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Photodynamic Effect of Glycochlorin Conjugates in Human Cancer Epithelial Cells

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A. T. P.C. Gomes,[a,b] M. A. F. Faustino,[a] M. G. P. M. S. Neves,[a,*] V. F. Ferreira,[c] A. Juarranz,[b] J. A. S. Cavaleiro,[a] F. Sanz-Rodríguez[b,*]

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The photodynamic effect of glycochlorins **1**-**4** was evaluated for the first time in human epithelial cells (HeLa) and their PDT efficiency was compared with the results obtained when using human normal skin keratinocytes cells (HaCaT). It was demonstrated that the galactose conjugate is the only photosensitizer that is selective for HeLa cells and without causing significant effect in the HaCaT cells. Morphological studies carried out after the photodynamic treatment attested such selectivity and showed that HeLa cells suffer a strong cytoplasmic vacuolization and a pronounced retraction, while in HaCaT cells the damage, under the same protocol conditions, is scarce.

Introduction

Among all lethal diseases, cancer is the second leading cause of death in the world and for this reason many research groups are working in the development of new methodologies of diagnosis and treatment and also in the prevention of those unhealthy situations.[1]

In general, cancer treatment includes surgery, chemotherapy and radiotherapy. Some of these approaches can cause some physical damage in patients and sometimes the disease is only put in a temporary state of latency.[1] Considering this, it is crucial to look for the development of therapeutic approaches that allow the eradication of cancer, with minor damages to patients. The photosensitizing ability demonstrated by porphyrins and analogues in Photodynamic Therapy (PDT) makes these tetrapyrrolic macrocycles special candidates to be applied on this type cancer therapy.[2] In PDT, the combination of the photosensitizing drug (PS), oxygen and visible light produces lethal cytotoxic agents (*e.g.* singlet oxygen ($^1O_2$) and/or other reactive species) that are responsible for the destruction of malignant cells.[3] This therapeutic approach presents as major advantage the fact that the selected PS is non-toxic in the absence of light and also it is a localized treatment with low accumulation in non-specific tissues. Additionally, it is a treatment confined to the irradiated area and can be repeated without the risk of harming neighbouring healthy tissue, since the effect of the non-ionizing activating light on tissues, in the absence of the PS drug, is harmless.[4] The clinical interest in formulations based on tetrapyrrolic macrocycles or its precursors for PDT is demonstrated by health agency approvals of Photofrin®, Levulan®, Visudyne®, Foscan®, Metvix® and Photosense® in US and other countries.[5]

One of the major challenges of PDT is to promote the selective PS localization on tumour cells in order to increase the efficiency of the neoplastic tissue damage when compared to the normal one.[6] One of the approaches to reach this selectivity is based on the synthesis of porphyrins or analogues bearing targeting moieties like carbohydrate ones.[7] There are many examples in literature involving the synthesis of carbohydrate-porphyrin conjugates based on the concept that carbohydrate moieties have the ability to target carbohydrate-recognized proteins, which are known for their high expression in certain tumours.[3,7,8,9,] Moreover, the conjugation of carbohydrates to porphyrin derivatives can improve their water solubility and bring better hydrophilic/hydrophobic ratios, this being very important in biodistribution and pharmacokinetic profile.[3,10] Whilst another important requirement for an ideal PS is related with the presence of a strong absorption in the visible region near or above 650 nm, where the light penetration in tissues is higher, it was decided to evaluate the photosensitizing ability of glycochlorins containing D-

glucose (**1**), D-ribose (**2**), D-fructose (**3**) and D-galactose (**4**) units (Figure 1) towards HeLa cells.

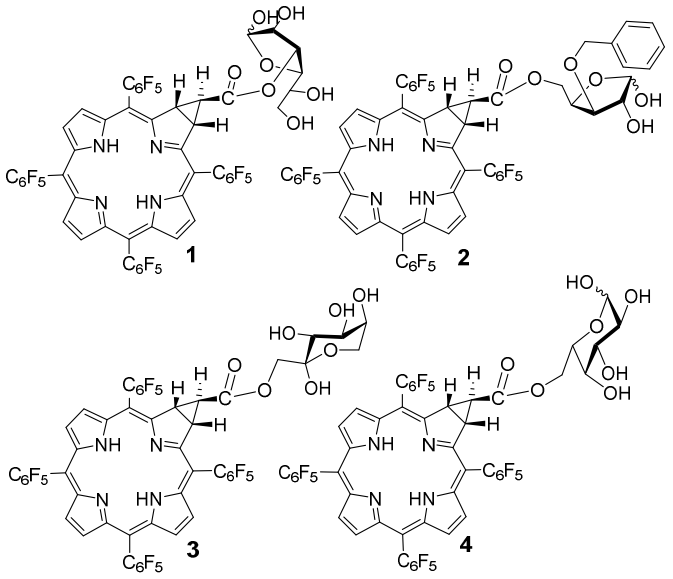
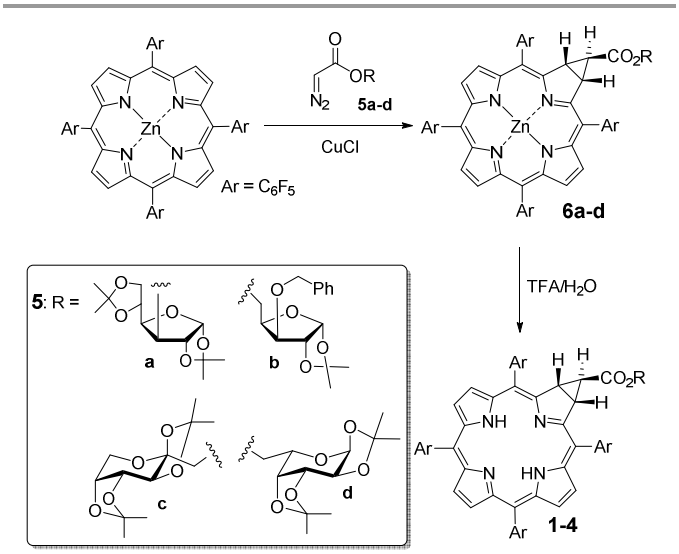


Figure 1. Structures of the glycochlorin derivatives **1**-**4**.

The synthesis of theses derivatives was previously reported by us and involved the reaction of *meso*-tetrakis(pentafluorophenyl)porphyrinatozinc(II) with the corresponding di-acetonides of carbohydrate-substituted α-diazoacetates **5a-d**, in the presence of catalytic amounts of CuCl (Scheme 1).[11] These reactions are selective for the products of mono-cyclopropanation, yielding preferentially the *trans* isomers **6a-d**. It is also shown that the products and their yields depend on the nature of the carbohydrate moiety. Glycochlorins **1-4** were obtained by demetalation and removal of the isopropylidene groups of the carbohydrate moieties of the cyclopropanation products **6a-d**. These derivatives, besides their strong absorption band at *ca* 660 nm, proved to be good $^1O_2$ producers.[11] In this work, we report for the first time the photodynamic effect of glycochlorin-conjugates **1-4** in a cervical cancer human cell line (HeLa) and in an human normal skin keratinocytes cell line (HaCaT).



Scheme 1

The efficiency of these glycochlorin conjugates was assessed by comparing the photodynamic effect in human cervical cancer cell line, HeLa, and in human normal skin keratinocytes cell line, HaCaT. Both cell types are of epithelial origin and are considered to be an excellent comparative model between non-tumoral *vs* tumoral studies *in vitro*. The photodynamic assays were performed with the glycochlorin conjugates incorporated in liposomal suspensions in order to avoid their aggregation in water. In fact, liposomes are considered valuable carriers and delivery systems for PDT. These vesicles composed by phospholipids have high loading capacity and flexibility to accommodate and deliver PSs with variable physicochemical properties.[12] It has been also demonstrated that the PDT outcome of liposomal PS is advantageous, when compared with the non-liposomal PS; a possible explanation is that a liposomal formulation can substantially decrease the extent of PS aggregation.[12]

Results

In vitro studies on HeLa and HaCaT cells

The photodynamic effect of glycochlorin-conjugates was evaluated in HeLa cells (cervical cancer human cell line) and in HaCaT cells (human normal skin keratinocytes cell line). Due to the low solubility in water and physiological medium shown by the glycochlorin-conjugates, they were administered to the cell cultures in liposomal suspensions. The incorporation of the conjugates **1**-**4** into DPCC liposomes was carried out as described in literature.[13]

During this process it was observed that all glycochlorin derivatives, with the exception of xylose derivative **2**, afforded stable photosensitizer-liposome suspensions. For the photodynamic assays, different concentrations of these suspensions were obtained by dilution of the original solution with DMEM with antibiotics. The photocytotoxicity of glycochlorin derivative **2** was not determined due to its inefficient incorporation into phospholipids layers. Several attempts to prepare stable liposomes suspension with this conjugate were performed, but without success.

Cell viability

The photodynamic effect of the glycochlorins **1**, **3** and **4** on the tumoral (HeLa) and non tumoral (HaCaT) human cells lines was studied at two different concentrations, $1.0x10^{-7}$ M and $1.0x10^{-6}$ M. After the addition of the PS-liposomal solutions to the cell cultures and incubation in the dark for 4 h, the cells were irradiated with red light for 5, 10, and 20 min with an irradiance of 9.15 mW/cm^2. The cell viability was evaluated using the MTT colorimetric assay,[14a,b] and the data of the cell survival are summarized in Table 1.

The results show that the photodynamic effect of glycochlorins **1**, **3** and **4** on the two epithelial cell lines was dependent on the sugar unit, concentration and irradiation time. With the glucose conjugate **1** the highest decrease in cell survival for both cell lines was achieved at the highest concentration and irradiation time. Additionally, it is possible to observe that the photodynamic effect of this conjugate on non tumoral HaCaT cells is similar or even more significant than the one observed for HeLa cells, principally when the low concentration was tested. These results indicate clearly that glycochlorin **1**, under the same experimental conditions, was not selective for tumoral cells, causing a similar photodynamic effect in both HeLa and HaCaT cells.

When the fructose conjugate **3** was used as photosensitizer, the highest decrease in cell survival was also attained at the highest concentration tested. In the case of the tumoral cell line HeLa, at the lowest concentration ($1.0x10^{-7}$ M) and after 20 min of irradiation it is observed a decrease of approximately 40% on cell survival while at the highest concentration ($1.0x10^{-6}$ M) and with the same irradiation time a decrease of 86% is attained. Although this value seems to be, in a first analysis, a very satisfactory reduction in the viability of the tumoral cell line (HeLa) when is compared with the one obtained in the non-tumoral cell line HaCaT, at the same experimental conditions, no significant selectivity was observed. So, in this case the presence of fructose moiety did not improve the chlorin selectivity on HeLa cells.

Table 1. Percentage survival of HeLa and HaCaT cell lines, at different irradiation times with an irradiance of 9.15mW/cm^2 in the presence of conjugates **1**, **3** and **4** at different concentrations.

Conjugate	Conc. (M)	Irradiation Time (min)	Survival fraction (% ± SD)	
			HaCaT	HeLa
1	$1.0x10^{-7}$	5	96.87 ± 1.50	113.25 ± 1.17
		10	89.53 ± 1.94	101.65 ± 3.74
		20	85.37 ± 1.58	102.50 ± 1.44
	$1.0x10^{-6}$	5	63.98 ± 3.33	42.53 ± 1.73
		10	32.80 ± 0.71	23.37 ± 5.51
		20	15.43 ± 1.74	16.29 ± 0.69
3	$1.0x10^{-7}$	5	70.68 ± 1.36	89.44 ± 0.89
		10	65.75 ± 4.42	84.57 ± 2.04
		20	49.97 ± 4.26	60.52 ± 2.61
	$1.0x10^{-6}$	5	25.93± 3.48	14.67 ± 0.90
		10	21.26 ± 0.19	13.50 ± 0.15
		20	22.87± 0.03	13.70 ± 0.37
4	$1.0x10^{-7}$	5	102.82 ± 1.40	93.72 ± 9.84
		10	102.60 ± 5.16	91.44 ± 6.85
		20	106.45 ± 2.37	78.68 ± 2.05
	$1.0x10^{-6}$	5	90.33 ± 9.42	62.12 ± 2.05
		10	82.14 ± 9.05	34.65 ± 3.58
		20	72.86 ± 3.85	30.52 ± 2.06

Each point corresponds to the mean value ± SD from 3 independent experiments with 4 replicates for each experimental condition.

However, the results summarized in Table 1 show that the photodynamic profile of galactose-chlorin **4** in both cell lines is completely different and very promising when compared with the previous data. In fact, at the lower concentration ($1.0x10^{-7}$ M) and after 20 min of irradiation, this photosensitizer is able to cause a decrease of approximately 21% on the survival of HeLa cells without affecting HaCaT cell line. This selectivity is maintained when the PDT experiments were performed in the presence of this glycochlorin at the highest concentration ($1.0x10^{-6}$ M) for all irradiation periods. For instance, after 20 min of irradiation, the decrease observed in the survival of the HeLa cells was approximately 70%, while the decrease in the survival of HaCaT cells under these conditions of treatment was approximately 27%. This result clearly indicates that this photosensitizer presented a high

selectivity for tumoral HeLa cell lines without significantly affecting the viability of non-tumoral HaCaT cell line.

Based on the results obtained and on the idea that a good photosensitizer must possess, high selectivity for tumoral cells, we can consider that conjugate **4** is the best candidate to be used as photosensitizer in PDT. The low selectivity of glycochlorins **1** and **3** towards the tumour cell line (HeLa), causing a similar cytotoxic effect in both non-tumoral and tumoral cells, precludes their use as PS. On the other hand, glycochlorin **4** showed high efficiency in photoinactivation of tumoral HeLa cells, causing minor cytotoxic effects on non-tumoral HaCaT cells. Due to this fact, only conjugate **4** was selected to develop further studies.

Dark Toxicity

Since the main action of a photosensitizer is related with the production of singlet oxygen and/or other cytoxic oxygen species generated under light irradiation, it is important to evaluate the toxicity of the PS in the absence of light. In fact, an important requirement for a PS to be considered in PDT is to be non-toxic in the absence of light.[16,17] This fact prompted us to evaluate the dark toxicity of the most promising conjugate **4**. The experiments were performed using the two PS concentrations ($1.0x10^{-7}$ M and $1.0x10^{-6}$ M) that were administered to cell cultures in liposomal suspensions. The cells were incubated in the dark for a period of 4 h and the cytotoxic activity of conjugate **4** (without the irradiation protocol) was evaluated using the MTT assay.

Table 2. Percentage survival of HeLa and HaCaT cells, incubated in the dark during 4 h with glycochlorin **4** at two different concentrations

PS	Conc. (M)	Cell survival (% ± SD)	
		HaCaT	HeLa
4	$1.0x10^{-7}$	102.35 ± 2.36	101.63 ± 4.19
	$1.0x10^{-6}$	108.73 ± 4.90	108.05 ± 6.42

Each point corresponds to the mean value ± SD from 3 independent experiments with 4 replicates for each experimental condition.

As it can be observed in table 2, the survival rates obtained show that chlorin **4** was not toxic to both cell types in the absence of light at the studied concentration, confirming that the cytotoxic effect is due to the reactive oxygen species produced under irradiation.

Subcellular localization and morphological studies

It is well known that the PS efficiency is dependent on its subcellular localization. The subcellular localization experiments and morphological studies of conjugate **4** were initially conducted at the concentration 1.0 x 10^{-6} M and after 20 min of irradiation with red light where it is observed a sublethal dose of LD_{30} for the non-tumoral HaCaT cell. However, under these HaCaT sublethal conditions (LD_{30}) the effects found in the tumoral cell line HeLa (70% lethality) showed to be too drastic (data not show) to allow a clear conclusion about chlorin **4** localization and morphological alterations. This fact prompted us to select a less drastic treatment condition in order to be able to analyze the photodynamic effect of conjugate **4** in both cell lines. Under the new conditions selected, 1.0 x 10^{-7} M and 20 min of irradiation, it was observed a lethality of 21% on the tumoral cell line whereas no cytotoxic effect was detected on non-tumoral cells (Table 1).

Morphologically, HaCaT cells tend to form microcolonies with well-developed adhesion systems.[18] When this non-tumoral cell line was incubated with conjugate **4** at concentration 1 x 10^{-7} M, it was possible to verify that the morphological cell changes are scarce and only partial cytoplasmic vacuolization was observed after 20 min of irradiation (Figure 2). It is important to note that these morphological changes are most apparent at the edge of the colony and no alterations were detected in the centre of the colony. This can be due to the fact that cells located at the edge of the colonies were more exposed to photosensitizer (Figure 2). It is also clear that this slight cytoplasmic vacuolization does not affect the viability of these cells, since no cytotoxic effect was seen in the MTT experiments.

After similar photodynamic treatment of HeLa cells, it was observed that after 5 min of irradiation the HeLa cells suffer strongly cytoplasmic vacuolization (Figure 2, inset) that is indicative of cell degeneration processes. After 20 min of irradiation, it was found that the morphology of HeLa cells was dramatically affected, being possible to observe cells presenting a severe cytoplasmic shrinkage as a consequence of the cell death process (Figure 2, inset). With these experimental conditions, 1.0 x 10^{-7} M and 20 min of irradiation, it was also possible to observe a reduction in the number of cells present in the preparation (in comparison with the control and after 5 min of irradiation). These results are in agreement with the reduction of 21% of viability found in MTT experiments.

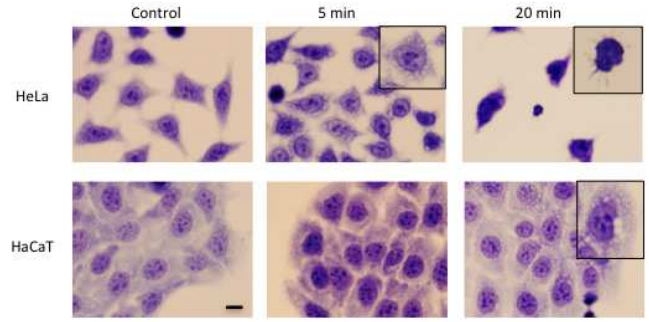


Figure 2. Morphological changes induced by glycochlorin derivative **4** on HaCaT and HeLa cell lines after being incubated for 4 h with a concentration of 1.0 x 10^{-7} M and subject to different irradiation times. After 24 h of light exposure, cells were fixed in cold methanol and stained with toluidine blue. Photographs are representative images of three different experiments for each condition. Scale bar: 10 µm.

The efficiency of a photosensitizer, both in cell cultures and in tumours, is directly related to its chemical structure, concentration, incubation time, light doses and cellular location.[19] A range of cellular components have been described as targets of cytotoxic oxygen species formed during the photodynamic process, such as mitochondria, lysosomes, Golgi apparatus and plasma membrane. Photosensitizers that are located in mitochondria and lysosomes are highly efficient in cell photoinactivation, causing necrosis and apoptosis.[20]

Having in mind the importance of this fact, we decided to carry out studies of subcellular localization with the most effective and selective photosensitizer, conjugate **4**, at the concentration of 1.0 x 10^{-7} M, the same concentration used for morphological studies. Thus, the cell lines were incubated with the conjugate **4** at 1.0 x 10^{-7} M for 2 (data not show), 4, and 6 h in the dark. After each incubation time, cells were washed with PBS in order to remove the photosensitizer that has not been incorporated into cells. After that, coverslips were mounted with PBS and the preparations were observed under blue light in a fluorescence microscope. Two controls were also performed where each cell line were incubated without conjugate **4**. The results are presented in Figure 3.

The distribution pattern of this conjugate was similar in HaCaT and Hela Cells. In both cell types the conjugate 4 was mainly located in granules that closely resemble endosomes/lysosomes (Figure 3, yellow arrows) since this fluorescent granular signal was similar to that observed after cell incubation with lysotracker. Some larger red fluorescent granules are also seen in the cytoplasm; these granules may correspond to other types of vesicles (White arrows).

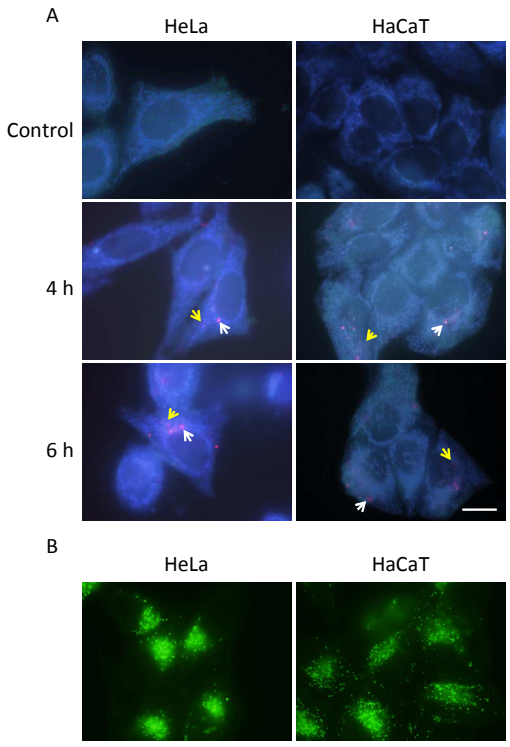


Figure 3. (A) Intracellular localization of glycochlorin **4** in HaCaT and HeLa cells after incubation for 4 and 6 h at concentration of 1.0x10^{-7} M. White arrows show the localization of the photosensitizer fluorescing in red and showing a lysosomal-like distribution. And yellow arrows show the red fluorescent granules of bigger size. (B) For comparative purpose, cell lysosomes were labelled with lysotracker marker. Blue autofluorescence of mitochondria's can be observed in both controls. Scale bar: 10 µm.

As discussed above, HaCaT cells tend to form microcolonies where the cells are very attached to others.[18] So, it is important to take into account that the location of the photosensitizer is always at the edge of colony of this cell line and it is only possible to find it within the colony when it is formed by a low number of cells.

Discussion and conclusions

Chlorin derivatives are currently being developed as new photosensitizers due to their interesting photophysical and photodynamic properties.[21] The present work reports the first study concerning the photosensitizing ability of glycochlorins containing D-glucose (**1**), D-ribose (**2**), D-fructose (**3**) and D-galactose (**4**) units towards a tumoral cell line. The synthesis, photochemical and photophysical studies of these conjugates have been recently described by our group.[11]

In the present study we show how photodynamic therapy with these glycochlorin conjugate affects cultured HeLa tumoral cells. HeLa cells are regarded as one of the best-known models of cell lines lacking functional p53 protein, due to activity of the host HPV16 viruses and to mutations in p53, which occur in over 50% of human tumours.[22] On the other hand, HaCaT cells are a transformed human epithelial cell line from adult skin, which maintains full epidermal differentiation capacity. This HaCaT cell line is obviously immortal and has a transformed phenotype *in vitro* but it remains non-tumorigenic, therefore this cell line is a good model of non-tumoral cells.[18] Using these two *in vitro* models of HaCaT and HeLa cells, we demonstrate high efficiency of PDT killing tumoral cells when our glycochlorin conjugates were applied.

However, not all compounds showed high selectivity for tumoral cells. First studies on cell viability after PDT, demonstrated that glucose- and fructose-chlorins (conjugates **1** and **3** have no cell selectivity, inactivating equally the non-tumoral HaCaT cells and the tumoral HeLa cells. A different situation was observed with the galactose-chlorin (conjugate **4)** that proved to be selective for the tumour cell line, inhibiting the growth of HeLa cells without affecting significantly the normal cell line (HaCaT). Interestingly the role of galactose in other PS (porphyrins, chlorins, corroles and phthalocyanines) was already highlighted.[23-27]

Tumour cells exhibit high levels of glycolysis, despite the presence of oxygen in a phenomenon termed aerobic glycolysis. During the last years it has been hypothesized that targeting glucose metabolism may provide a selective mechanism to kill cancer cells.[28] However, glucose (and consequently fructose) is also metabolized in healthy cells through glycolysis, therefore it is expected that HaCaT and HeLa cells recognize equally glucose-chlorin **1** and fructose-chlorin **3**.

The action of a PS in the presence of light is in general directly related to the $^1O_2$ production. So, the lack of photodynamic effect in the absence of light can provide useful information about the mechanism that is responsible for the decrease of cellular viability of the studied cells.[15,16] In this study it was confirmed that galactose-chlorin **4** is not toxic in the absence of light confirming that the principal mechanism of action is related to the $^1O_2$ production.

Considering the morphological alterations, after the photodynamic treatment with conjugate **4** at concentration of $1.0x10^{-7}$ M, it was observed that HeLa cells suffer strongly cytoplasmic vacuolization and pronounced retraction, whereas in HaCaT cells the damage observed is scarce, showing only partial cytoplasmic vacuolization of the cells located at the edge of the colonies. These results strongly corroborate the high selectivity of galactose-chlorin **4** for the tumoral cells.

At the same conditions, the subcellular localization studies showed that conjugate **4** localizes in cytoplasmic membranes and into lysosomes both in HeLa cells and in HaCaT. However, in the case of non-tumoral cell lines this conjugate is always present at the edge of the colony. This specific localization of conjugate **4** may be related to the composition of the liposome formulation used in the administration of the PSs. In fact, the liposome formulations can modulate the intracellular localization of PSs by controlling the interaction of liposomes with the subcellular membrane.[12,29] The liberation of PSs from liposomes can be affected by the structure of liposome, temperature, pH, etc. The DPPC liposomes used in this work are pH-sensitive and it is known that they destabilize between pH 5 and 6.3. Since the endosomal and lysosomal pH as well the interstitial pH in tumour tissue is lower than the normal physiological pH,[12,29] the release of these liposomes content in cytoplasm of tumoral cells is more effective than in non-tumoral cells.

In conclusion, these promising results on the selectivity demonstrated by galactose-chlorin conjugate towards HeLa cell line will lead us to perform further studies on the mechanism of action of this conjugate.

Experimental

Glycoclorin derivatives 1-4

The glycochlorin conjugates 1-4 were synthesized according to literature data.[12]

Cell cultures

Human cervical cancer cell line (HeLa) and human normal skin keratinocytes (HaCaT) were obtained from ATCC. Cell cultures were constantly monitored to ensure that they were free of mycoplasma, by staining with DAPI. The cells were grown as a monolayer employing Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) and 50 units/mL penicillin and 50 μg/mL streptomycin (Sigma). The cells, were incubated at 37 ^{0}C in a humidified 5% CO_2 atmosphere and the medium was changed daily. For the photocytotoxicity experiments,

cells were plated on 24-well plates and for fluorescence observation, cells were plated onto round coverslips placed into wells.

*Liposomes incorporation of glycochlorin conjugates **1-4***

Glycochlorin conjugates were dissolved in pyridine in a concentration of 0.5 mg/mL and stored in the dark at 4˚C. All conjugates were incorporated at 55˚C into liposomes of dipalmitoyl-α-phosphatidylcholine (DPPC), according to the procedure, described in literature.[12,13] Subsequently, glycochlorin-liposomal solutions were sterilized by filtration through a Millipore© (Millipore Corporation, Bedford, MA) filter (0.22 µm pore diameter), kept at 4˚C and used within 48 h after preparation.

Photodynamic therapy treatment in vitro

Cells seeded in 24-well plates were incubated with an appropriate volume of each glycochlorin conjugate-liposome suspension and were incubated at 37^0C in a humidified 5% CO_2 atmosphere for 4 h. After this period the plates were irradiated with red light (λ = 654 nm ± 20 nm) with an irradiance of 9.15 mW/cm^2 for 5, 10, 15 and 20 min. After irradiation, the medium of each well was replaced with DMEM with antibiotics and FCS, and maintained in the dark in a sterile environment in the incubator for 24 h. Cell viability was estimated using the MTT colorimetric method.

To assess the possible cytotoxic effect of glycochlorin **4**, cells were incubated in the presence of this PS, under the same conditions as for the PDT experiments, but without performing irradiation with red light. Cell viability was also estimated using the MTT colorimetric method. All results obtained represents three independent experiments with replicates.

Cell Morphology studies

For evaluation of cell morphology, the cells were seeded in 24-well plates containing sterile round coverslips and were kept in a sterile environment in the incubator for 24 h until the experiment start. After PDT treatments, cells were fixed with cold methanol (-20˚C) for 10 min. After three washes with PBS, cells were stained with toluidine blue (TB, Merck, Darmstadt, Germany) for 5 min (0.05 mg/mL in distilled water). After being washed and air-dried, samples were mounted in DePeX (Serva, Heidelberg, Germany) and observed under bright field illumination.

Light source

A LED array was constructed and used for irradiation. It is composed of a matrix of 24 x 16 LEDs (Kingbright, model L53 SRCE), which makes a total of 384 light sources with an emission peak at 654 nm and a bandwidth at half maximum (BWHM) of ± 20 nm, as measured with a spectrometer (Fiberoptic, Avantes). The array works with alternate current, fed by integrated circuits (MBI6001 N2D, Macroblock Inc.), which provides current pulses of 1.66 ms duration followed by a non-emitting period of 7 ms. So, the emission of the LED array has a pulsed light pattern. The irradiance at 1.5 cm distance from the array surface is 9.15 mW/cm^2 (photocell PAR 190 Li-1000, Li-Cor).

MTT viability assay

Cell viability was documented by the MTT assay.[14] So, following appropriate treatments, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) solution was added to each well at a concentration of 0.5 ng/mL, and plates were incubated at 37˚C for 2–3 h. The resulting formazan crystals were dissolved in DMSO and absorbance was measured at 542 nm in an automatic way on a plate reader (spectra fluor 4, TECAN). Measurements of total absorption controls were taken as 100% cell viability and the percentage viability for the rest of the samples undergoing complete treatment was estimated.

Intracellular localization studies

The cells were cultured in 6-well plates containing sterile coverslips and were allowed to grow for 24 h. After that, cells were incubated with the conjugate-liposome suspension for 2, 4 and 6 h. Subsequently, the coverslips were washed twice with PBS and mounted on slides with PBS and examined by fluorescence microscopy. Microscopic observations and photographs were performed in an Olympus photomicroscope IMT-2, equipped with a HBO 100 W mercury lamp and the corresponding filter sets for fluorescence microscopy: UV (365 nm, exciting filter UG-1), blue (450-490 nm, exciting filter BP 490) and green (545 nm, exciting filter BP 545). Photographs were processed using Adobe Photoshop CS software (Adobe Systems, San Jose, USA).

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Notes and references

[a] *QOPNA & Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal*

[b] *Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain*

[c] *Departamento de Química Orgânica, Universidade Federal Fluminense, 24020-150, Niterói, RJ, Brazil*

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