT

Supporting Information

NMR spectra, UV-visible absorbance spectra, fluorescence emission spectra, mass spectra, DLS, uptake, dark toxicity.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. #These authors contributed equally.

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ABBREVIATIONS

GLUT1, glucose transporter 1; PDT, Photodynamic Therapy; PS, Photosensitizer; ROS, Reactive Oxygen Species, DMSO dimtheylsulfoxide.

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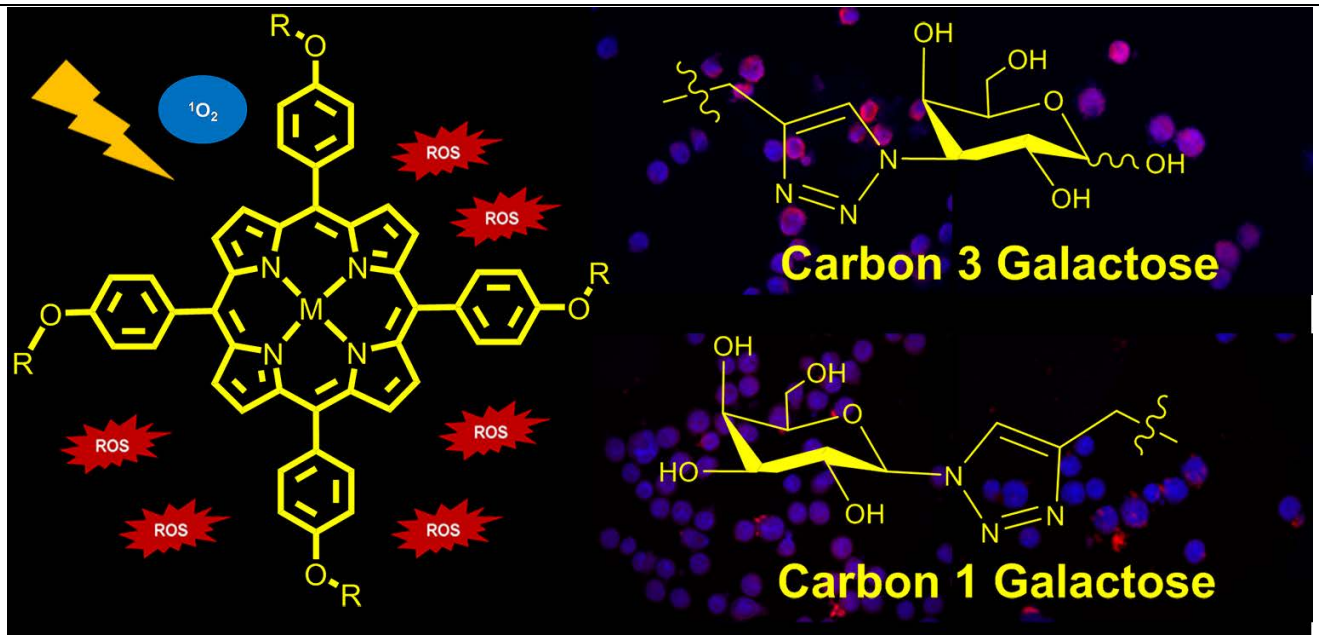
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