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Synthesis of neutral and cationic tripyridylporphyrin-D-galactose conjugates and the photoinactivation of HSV-1

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Abstract—Neutral and cationic tripyridylporphyrin-D-galactose conjugates were synthesized and their antiviral activity against herpes simplex virus type 1 (HSV-1) was evaluated. At non-cytotoxic concentrations the studied compounds show significant antiviral activity after photoactivation. The influence of photoactivation on drug treated cells was also analyzed, at different times of infection with HSV-1, for a neutral (**1b**) and a cationic glycoporphyrin (**3b**) derivative. The results show that the inhibition of the viral yield is more dependent on photoactivation for compound **1b** than for compound **3b**. These two compounds also differ in the inhibitory effect during the viral replicative cycle: while compound **3b** inhibits the viral yield at all the addition times assayed, compound **1b** is more efficient in later times of infection.

1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are members of the family *Herpesviridae*, subfamily *Herpesvirinae*[1] and are responsible for a silent disease that has plagued humanity since ancient times.[2–5] Disease symptoms often interfere with human everyday activities and are of special concern when they are responsible for life-threatening or sight-impairing disease, especially in neonates and in immunocompromised host population.[2] After primary or initial infection, the virus persists for life in a latent form in neurons of the host, periodically reactivating and often resulting in significant psychosocial distress for the patient. Currently, only few antiviral drugs of proven effectiveness exist[6,7] and viral resistance to some antiviral drugs, especially in the immunocompromised host, has encouraged research for new drugs and methodologies that could actually cure these diseases triggered by viruses. One of such methodologies is the photoinactivation of viruses by different photosensitizers.[8–13] This promising methodology, inspired in the photodynamic therapy (PDT),[14,15] is being studied for the inactivation of viruses in biological fluids and blood products.[16–18] In PDT, light, oxygen and a photosensitizing drug are combined to produce a selective therapeutic effect. Despite all the three parameters are important, the key point of the PDT success is centred on the photosensitizer (photoactive dye). Thus, new photosensitizers, namely porphyrin-like compounds, have been developed in order to find the best candidates for a specific PDT purpose.[14,15,19]

Both the photophysical and the hydrophobic/hydrophilic properties of a photosensitizer are important parameters to have in consideration. Because simple porphyrins are hydrophobic, the addition of polar groups, such as carbohydrate residues and/or pyridinium groups, is required to increase their hydrophilic character. Studies with a range of cationic porphyrins and porphyrin–carbohydrate conjugates have shown that these types of compounds are efficient photosensitizers in PDT, not only against cancer cells[20,21] but also against bacteria[22,23] and viruses.[16,24–26]

Keywords: Cationic porphyrins; Sugars; Antiviral compounds; HSV-1.
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Despite the mechanism of viral photoinactivation being still unclear, it is known that enveloped viruses are significantly more photosensitive than non-enveloped ones.[8,27] It is thought that the constituents of the envelope (lipids and proteins) can act as recognition and binding-sites for the photosensitizer.[28] Knowing that some carbohydrates have a specific affinity for those binding-sites,[29,30] many glycoporphyrins have been prepared and studied.[31]

Recently we reported that neutral porphyrin–carbohydrate conjugates exhibit strong antiviral activity against HSV-1 and HSV-2.[25] Other studies have shown that cationic porphyrin derivatives also display significant antiviral activity after photoactivation.[16,24,26] In that way, we decided to prepare neutral and cationic tripyridylporphyrins, and the corresponding D-galactose conjugates, to study, simultaneously, the influence of the charge and the presence of the carbohydrate moiety on the antiviral activity of the compounds. D-Galactose was selected simply because the required derivative (1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose) is a commercial compound; we have previously used it to prepare other porphyrin-D-galactose conjugates.[25]

The activity of the tripyridylporphyrin derivatives against HSV-1, at non-cytotoxic concentrations, was evaluated under dark conditions and upon photoactivation. The influence of the neutral or cationic character of the drugs and the presence of a D-galactose moiety with protected or unprotected hydroxyl groups was studied. The influence of drug addition time on the viral yield was also analyzed in infected cells, with and without photoactivation.

2. Chemistry

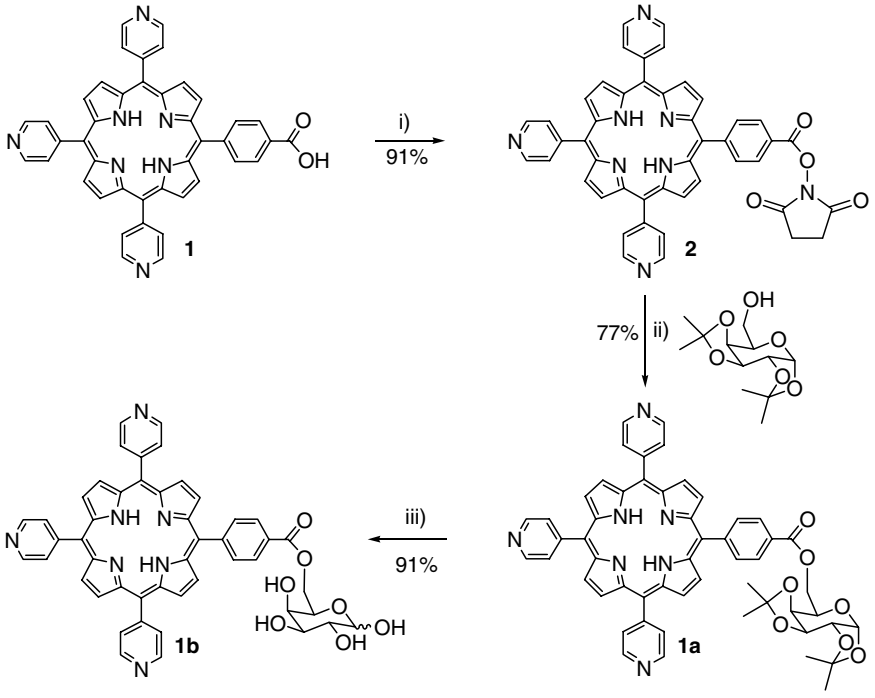
2.1. Results and discussion

Porphyrin **1**, the key compound in the synthesis of the other porphyrin derivatives, was obtained from a crossed Rothemund reaction as described in the literature.[23,32] The activated ester **2** was obtained in a one-pot procedure: reaction of porphyrin **1** with thionyl chloride (to generate the corresponding acyl chloride) followed by the addition of *N*-hydroxysuccinimide.[23] The coupling of the activated ester **2** with 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose was carried out in dry toluene at room temperature in the presence of sodium hydride (Scheme 1). After purification by flash chromatography on silica gel and crystallization, the ester **1a** was obtained in 77% yield. Deprotection of the sugar moiety with aqueous trifluoroacetic acid at room temperature afforded, after purification, porphyrin **1b** in 91% yield.[25]

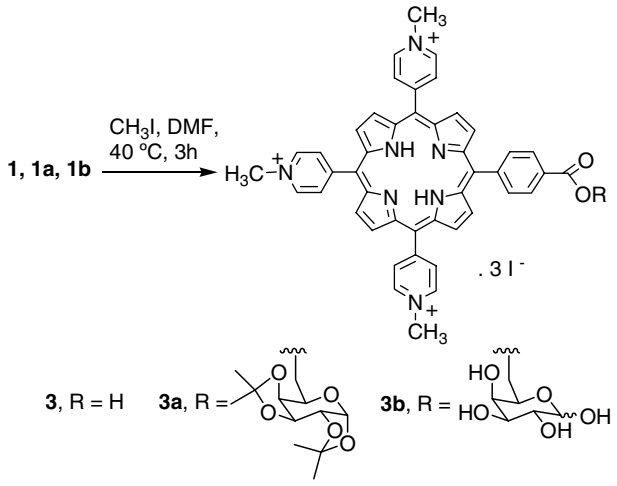
Methylation of porphyrins **1**, **1a** and **1b** with methyl iodide in dry DMF gave the expected Tris(*N*-methylpyridinium)porphyrins **3**,[23] **3a** and **3b** in ca. 90% yield (Scheme 2).

The structures of the neutral and cationic glycoporphyrin derivatives were confirmed by NMR, UV–visible spectroscopy, mass spectrometry and HRMS or elemental analysis.

The ^{1}H and ^{13}C NMR spectra of compounds **1** and **1a** differ only in the aliphatic region, where the resonances corresponding to the protons and carbons of the carbohydrate moiety are observed. In particular, the ^{1}H NMR



Scheme 1. Reagents and conditions: (i) $SOCl_2$, pyridine, 50 °C, 30 min, then *N*-hydroxysuccinimide, 50 °C, 3 h; (ii) NaH, toluene, rt, 90 min; (iii) TFA, H_2O, rt, 30 min.



Scheme 2.

spectrum of **1a** shows the resonances due to the anomeric proton as a doublet at δ 5.67 ppm (J 5.0 Hz), the isopropylidene methyl groups as four singlets between 1.39 and 1.63 ppm and the remaining protons of the sugar unit as two multiplets between 4.36 and 4.76 ppm. Comparing the ^{1}H NMR spectra of compounds **1a** and **1b**, the absence of the four singlets due to the resonance of the isopropylidene protons is the main difference and confirms the deprotection of the sugar moiety.

Moreover cationic porphyrins **3a** and **3b** have two sets of unequivalent *N*-methyl groups, their ^{1}H NMR spectra show only one singlet at ca. 4.7 ppm corresponding to the resonances of nine *N*-methyl protons; their mass spectra confirm that the three pyridyl groups are methylated.

3. Photophysical studies

3.1. Photostability of the compounds

In order to establish the rate of photodegradation of the target compounds, we performed the photostability studies at the same conditions of irradiation used in the biological assays (white light, (50 mW/cm^2). The results are shown in Table 1 and were calculated by the ratio of residual absorbance after irradiation for different periods of time and the absorbance before irradiation. Under these conditions, all compounds show a high photostability during the first 15 min of irradiation. The low photobleaching of the compounds indicates that they do not undergo an extensive destruction of the tetrapyrrolic macrocycle.[8] The photostability of the compounds is an important parameter to certify that the results are directly related with the initial concentration of the drugs.

3.2. Singlet oxygen production

The capacity of singlet oxygen generation by porphyrin derivatives is the basis of their application in PDT. In order to test the potential of the new porphyrin derivatives

Table 1. Photostability of the photosensitizers after irradiation with white light (50 mW/cm^2) for different periods of time

Compound	Irradiation time (min)					
	0	**1**	**3**	**5**	**10**	**15**
1	100	99	99	96	92	87
1a	100	98	98	98	94	93
1b	100	100	98	97	88	83
3	100	98	98	98	98	97
3a	100	99	98	98	96	95
3b	100	99	98	98	98	98

The absorbance was measured at the corresponding Soret band wavelength. The results are presented in percentage calculated by the ratio of residual absorbance at different periods of time and the absorbance before irradiation.

to act as photosensitizers, their capacity to generate singlet oxygen was qualitatively estimated by measuring the absorption decay of 1,3-diphenylisobenzofuran (DPBF) at 415 nm in DMF/H_2O (90:10).[33–35] A solution containing DPBF (75 μM) and the porphyrin derivative (1 μM) was irradiated over a period of 7 min using the white lamp with a cut-off filter for wavelengths <540 nm, at a fluence rate of 50 mW/cm^2. The absorption decay of DPBF at 415 nm was measured at irradiation intervals of 1 min. The results summarized in Figure 1 show that the DPBF photodegradation is highly enhanced in the presence of the photosensitizers. These results confirm that they are good singlet oxygen generators, although the cationic porphyrin derivatives (**3**, **3a** and **3b**) are slightly more efficient than the neutral ones (**1**, **1a** and **1b**). Since neutral porphyrins are expected to be less soluble in DMF/H_2O (9:1) than the cationic ones, the difference observed in the singlet oxygen generation might result, eventually, from aggregation phenomena. However, absorption studies with solutions of the neutral porphyrins at the range 0.25–1.5 μM show that the intensity of the Soret band is linearly proportional to the concentration, indicating that there is no aggregation.

4. Biological evaluation

The antiviral efficiency of the neutral porphyrin derivatives **1**, **1a** and **1b** and the corresponding cationic forms

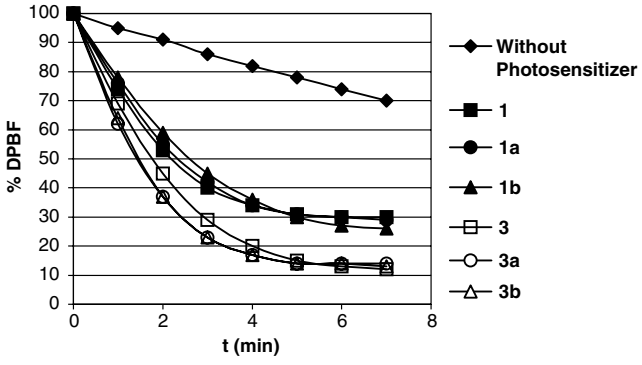
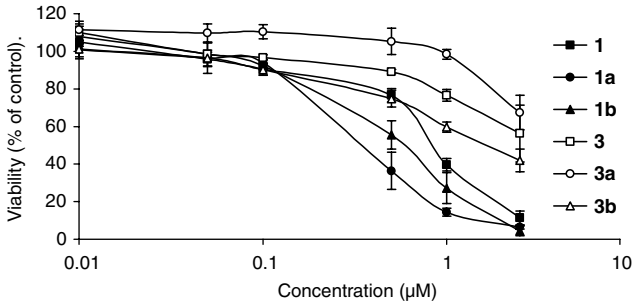


Figure 1. Comparative photooxidation of DPBF (75 μM) in DMF/H_2O (9:1) with or without photosensitizer (1 μM); irradiation with white light filtered through a cut-off filter for wavelengths <540 nm (50 mW/cm^2).



	Cell viability (% of control ± SD)					
	Compound					
Conc. (μM)	1	1a	1b	3	3a	3b
0.01	105.0 ± 0.9	100.8 ± 4.7	108.0 ± 5.4	110.0 ± 6.0	111.5 ± 3.0	101.3 ± 4.3
0.05	95.7 ± 3.8	96.4 ± 1.3	98.4 ± 6.1	98.6 ± 2.0	109.8 ± 4.8	96.4 ± 8.1
0.1	90.6 ± 3.1	93.9 ± 1.7	92.3 ± 1.5	96.7 ± 1.4	110.4 ± 3.8	90.2 ± 2.9
0.5	76.6 ± 3.5	36.4 ± 9.9	55.5 ± 7.6	89.2 ± 1.2	105.2 ± 7.0	74.8 ± 4.4
1.0	39.7 ± 3.3	14.4 ± 2.0	27.2 ± 8.2	76.6 ± 3.2	98.4 ± 2.6	59.7 ± 2.8
2.5	11.4 ± 3.7	6.4 ± 0.9	4.4 ± 2.6	56.3 ± 15.1	67.4 ± 9.1	42.0 ± 6.0

Figure 2. Photocytotoxicity profile of studied compounds in Vero cells. Each value represents mean ± standard deviation of three experiments, with three replicates each. Error bars represent standard deviations.

3, **3a** and **3b** was assayed against HSV-1, either directly (virucidal effect) or on Vero cells infected with HSV-1 (effect on virus yield).

4.1. Photocytotoxicity studies

The photocytotoxic effect of the porphyrins was evaluated on Vero cells using different concentrations of photosensitizers (0.01–2.5 μM). After 10 min of incubation with the compounds, the cells were irradiated for 15 min with white light at a fluence rate of 50 mW/cm^2. Control cells were irradiated in the absence of photosensitizer. The cellular viability was determined 48 h post-irradiation, by the MTT colorimetric assay.[36] No decrease in cell survival due to irradiation was detected, in comparison to non-irradiated cells (another control of the experiment). The results presented in Figure 2 (where the viability of the treated cells is referred to the 100% viability of control cells) show that none of the studied compounds has photocytotoxic effect at the concentration of 0.01 μM; compound **3a** shows the lower cytotoxicity, which is below 0.5 μM. In fact, all the studied compounds, except compound **1a**, show IC_{50} values above 0.5 μM. It was also noticed that the photocytotoxicity profiles of the neutral and cationic derivatives are significantly different. In general, the neutral porphyrins show higher toxicity, with IC_{50} values below 1.0 μM.

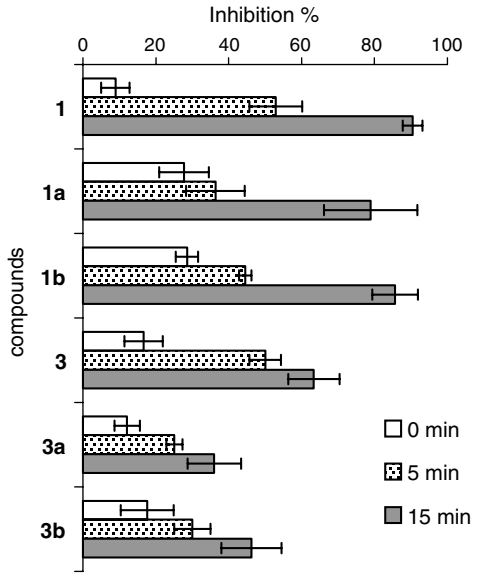
4.2. Virucidal effect

In order to establish whether the synthesized compounds could act directly on the HSV-1 virions, their virucidal activity was determined. The virucidal activity tests were performed in the presence of the photosensitizer at a concentration of 0.02 μM (close to the maximum non-cytotoxic studied concentration—MNCSC). These experiments were performed by incubating viral suspensions of HSV-1 with each compound for 10 min under dark conditions at 37 °C. After that, the samples were irradiated for 5 or 15 min with white light at a fluence rate of 50 mW/cm^2 or not exposed to light (0 min in Fig. 3). As controls of these experiments, non-treated viral suspensions were either irradiated or non-irradiated. A plaque reduction assay was used to determine the titres of the drug treated viral suspensions as well as the controls.

The data summarized in Figure 3 (where the loss of infectivity is expressed as a percentage relative to the titres of the control viral suspensions) indicate that at the concentration assayed, all compounds exhibit some virucidal effect in the absence of light. Neutral compounds **1a** and **1b** display the highest values. However, the virucidal activity increases with photoirradiation, this increase being dependent on the time of exposure to light. The values of photoinactivation observed after 15 min of irradiation are 2.5–4 times the values of inactivation in the dark, except for compound **1**, which is 10 times more active. It was also observed that the neutral derivatives (**1**, **1a** and **1b**) are more effective than the cationic ones, inducing viral inactivation up to 90% under the most favourable conditions (15 min, white light, 50 mW/cm^2). Photoinactivation differences are also observed between glycosylated porphyrins and sugar-free derivatives **1** and **3**.

4.3. Influence of the drug addition time on virus yield during infection

In order to elucidate which event of the HSV-1 replication cycle is affected by the photosensitizers, time



Virucidal effect (viral inhibition % ± SD)			
	Irradiation time (min)		
Comp.	0	5	15
1	8.9 ± 3.9	52.9 ± 7.2	90.5 ± 2.7
1a	27.7 ± 6.9	36.4 ± 8.1	78.9 ± 12.7
1b	28.6 ± 3.1	44.5 ± 1.7	85.6 ± 6.3
3	16.7 ± 5.3	50.0 ± 4.4	63.3 ± 7.1
3a	12.1 ± 3.5	25.1 ± 2.2	36.0 ± 7.3
3b	17.6 ± 7.2	30.0 ± 5.0	46.2 ± 8.2

Figure 3. Virucidal effect of the target compounds (0.02 μM) on HSV-1, non-irradiated and irradiated for 5 or 15 min with white light (50 mW/cm^2). The inhibition values (in %) were calculated in relation to the non-treated controls. Data are reported as means of three independent assays, each run in triplicate. Error bars represent standard deviations.

addition experiments were carried out with the neutral and cationic target compounds **1b** and **3b**, respectively. These compounds were selected based on our previous results, which have shown that glycosylporphyrins with the free hydroxyl groups display the highest virus yield reduction.[25] Vero cells were infected with HSV-1 and, after allowing virus adsorption at 4 °C for 2 h, they were incubated again at 37 °C. The cell monolayers were then treated with compounds **1b** or **3b** (0.02 μM) at different post-adsorption times (0, 2, 4 and 16 h). After a 30 min period of incubation with the compound, they were irradiated or not (control cultures) with white light for 15 min, with a fluence rate of 50 mW/cm^2. The virus yield was determined by titration of samples collected 24 h post-infection, when extensive cytopathic effect was observed in the control cells.

The data presented in Table 2 clearly show that the photoactivation of both compounds in infected cells largely reduced the HSV-1 yield. However, the cationic derivative **3b** also shows activity in the absence of light, all along the viral replication cycle. The higher inhibition was observed at 16 h post-adsorption (the later addition time assayed). For the neutral compound **1b**, under dark conditions, only at this addition time is some activity evident.

Table 2. Influence of the drug addition (0.02 μM) at different post-adsorption times on the inhibition of the virus yield

Inhibition of HSV-1 yield (% of control ± SD)				
Addition time of drug (h post-adsorption)	Dark		15 min, 50 mW/cm^2	
	1b	3b	1b	3b
0	0	48.9 ± 4.0	71.1 ± 9.5	88.6 ± 3.6
2	0	40.9 ± 6.3	79.2 ± 5.5	83.9 ± 7.2
4	0	37.6 ± 2.6	93.6 ± 3.4	88.2 ± 5.3
16	45.8 ± 5.2	76.3 ± 3.0	95.7 ± 4.1	99.8 ± 2.3

Each value represents mean ± standard deviation of two experiments, with three replicates each.

The kinetic experiments carried out in the absence and in the presence of light indicate that neutral compound **1b** is more efficient at the later times of the viral replication cycle assayed (4 and 16 h post-infection), showing very little inhibition at the immediate early time of infection (time 0). On the other hand, cationic compound **3b** highly inhibits the viral yield at all the addition times assayed. However, similarly to compound **1b**, the highest inhibition is also observed at the addition time 16 h post-adsorption.

The higher photodynamic effect is observed with compound **1b**, which presents the highest light/dark ratios concerning viral yield inhibition.

5. Discussion and conclusions

New neutral and cationic glycosylated porphyrins were efficiently prepared in high yields. These compounds were assayed for anti-herpetic activity at non-cytotoxic concentrations previously determined, with and without photoactivation. The virucidal effect of the neutral porphyrin derivatives was largely enhanced after 15 min of irradiation. Identical results, although with lower values, were obtained with the cationic derivatives. Nevertheless, the results obtained with DPBF photooxidation indicate that both neutral and cationic derivatives are efficient singlet oxygen generators. In such way, the higher virucidal effect observed with neutral porphyrins can be related to multiple factors, namely different affinity towards the viral envelope.

The drug addition experiments were carried out in different stages of HSV-1 replication cycle. In these experimental conditions (high multiplicity of infection and a 2-h period of adsorption at 4 °C), it is expected that viral DNA replication has already started at 4 h post-adsorption. Therefore, times 0 and 2 h post-adsorption correspond to the immediate early and early times of infection, respectively, and it can be assumed that the other experimental points refer to the late time of infection.[1]

The results obtained with compounds **1b** and **3b** at different post-adsorption times show their antiviral activity and that it is enhanced by photoactivation. However, they also suggest two distinct action modes on virus yield inhibition. While the cationic derivative **3b** is active in the absence of irradiation and all along the viral replication cycle, the neutral compound **1b** does not show any activity in the dark, except in the later time assayed (16 h post-adsorption). Photoactivation of compound **3b** results in inhibitions of the viral yield by more than 83% at all the addition times assayed. Compound **1b**, when photoactivated, has a low inhibitory effect in the early times of infection (mainly at 0 h post-adsorption) and a very high inhibitory effect (identical to compound **3b**) in the late period of infection (mainly at 16 h post-adsorption).

The best photodynamic effect is observed with compound **1b**, as a consequence of the activity of compound **3b** in dark conditions.

The very high values of inhibition of HSV-1 yield after photoactivation of both compounds, at 16 h post-adsorption, may be due to a direct action on the new viral particles already present in the cells or undergoing the last steps of morphogenesis. Actually this may be the case, especially for compound **1b** which also exhibited a high virucidal effect.

In what concerns the high inhibitory effects observed in periods of infection in which there are no viral particles yet, and especially high with compound **3b**, other steps of the viral replication cycle must be involved, such as DNA or RNA synthesis and/or processing, as well as synthesis, processing and/or transport of viral proteins. A direct action on the host cell metabolism with repercussions on viral morphogenesis can also be hypothesised. In this case, uninfected cells should be affected too, although in a reversible way, therefore without consequences on cell viability.

Photoactivation of these compounds seems to be crucial for their activity, even though the photoactivation-independent inhibitory effect obtained with compound **3b**.

The results obtained with compounds **1b** and **3b** on the direct effect and on virus yield show a significant inhibitory effect on HSV-1 at non-cytotoxic concentrations. Therefore, although these are results from in vitro studies, they evidence the potential anti-herpetic activity of these compounds.

6. Experimental

6.1. General

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 300 spectrometer at 300.13 and 75.47 MHz, respectively. DMSO-d_6 was used as solvent (except when indicated) and tetramethylsilane as internal reference; the chemical shifts are expressed in δ (ppm) and the coupling constants (J) in Hz. Mass spectra and HRMS were recorded on VG AutoSpec Q and M mass spectrometers. For the cationic compounds DMSO was used as solvent and 1-thioglycerol as matrix. Elemental analyses were performed with a Leco 932 CHNS analyzer. The UV–vis spectra were recorded on an Uvikon spectrophotometer. Melting points were measured on a Reichert Thermovar apparatus fitted with a microscope and are uncorrected. Flash chromatography was carried out in silica gel (Merck, 35–70 mesh). Analytical TLC was carried out on precoated sheets with silica gel (0.2 mm thick, Merck).

6.2. Synthesis

6.2.1. 5-[4-(1,2:3,4-Di-*O*-isopropylidene-α-D-galactopyranosyl-6-oxycarbonyl)phenyl]-10,15,20-tri(4-pyridyl)porphyrin (1a). To a solution of porphyrin **2**[23] (30.0 mg, 39.6 μmol) and 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose (150 mg, 0.58 mmol, 14.6 equiv) in dry toluene (2.5 mL) was added an excess of sodium hydride (≈25 mg). The reaction mixture was stirred in the dark, and under nitrogen, during 90 min at room temperature. After that, water was added and the mixture was then neutralized with aqueous saturated citric acid. The mixture was extracted with chloroform, the organic phase was washed with water (2 × 100 mL), dried (Na_2SO_4) and the solvent removed. The residue was purified by flash chromatography (silica gel) using chloroform/acetone (1:1) as eluent. Porphyrin **1a** was crystallized from chloroform/petroleum ether (27.5 mg, 77% yield). mp >300 °C; ^{1}H NMR ($CDCl_3$): δ −2.90 (s, 2H, NH), 1.39, 1.43, 1.56, 1.63 (4s, 12H, CH_3), 4.36–4.48, 4.60–4.76 (2m, 6H, Gal-H2, 3, 4, 5, 6), 5.67 (d, J = 5.0, 1H, Gal-H1), 8.17 (d, J = 5.7, 6H, 10,15,20-Ar-*o*-H), 8.29 (d, J = 8.1, 2H, 5-Ar-*o*-H), 8.48 (d, J = 8.1, 2H, 5-Ar-*m*-H), 8.84-8.89 (m, 8H, β-H), 9.06 (d, J = 5.7, 6H, 10,15,20-Ar-*m*-H); ^{13}C NMR ($CDCl_3$): δ 24.6, 25.0, 26.0, 26.1 [4 × C($\underline{C}H_3$)$_2$], 64.4 (Gal-C6), 66.2 (Gal-C5), 70.5, 70.8 (Gal-C3, 4), 71.2 (Gal-C2), 96.4 (Gal-C1), 108.9, 109.8 [2 × $\underline{C}$(CH$_3$)$_2$], 117.4, 117.6, 120.1, 128.2, 129.3, 129.8, 131.4, 134.5, 146.3, 148.4, 149.9, 166.5 (CO); UV–vis ($CHCl_3$) λ_{max} (log ε): 420 (5.63), 514 (4.26), 548 (3.66), 589 (3.67), 644 (2.96) nm; HRMS (FAB^+) m/z: calcd for $C_{54}H_{46}N_7O_7$ $(M+H)^+$: 904.3459; Found: 904.3433.

6.2.2. 5-[4-(α/β-D-Galactopyranosyl-6-oxycarbonyl)phenyl]-10,15,20-tri(4-pyridyl)porphyrin (1b). Porphyrin **1a** (20 mg, 22.1 μmol) in trifluoroacetic acid and water (9:1) (4 mL) was stirred in the dark at room temperature for 30 min. Chloroform (20 mL) and water (20 mL) were then added, and the mixture was neutralized with aqueous sodium carbonate. The mixture was extracted with $CHCl_3$/MeOH (85:15), and then the organic phase was washed with water (100 mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to dryness and the product was crystallized from $CHCl_3$/MeOH (85:15)/petroleum ether (16.5 mg, 91% yield). mp >300 °C; ^{1}H NMR: δ −3.05 (s, 2H, NH), 3.63–4.01, 4.33–4.57 (2m, 7H, Gal-H and OH), 4.66–4.88 (m, 2H, Gal-H and OH), 5.02–5.06, 5.30–5.34 (2m, 1H, Gal-Hα and β), 6.37–6.38 and 6.72 (m and d, J = 6.7, 1H, Gal-

OHα and β), 8.23 (d, $J = 4.5$, 6H, 10,15,20-Ar-*o*-H), 8.31–8.43 (m, 4H, 5-Ar-*m*- and *o*-H), 8.87-8.89 (m, 8H, β-H), 9.02 (d, $J = 4.5$, 6H, 10, 15, 20-Ar-*m*-H); UV–vis [$CHCl_3$/MeOH (85:15)] λ_{max} ($\log\varepsilon$): 420 (5.47), 514 (4.14), 548 (3.63), 589 (3.63), 644 (3.16) nm; HRMS (FAB^+) *m*/*z*: calcd for $C_{48}H_{38}N_7O_7$ $(M+H)^+$: 824.2833; Found: 824.2813.

6.2.3. Methylation of porphyrins 1a and 1b. General procedure: To a stirred solution or suspension of porphyrin **1a** (or **1b**) in dry DMF (5 mL for 25 mg of porphyrin) was added a large excess of methyl iodide and the reaction mixture was heated at 40 °C in one flask equipped with a condenser. The reaction progress was monitored by TLC using acetic acid/methanol/water (5:2:1) as the eluent. When the reaction was complete (after ca. 3 h), diethyl ether was added to the reaction mixture and the Tris(*N*-methylpyridinium)porphyrin **3a** (or **3b**) precipitated. The solid was filtered and washed with diethyl ether. The compound was dissolved in acetone/water (1:1) and re-precipitated with acetone. The products were dried under reduced pressure. The synthesis of porphyrin **3** has already been described.[23]

6.2.4. 5-[4-(1,2:3,4-Di-*O*-isopropylidene-α-D-galactopyranosyl-6-oxycarbonyl)phenyl]-10,15,20-Tris(*N*-methylpyridinium-4-yl)porphyrin tri-iodide (3a). Compound **3a** (33.5 mg, 91% yield) was obtained from porphyrin **1a** (25.0 mg, 27.7 μmol) and methyl iodide (1 mL, 16.1 mmol). mp 272–274 °C; ^{1}H NMR: δ −3.05 (s, 2H, NH), 1.33, 1.38, 1.46, 1.54 [4s, 12H, $C(CH_3)_2$], 4.22–4.35 (m, 1H, Gal-H), 4.47–4.57 (m, 4H, Gal-H), 4.71 (s, 10H, $3\times$N–CH_3 and Gal-H), 5.58 (d, $J = 4.9$, 1H, Gal-Hα), 8.40 (d, $J = 8.3$, 2H, 5-Ar-*o*-H), 8.45 (d, $J = 8.3$, 2H, 5-Ar-*m*-H), 9.00–9.17 (m, 14H, 10,15,20-Ar-*o*- and β-H), 9.48 (d, $J = 4.6$, 6H, 10,15,20-Ar-*m*-H); ^{13}C NMR: δ 24.5, 25.0, 26.0, 26.1 [$4\times C(\underline{C}H_3)_2$], 47.96 ($3\times CH_3$), 64.3 (Gal-C6), 65.7 (Gal-C5), 69.8, 70.2 (Gal-C3, 4), 70.5 (Gal-C2), 95.8 (Gal-C1), 108.2, 108.9 [$2\times\underline{C}(CH_3)_2$], 114.9, 115.5, 121.4, 128.1, 129.6, 132.2, 134.7, 144.3, 145.4, 156.4, 156.5, 165.9 (CO); anal. calcd for $C_{57}H_{54}I_3N_7O_7.6H_2O$: C, 47.61; H, 4.63; N, 6.82; Found: C, 47.34; H, 4.12; N, 6.82; UV–vis (DMSO) λ_{max} ($\log\varepsilon$): 426 (5.58), 517 (4.38), 552 (3.93), 589 (3.94), 644 (3.47) nm; MS (FAB^+) *m*/*z*: 948 $(M-3I)^+$.

6.2.5. 5-[4-(α/β-D-Galactopyranosyl-6-oxycarbonyl)phenyl]-10,15,20-Tris(*N*-methylpyridinium-4-yl)porphyrin tri-iodide (3b). Compound **3b** (34.0 mg, 90% yield) was obtained from porphyrin **1b** (25.0 mg, 30.4 μmol) and methyl iodide (1 mL, 16.1 mmol). mp >300 °C; ^{1}H NMR: δ −3.05 (s, 2H, NH), 3.65–4.15, 4.31–4.63 (2m, 7H, Gal-H and OH), 4.72 (s, 9H, CH_3), 4.77–4.90 (m, 2H, Gal-H and OH), 5.02–5.07, 5.28–5.34 (2m, 1H, Gal-Hα and β), 6.33–6.38 and 6.69 (m and d, $J = 6.9$, 1H, Gal-OHα and β), 8.40 (d, $J = 8.2$, 2H, 5-Ar-*o*-H), 8.46 (d, $J = 8.2$, 2H, 5-Ar-*m*-H), 8.99–9.18 (m, 14H, 10,15,20-Ar-*o*- and β-H), 9.48 (d, $J = 6.6$, 6H, 10,15,20-Ar-*m*-H); anal. calcd for $C_{51}H_{46}I_3N_7O_7{\cdot}5H_2O$: C, 45.72; H, 4.21; N, 7.32; Found: C, 46.04; H, 4.24; N, 7.22; UV–vis [DMSO/H_2O (9:1)] λ_{max} ($\log\varepsilon$): 426 (5.43), 519 (4.24), 552 (3.80), 590 (3.80), 644 (3.33) nm; MS (FAB^+) *m*/*z*: 868 $(M-3I)^+$.

6.3. Photostability of the porphyrin derivatives

In a typical experiment, 2 mL of a solution of the porphyrin (1 μM) in PBS in a glass cuvette was irradiated with white light (50 mW/cm^2) at room temperature and under gentle magnetical stirring for different periods of time. The intensity of the Soret band was registered at intervals of 1 minute by UV–visible spectroscopy, and the photostability was expressed as I_t/I_0 (%) (I_t = intensity of the band at given time of irradiation, I_0 = intensity of the band before irradiation).

6.4. Singlet oxygen generation

A solution containing 1,3-diphenylisobenzofuran (75 μM) and photosensitizer (1 μM) in DMF/water (9:1) in a glass cuvette was irradiated at room temperature with white light filtered through a cut-off filter for wavelengths <540 nm, at a fluence rate of 50 mW/cm^2. The absorption decay of DPBF at 415 nm was measured at irradiation intervals of 1 min.

6.5. Photosensitizers

Stock solutions of photosensitizers were prepared at a concentration of 8 mg/ml in dimethylsulfoxide (DMSO) and diluted to the final concentration in Dulbecco's modified Eagle's medium (DMEM) or in phosphate-buffered saline (PBS).

6.6. Cells and virus

African green monkey kidney (Vero) cells were grown as monolayers in DMEM supplemented with 10% foetal calf serum (FCS) and gentamycin (50 μg/mL), both purchased from Gibco, Scotland, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. HSV-1, strain SC16 from ATCC (USA) adapted to grow in Vero cells, was produced as described elsewhere.[37]

6.7. Photocytotoxicity

Confluent monolayer Vero cells in 96-well plates (10^5 cells/well) were incubated for 10 min with different concentrations of photosensitizers (0.01–2.5 μM) diluted in DMEM-8% FCS. All samples were then irradiated with white light at a fluence rate of 50 mW/cm^2 for 15 min, and subsequently incubated for 48 h in the dark (37 °C, 5% CO_2). The viability of the cells was then determined by the colorimetric MTT assay.[36] The maximum non-cytotoxic studied concentration (MNCSC) is the highest concentration of the drug which gives identical results as the control. For each concentration the average cell viability was calculated from the data of three experiments with three replicates each and was expressed as a percentage compared to the untreated cells (100%). Data are reported as means of three independent assays, each run in triplicate.

6.8. Virucidal effect

HSV-1 (10^7 PFU in a 1 mL volume) was incubated with the compounds, at 0.02 μM in PBS, for 10 min at 37 °C

and then irradiated for 5 or 15 min with white light at a fluence rate of 50 mW/cm^2. The viral particles were concentrated by high speed centrifugation (44,000*g*) and the pellets were resuspended in PBS. This procedure also allows the removal of the drugs in the rejected supernatant. Controls were similarly processed. Virucidal effect was determined by plaque titration: confluent Vero cells in a 24-well plate were infected with 100 μL of serial dilutions of the viruses in PBS. After a 2-h adsorption at 37 °C the inoculum was removed, the cells were washed and covered with 2% of Sephadex G50 in DMEM with 2% FCS. On the 4th day post-infection, the cells were fixed with 10% formaldehyde and stained with a solution of 0.1% crystal violet. Viral plaques were counted under the microscope. The inhibitory activity of each compound was calculated in relation to the control. Data are reported as means of three independent assays, each run in triplicate.

6.9. Influence of the drug addition time on virus yield during infection

Confluent Vero cells in 35-mm diameter Petri dishes were cooled at 4 °C for 15 min and then infected with an ice-cold suspension of HSV-1 (10^7 PFU/mL) at a MOI of approximately 10 PFU/cell. The dishes were then gently shaken for 2 h at 4 °C. The free viruses were then removed and the cell monolayers were washed twice with cold PBS and new 2% FCS medium was added. At different post-adsorption times (0, 2, 4, and 16 h) the medium was removed and new medium containing compounds **1b** or **3b** at 0.02 μM was then added. The cells were kept under dark for 30 min at 37 °C and then they were irradiated or not (controls) with white light at a fluence rate of 50 mW/cm^2 for 15 min. The infected cultures (scrapped cells plus culture medium) were collected 24 h post-infection, subjected to two cycles of freeze and thawing, and then clarified by low speed centrifugation. The supernatants were subjected to a plaque reduction assay in order to determine the virus yields of the different cultures. Data are reported as means of two independent assays, each run in triplicate.

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