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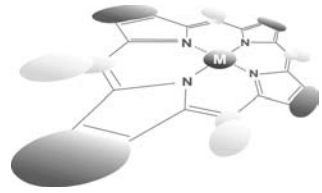

Synthetic porphyrins bearing β-propionate chains as photosensitizers for photodynamic therapy

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ABSTRACT: Porphyrins with different numbers of β-propionate chains mimicking natural porphyrins were prepared *via* the 2+2 MacDonald type approach. Photodynamic activity against WiDr colon adenocarcinoma cells showed that activity is related to the number of β-propionate chains, with the derivatives with two carboxylic groups showing higher activity.

KEYWORDS: photodynamic therapy, colon adenocarcinoma, β-propionate porphyrins, anti-cancer, WiDr cancer cells.

INTRODUCTION

Natural porphyrins, their chemically modified derivatives or purely synthetic structures are the source of photosensitizers for PDT [1]. Among the photosensitizers approved for use in PDT, some are based on naturally occurring porphyrins or their chemically modified derivatives: polyhematoporphyrin ether/ester, mono-*L*-aspartylchlorin e6, benzoporphyrin derivative monoacid ring A, Tookad® and Photofrin® [2–4]. Typically, they have free *meso*-positions and alkyl acid chains at the β-positions. Studies with synthetic porphyrin sensitizers have concentrated on the more easily available *meso*-aryl substituted β-free structures. Consistent results obtained with cell cultures, animals and membrane models present strong evidence that the extracellular-intracellular pH gradient in the tumor cells might play an important role in the accumulation and selectivity of sensitizers that possess carboxylic chains [5–7]. Hydrophilic and hydrophobic characteristics of the sensitizer affect the cellular uptake and localization inside cells and can play a decisive role on the results obtained in the photodynamic treatment [8]. We decided to make use of our synthetic capacities to prepare porphyrinic photosensitizers having β-propionic side-chains to exploit the effect of the presence of halogen on the photodynamic activity previously demonstrated for *meso*-tetraphenyl porphyrins [9]. In this work, we describe the preparation of porphyrins incorporating halogenated *meso*-phenyl groups and also propionic acid chains at β-positions, synthetic structures more similar to natural porphyrins than those previously studied. The photodynamic activity of these new porphyrins was tested against WiDr adenocarcinoma cells.

EXPERIMENTAL

General

Solvents were purified by standard methods before use. Dichloromethane was dried over CaH_2 and distilled. Chloroform was filtered through neutral active alumina. 9,10-dimethylanthracene (DMA) was obtained from Aldrich and used without further purification. Methylene Blue was used as received (Riedel-de Haën). Pyrrole was distilled before used. For column chromatography, silica gel 60–220 mesh/0.035–0.070 mm, from Fluka was used. Photofrin® was kindly offered by the Gastroenterology

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Service of the Hospitais da Universidade de Coimbra. Absorption spectra were recorded with a Shimadzu UV-2100 spectrophotometer. Fluorescence spectra were measured with a Spex Fluorolog 3 spectrophotometer. NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. Mass spectra of EI were obtained with GC-MSD HP 6890/5973 and Finnigan Advantage for the MS (ESI) spectra. High resolution mass spectra were recorded on a Bruker FTMS APEXIII instrument under electrospray ionization (ESI) (Vigo University).

Porphyrin synthesis

4-acetyl-5-oxo-hexanoate (1). Obtained as described [10] as a colorless oil (50.2 g, 55% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si; enol-ketone tautomers): δ_H, ppm ketone: 8.55 (1H, t, J = 6.9, COCH), 3.68 (3H, s, CO_2CH_3), 2.32 (2H, t, J = 7.3, $CH_2CH_2CO_2$), 2.21 (6H, s, 2 × CH_3), 2.13–2.15 (2H, m, $CH_2CH_2CO_2$), enol: 3.69 (3H, s, CO_2CH_3), 2.59–2.62 (2H, m, $CH_2CH_2CO_2$), 2.39–2.43 (2H, m, $CH_2CH_2CO_2$), 2.17 (6H, s, 2 × CH_3). GC-MS (EI): *m/z* 186, calcd. for $[M]^+$ 186.

Benzyl 4-(2-methoxycarbonylethyl)-3,5-dimethylpyrrole-2-carboxylate (3). Obtained as described [10] as colorless needles (44.2 g, 52% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 8.55 (1H, br s, NH), 7.32–7.46 (5H, m, Ph), 5.28 (2H, s, CH_2Ph), 3.66 (3H, s, CO_2CH_3), 2.70 (2H, t, J = 7.7, $CH_2CH_2CO_2$), 2.42 (2H, t, J = 7.7, $CH_2CH_2CO_2$), 2.29 (3H, s, CH_3), 2.20 (3H, s, CH_3).

Benzyl 5-acetoxymethyl-4-(2-methoxycarbonylethyl)-3-methylpyrrole-2-carboxylate (4). Obtained as described [11] (4.7 g, 88% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 9.07 (1H, br s, NH), 7.34–7.40 (5H, m, Ph), 5.30 (2H, s, CH_2Ph), 5.05 (2H, s, CH_2OAc), 3.65 (3H, s, CO_2CH_3), 2.78 (2H, t, J = 7.7, $CH_2CH_2CO_2$), 2.46 (2H, t, J = 7.7, $CH_2CH_2CO_2$), 2.29 (3H, s, CH_3), 2.06 (3H, s, $COCH_3$).

Dibenzyl 3,7-bis(2-methoxycarbonylethyl)-2,8-dimethyldipyrrylmethane-1,9-dicarboxylate (5). Obtained as described [12] as a solid (1.82 g, 74% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 9.05 (2H, br s, 2 × NH), 7.30–7.39 (10H, m, Ph), 5.25 (4H, s, CH_2Ph), 3.97 (2H, s,), 3.57 (6H, s, 2 × CO_2CH_3), 2.76 (4H, t, J = 6.9, 2 × $CH_2CH_2CO_2$), 2.51 (4H, t, J = 6.9, 2 × $CH_2CH_2CO_2$), 2.28 (6H, s, 2 × CH_3).

3,7-bis(2-methoxycarbonylethyl)-2,8-dimethyldipyrrylmethane-1,9-dicarboxylic acid (6) [12]**.** Obtained from hydrogenolysis of the dipyrrylmethane **5** [12] (1.12 g, 87% yield). ^{1}H NMR (400 MHz; $CDCl_3$/$(CD_3)_2SO$; Me_4Si): δ_H, ppm 10.49 (2H, br s, 2 × NH), 3.87 (2H, s,), 3.67 (6H, s, 2 × CO_2CH_3), 2.76 (4H, t, J = 7.7, 2 × $CH_2CH_2CO_2$), 2.40 (4H, t, J = 7.7, 2 × $CH_2CH_2CO_2$), 2.25 (6H, s, 2 × CH_3).

5-(4-bromophenyl)dipyrrylmethane (7a) [13]**.** A mixture of 4-bromobenzaldehyde (1.0 g, 5.40 mmol) and pyrrole (10 mL, 0.15 mol) was bubbled with dry N_2 for 15 min. Trifluoroacetic acid (50 µl, 0.65 mmol) was added and the mixture was stirred under N_2 for 15 min at room temperature. A NaOH solution (25 mL, 0.1 M) was carefully added and the mixture was extracted two times with ethyl acetate. The extracts were washed with water, dried over anhydrous sodium sulfate, and the solvent evaporated. The excess of pyrrole was removed under reduced pressure with slight heating to yield a dark oily residue. This was purified by column chromatography (silica gel; eluent: CH_2Cl_2) to give, after evaporation of the solvent *in vacuo*, the desired product as a light brown oil that solidified upon standing (1.19 g, 73% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 7.89 (2H, br s, 2 × NH), 7.42 (2H, d, J = 8.2, PhBr), 7.07 (2H, d, J = 8.2, PhBr), 6.68 (2H, s), 6.15 (2H, d, J = 2.8), 5.88 (2H, s), 5.41 (1H, s).

5-(2-bromophenyl)dipyrrylmethane (7b). The dipyrrylmethane **7b** was prepared according to the procedure described above for **7a** using 2-bromobenzaldehyde (94% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 7.97 (2H, br s, 2 × NH), 7.56 (1H, d, J = 7.6, PhBr), 7.23 (1H, d, J = 7.2, PhBr), 7.09–7.12 (2H, m, PhBr), 6.70 (2H, s), 6.15 (2H, d, J = 2.8), 5.89 (2H, s), 5.88 (1H, s).

5-phenyldipyrrylmethane (7c). The dipyrrylmethane **7c** was prepared according to the procedure described above for **7a** using benzaldehyde (54% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 7.88 (2H, br s, 2 × NH), 7.17–7.32 (5H, m, Ph), 6.67 (2H, s), 6.14 (2H, d, J = 6.1), 5.90 (2H, s), 5.45 (1H, s).

5-(4-bromophenyl)-1,9-diformyldipyrrylmethane (8a) [14]**.** The Vilsmeier reagent used in the formylation was prepared by adding $POCl_3$ (3.0 mL, 32 mmol) dropwise under N_2 to *N,N*-dimethylformamide (20 mL) at 0 °C. Dipyrrylmethane **7a** (1.35 g, 4.5 mmol) was dissolved in *N,N*-dimethylformamide (15 mL) and the solution was cooled to 0 °C. To this stirred solution was added the Vilsmeier reagent dropwise (7 mL, 2.4 equiv.) and the mixture was stirred for 1.5 h at 0 °C under N_2. Saturated aqueous sodium acetate (50 mL) was carefully added and the mixture was stirred for a further 4 h at room temperature. The solution was extracted three times with ethyl acetate and the extracts were washed with brine and water, dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo* to yield a brown oil. This was purified by column chromatography (silica gel; eluent: CH_2Cl_2/10% ethyl acetate) to give, after evaporation of the solvent *in vacuo*, the desired product as a brown solid (0.60 g, 47% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 10.20 (2H, br s, 2 × NH), 9.27 (2H, s, 2 × CHO), 7.48 (2H, d, J = 8.3, PhBr), 7.14 (2H, d, J = 8.3, PhBr), 6.88 (2H, s), 6.05 (2H, s), 5.52 (1H, s); GC-MS (EI): *m/z* 358. Calcd. for $[M^+]$ 358.

5-(2-bromophenyl)-1,9-diformyldipyrrylmethane (8b). The dipyrrylmethane **8b** was prepared according to the procedure described above for **8a** (18% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 9.82 (2H,

br s, 2 × NH), 9.30 (2H, s, 2 × CHO), 7.62 (1H, d, *J* = 7.9, PhBr), 7.28–7.32 (1H, m, PhBr), 7.17–7.21 (2H, m, PhBr), 6.88 (2H, s), 6.05 (1H, s) 6.04 (2H, s). GC-MS (EI): *m/z* 356, calcd. for $[M]^+$ 356.

1,9-diformyl-5-phenyldipyrrylmethane (8c). The dipyrrylmethane **8c** was prepared according to the procedure described above for **8a** (46% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 10.07 (2H, br s, 2 × NH), 9.25 (2H, s, 2 × CHO), 7.23–7.66 (5H, m, Ph), 6.87 (2H, s), 6.08 (2H, s), 5.57 (1H, s). GC-MS (EI): *m/z* 278, calcd. for $[M]^+$ 278.

5,15-di(4-bromophenyl)-3,7,13,17-tetra(2-methoxycarbonylethyl)-2,8,12,18-tetramethylporphyrin (9) [15]**.** A mixture of 4-bromobenzaldehyde (0. 320 g, 1.7 mmol) and dipyrrylmethane dicarboxylic acid **6** (0.500 g, 1.2 mmol) in dichloromethane (90 mL) was bubbled with dry N_2 for 15 min. A solution of *p*-toluenesulfonic acid (1 g) in methanol (17 mL) was added to the mixture and stirred under N_2 for 24 h at room temperature. A solution of zinc acetate (1 g) in methanol (15 mL) was added to the mixture, bubbled with air and stirred for a further 24 h under air at room temperature. The solution was washed with water and saturated aqueous sodium bicarbonate, dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo*. The residue was chromatographed over silica gel (eluent: dichloromethane/10% ethyl acetate). To the fractions with the zinc porphyrinate dissolved in dichloromethane (30 mL) was added trifluoroacetic acid (1 mL) and the mixture stirred for 30 min. The solution was washed with water and saturated aqueous sodium bicarbonate, dried over anhydrous sodium sulfate and the solvent evaporated. The resulting Zn-free porphyrin was chromatographed over a short alumina column eluted with dichloromethane and the solvent evaporated. The porphyrin was recrystallized from dichloromethane/methanol, isolated and dried *in vacuo* (0.24 g, 40% yield.). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 10.29 (2H, s, *meso*), 7.94 (4H, d, *J* = 8.5, PhBr), 7.90 (4H, d, *J* = 8.0, PhBr), 4.36 (8H, t, *J* = 7.8, 4 × $CH_2CH_2CO_2$), 3.66 (12H, s, 4 × CO_2CH_3), 3.16 (8H, t, *J* = 7.8, 4 × $CH_2CH_2CO_2$), 2.54 (12H, s, 4 × CH_3), -2.47 (2H, br s, 2 × NH). MS (ESI): *m/z* 1021.27 $[M + H]^+$. Anal. calcd. for $C_{52}H_{52}Br_2N_4O_8 \cdot 6H_2O$: C, 55.32; H, 5.71; N, 4.96. Found: C, 54.75; H, 4.07; N, 3.91. UV-vis (CH_2Cl_2): λ_{max}, nm (ε %) 408 (100), 505 (15), 538 (8), 573 (8), 624 (5).

5-(4-bromophenyl)-13,17-bis(2-methoxycarbonylethyl)-12,18-dimethyl-porphyrin (11a). Porphyrin **11a** was prepared by reacting dipyrrylmethane dicarboxylic acid **6** (1.1 mmol) with diformyldipyrrylmethane **8a** (1.3 mmol) by following the procedure used for porphyrin **9** (0.081 g, 11% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 10.66 (1H, s, *meso*), 10.59 (2H, s, *meso*), 9.33 (2H, d, *J* = 4.7, *beta*), 8.86 (2H, d, *J* = 4.7, *beta*), 8.33 (2H, d, *J* = 7.3, PhBr), 8.12 (2H, d, *J* = 8.2, PhBr), 4.41 (4H, t, *J* = 7.4, 2 × $CH_2CH_2CO_2$), 3.65 (6H, s, 2 × CO_2CH_3), 3.58 (6H, s, 2 × CH_3), 3.20 (4H, t, *J* = 7.4, 2 × $CH_2CH_2CO_2$), -3.40 (2H, br s, 2 × NH). MS (ESI): *m/z* 667.27 $[M + H]^+$. Anal. calcd. for $C_{36}H_{33}BrN_4O_4 \cdot 1/2H_2O$: C, 64.10; H, 5.08; N, 8.31. Found C, 63.89; H, 4.98; N, 8.35. UV-vis (CH_2Cl_2): λ_{max}, nm (ε %) 404 (100), 499 (10), 530 (4), 569 (5), 622 (2).

5-(2-bromophenyl)-13,17-bis(2-methoxycarbonylethyl)-12,18-dimethyl-porphyrin (11b). Porphyrin **11b** was prepared by reacting dipyrrylmethane dicarboxylic acid **6** (1.1 mmol) with diformyldipyrrylmethane **8b** (1.3 mmol) by following the procedure used for porphyrin **9** (0.044 g, 6% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 10.18 (2H, s, *meso*), 10.09 (1H, s, *meso*), 9.32 (2H, d, *J* = 4.6, *beta*), 8.85 (2H, d, *J* = 4.6, *beta*), 8.19 (1H, dd, J_3 = 6.9, J_4 = 2.2, PhBr), 8.04 (1H, dd, J_3 = 7.3, J_4 = 1.8, PhBr), 7.66–7.75 (2H, m, PhBr), 4.43 (4H, t, *J* = 7.6, 2 × $CH_2CH_2CO_2$), 3.65 (12H, s, 4 × CH_3), 3.31 (4H, t, *J* = 7.7, 2 × $CH_2CH_2CO_2$), -3.40 (2H, br s, 2 × NH). MS (ESI): *m/z* 667.33 $[M + H]^+$. Anal. calcd. for $C_{36}H_{33}BrN_4O_4 \cdot 3H_2O$: C, 60.09; H, 5.46; N, 7.79. Found C, 60.79; H, 5.01; N, 7.31. UV-vis (CH_2Cl_2): λ_{max}, nm (ε %) 403 (100), 499 (9), 533 (5), 569 (6), 622 (4).

13,17-bis(2-methoxycarbonylethyl)-12,18-dimethyl-5-phenylporphyrin (11c). Porphyrin **11c** was prepared by reacting dipyrrylmethane dicarboxylic acid **6** (1.1 mmol) with diformyldipyrrylmethane **8c** (1.3 mmol) by following the procedure used for porphyrin **9** (0.018 g, 28% yield). ^{1}H NMR (400 MHz; $CDCl_3$; Me_4Si): δ_H, ppm 10.18 (2H, s, *meso*), 10.05 (1H, s, *meso*), 9.32 (2H, d, *J* = 4.6, *beta*), 9.02 (2H, d, *J* = 4.6, *beta*), 8.24–8.27 (2H, m, Ph), 7.78–7.80 (3H, m, Ph), 4.42 (4H, t, *J* = 7.7, 2 × $CH_2CH_2CO_2$), 3.66 (6H, s, 2 × CO_2CH_3), 3.65 (6H, s, 2 × CH_3), 3.31 (4H, t, *J* = 7.7, 2 × $CH_2CH_2CO_2$), -3.39 (2H, br s, NH). MS (ESI): *m/z* 587.40 $[M + H]^+$. Anal. calcd. for $C_{36}H_{34}N_4O_4$: C, 73.70; H, 5.84; N, 9.55. Found C, 73.23; H, 5.87; N 9.53. UV-vis (CH_2Cl_2): λ_{max}, nm (ε %) 401 (100), 498 (10), 530 (4), 569 (5), 621 (2).

5,15-di(4-bromophenyl)-3,7,13,17-tetra(2-hydroxycarbonylethyl)-2,8,12,18-tetramethylporphyrin (10). To porphyrin **9** (50 mg) dissolved in tetrahydrofuran (30 mL) a solution of potassium hydroxide (250 mg) in water (1 mL) was added and the mixture was stirred overnight under N_2 at room temperature. The potassium salt of the porphyrin precipitated, was filtered off, washed with tetrahydrofuran and dissolved in water. The carboxylic acid porphyrin was precipitated by neutralization of the aqueous solution with acetic acid. The porphyrin was collected by filtration, washed well with water and dried *in vacuo* (0.036 g, 76% yield). ^{1}H NMR (400 MHz; $CDCl_3$/TFA; Me_4Si): δ_H, ppm 10.55 (2H, s, *meso*), 8.07 (8H, mL, Ph), 4.06 (8H, t, *J* = 6.2, 4 × $CH_2CH_2CO_2$), 2.90 (8H, t, *J* = 6.2, 4 × $CH_2CH_2CO_2$), 2.31 (12H, s, 4 × Me(β)), -2.13 (4H, s, 2 × NH). MS (ESI): *m/z* 965.27 $[M + H]^+$. HRMS (FAB): *m/z* calcd. for $C_{48}H_{45}Br_2N_4O_8$ $[M + H]^+$, 963.15987; found 963.16013; Δ = -0.26 mmu $[M + H]^+$.

5-(4-bromophenyl)-13,17-bis(2-hydroxycarbonylethyl)-12,18-dimethyl-porphyrin (12a). Porphyrin **12a**

was prepared by hydrolysis of porphyrin **11a** using the procedure followed for porphyrin **10** (90% yield). ^{1}H NMR (400 MHz; $CDCl_3$/TFA; Me_4Si): δ_H, ppm 10.80 (1H, s, *meso*), 10.75 (2H, s, *meso*), 9.46 (2H, d, *J* = 4.2, *beta*), 8.98 (2H, d, *J* = 4.2, *beta*), 8.34 (2H, d, *J* = 7.8, PhBr), 8.19 (2H, d, *J* = 7.7, PhBr), 4.46 (4H, t, *J* = 5.8, 2 × $CH_2CH_2CO_2$), 3.69 (6H, s, 2 × CH_3), 3.27 (4H, t, *J* = 5.8, 2 × $CH_2CH_2CO_2$). MS (ESI): *m/z* 639.33 $[M + H]^+$. HRMS (FAB): *m/z* calcd. for $C_{34}H_{30}BrN_4O_4$ $[M + H]^+$, 637.14449; found 637.14530; Δ = -0.81 mmu $[M + H]^+$.

5-(2-bromophenyl)-13,17-bis(2-hydroxycarbonylethyl)-12,18-dimethyl-porphyrin (12b). Porphyrin **12b** was prepared by hydrolysis of porphyrin **11b** using the procedure followed for porphyrin **10** (87% yield). ^{1}H NMR (400 MHz; $CDCl_3$/TFA; Me_4Si): δ_H, ppm 10.86 (1H, s, *meso*), 10.71 (2H, s, *meso*), 9.49 (2H, d, *J* = 4.7, *beta*), 8.96 (2H, d, *J* = 4.7, *beta*), 8.19–8.24 (2H, m, Ph), 7.89–7.93 (2H, m, Ph), 4.49 (4H, t, *J* = 6.7, 2 × $CH_2CH_2CO_2$), 3.68 (6H, s, 2 × CH_3), 3.22 (4H, t, *J* = 6.7, 2 × $CH_2CH_2CO_2$). MS (ESI): *m/z* 639.33 $[M + H]^+$. HRMS (FAB): *m/z* calcd. for $C_{34}H_{30}BrN_4O_4$ $[M + H]^+$, 637.14449; found 637.14274; Δ = +1.75 mmu $[M + H]^+$.

13,17-bis(2-hydroxycarbonylethyl)-12,18-dimethyl-5-phenylporphyrin (12c). Porphyrin **12c** was prepared by hydrolysis of porphyrin **11c** using the procedure followed for porphyrin **10** (73% yield). ^{1}H NMR (400 MHz; $CDCl_3$/TFA; Me_4Si): δ_H, ppm 10.90 (1H, s, *meso*), 10.71 (2H, s, *meso*), 9.42 (2H, d, *J* = 4.6, *beta*), 8.98 (2H, d, *J* = 4.6, *beta*), 8.47 (2H, s, Ph), 8.03 (3H, s, Ph), 4.43 (4H, t, *J* = 5.8, 2 × $CH_2CH_2CO_2$), 3.68 (6H, s, 2 × CH_3), 3.33 (4H, t, *J* = 5.8, 2 × $CH_2CH_2CO_2$), -2.00 (2H, br s, NH). MS (ESI): *m/z* 559.53 $[M + H]^+$. UV-vis (CH_3OH): λ_{max}, nm (ε %) 398 (100), 497 (12), 527 (6), 567 (7), 618 (4). HRMS (FAB): *m/z* calcd. for $C_{34}H_{31}N_4O_4$ $[M + H]^+$, 559.23398; found 559.23219; Δ = +1.79 mmu $[M + H]^+$.

Cell culture conditions

The human colon carcinoma cell line WiDR was purchased from American Type Culture Collection. The cell lines were cultured with Dulbecco's Modified Eagle medium (Sigma-Aldrich, Inc; Sigma D-5648) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Invitrogen Life Technologies; Gibco 2010-04), 1% Penicillin-Streptomycin (Gibco Invitrogen Life Technologies; 100 U/mL penicillin and 10 μg/mL streptomycin – Gibco 15140-122) and 100 μM Sodium Pirutvate (Gibco Invitrogen Life Technologies; Gibco 1360) at 37 °C, in a humidified incubator with 95% air and 5% CO_2.

Photodynamic treatment

For each experiment, cells were plated in 48 multiwells (Corning Costar Corp), in a concentration of 40,000 cells/mL and kept in the incubator overnight, in order to allow the attachment of the cells. The formulation of these sensitizers consisted of a 1 mg/mL solution in a ternary mixture of H_2O:PEG_{400}:EtOH (50/30/20, v/v/v), the desired concentrations being achieved by successive dilutions. The sensitizers were administered in several concentrations and cells were incubated for 24 hours. Cells were washed with PBS and new drug-free medium was added. Each plate was irradiated with a fluence rate of 7.5 mW/cm^2 until a total of 10 J was reached. Cell viability was measured 24 hours after the photodynamic treatment. Dark controls were made as described above except that no irradiation were made.

Measurement of cell viability

The sensitivity of the cell lines to the sensitizers was analyzed using the MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, colorimetric assay (Sigma-Aldrich, Inc; Sigma M2128) to measure cell proliferation. Cytotoxicity was expressed as the percentage of inhibition of cell proliferation correlated with irradiated cells with only the ternary solvent mixture. This allows the calculation of the concentration that inhibits the culture cell proliferation in 50% (IC_{50}). Each experiment was performed in duplicate or triplicate and repeated in three sets of tests.

Cellular uptake

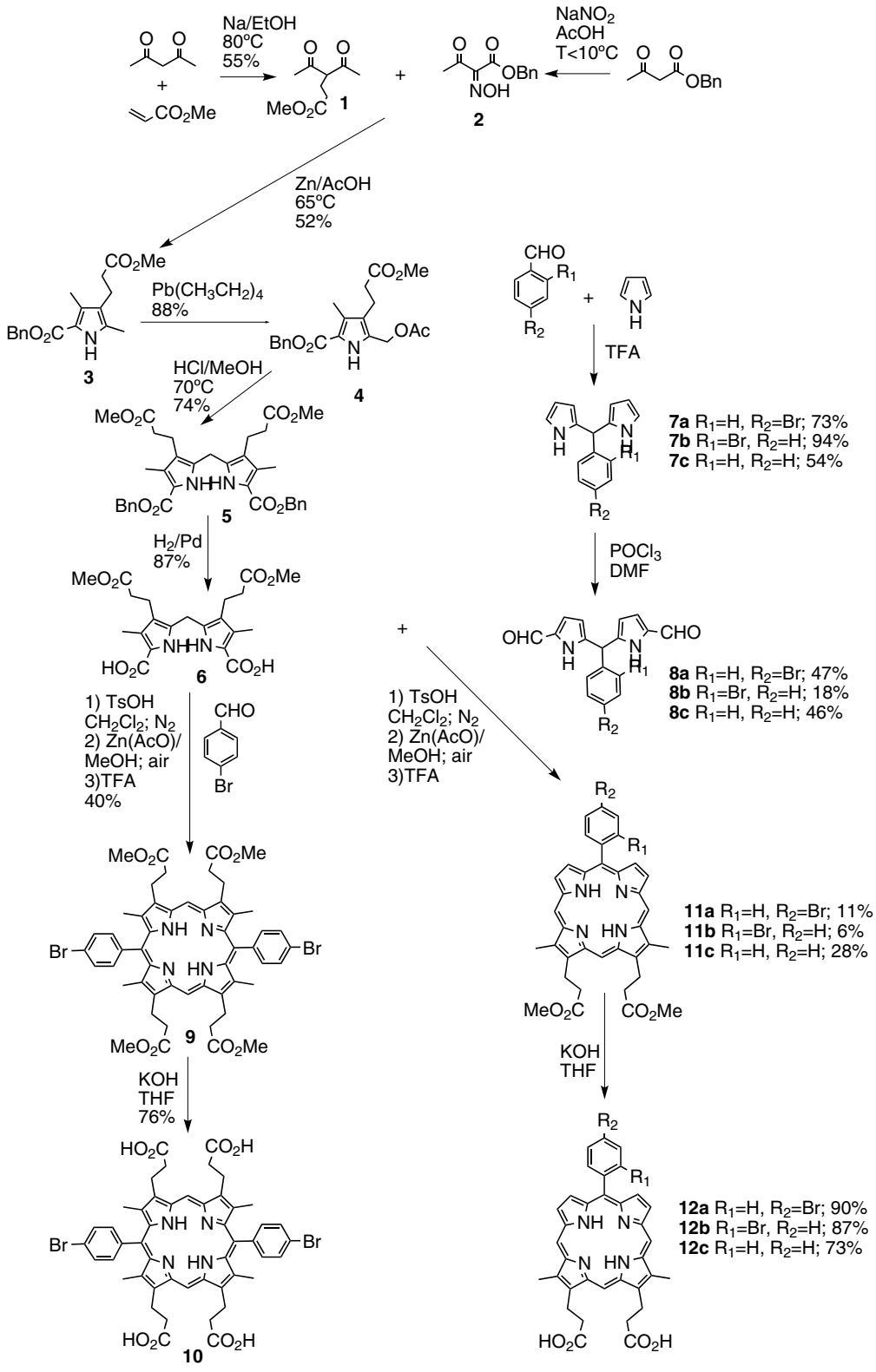
Cells, 1 × 10^5, were incubated with solutions of the sensitizer of 5 μM, 1 μM and 0.5 μM concentrations for 24 hours, released by trypsinization and washed with cold PBS. To ensure full disaggregation and cell rupture, 5 mL of methanol was added and the resulting suspension left in the dark for 24 hours. The fluorescence intensity of the supernatant was determined by fluorescence spectroscopy with a SPEX fluoromax 322-2 spectrophotometer using 410 nm excitation wavelength. The intracellular concentration was determined using a calibration curve obtained from the fluorescence intensity in methanol solutions for each sensitizer.

RESULTS AND DISCUSSION

Synthesis of porphyrins

The synthesis of porphyrins having four β-propionic acid chains **10** and two β-propionic acid chains **12a–c** is outlined in Scheme 1.

The synthesis starts with the preparation of the propionate substituted pyrrolic precursors. For the preparation of the methyl 4-acetyl-5-oxohexanoate (**1**) we used a Michael addition of acetylacetone to methyl acrylate, catalyzed by sodium ethoxide [10]. The oxime **2** was prepared from the addition of a solution of sodium nitrite to benzyl acetoacetate. The tetrasubstituted pyrrole **3** was synthesized from the reaction of dione **1** with benzyl oxyiminoacetate (**2**) in the presence of zinc dust and sodium acetate. Reduction of the compound **2** to the



Scheme 1. Synthetic routes to porphyrins **10** and **12a–c**

respective amine, followed by the condensation *in situ* with **1** gave the intended pyrrole **3** in 52% [10]. Pyrrole **3** was α-acetoxylated with lead tetraacetate originating **4** [11] and underwent a self-condensation process in acid medium to give the dipyrromethane **5** in 77% yield [12]. Hydrogenolysis of the benzyl group with 10% palladium on charcoal at room temperature and 1 atm of hydrogen originated the diacid dipyrrylmethane **6**. Condensation of the dipyrrylmethane **6** with 4-bromobenzaldehyde in the presence of *p*-toluenesulfonic acid using zinc acetate

template originated the tetrasubstituted propionate porphyrin which was demetalated with TFA and hydrolyzed to give the tetraacid derivative **10** [15, 16].

For the synthesis of porphyrins **12a–c** a different approach was required. Substituted dipyrrylmethanes **7a–c** were obtained by reaction of the substituted benzaldehyde with an excess of pyrrole and a catalytic amount of trifluoroacetic acid [13]. These dipyrrylmethanes were diformylated to dipyrrylmethanes **8a–c** by treatment with Vilsmeyer reagent ($POCl_3$/dimethylformamide) [14]. Condensation of the dipyrrylmethane **6** with dipyrrylmethanes **8a–c** catalyzed by *p*-toluenesulfonic acid in the presence of zinc acetate [15] gaves, after demetalation, porphyrins **11a–c**. In order to increase water solubility of the products for the photodynamic studies, the ester function was hydrolysed with KOH to the carboxyl derivatives porphyrins **12(a,b,c)**.

A complete spectroscopic characterization of sensitizers **10** and **12a–c** by UV-vis spectroscopy and fluorescence was carried out (Table 1).

All sensitizers present absorption spectra of the *phyllo* type [17]. The absorption coefficients were calculated using the Beer-Lambert law from methanolic solutions with concentrations between 10^{-5}-10^{-7} M. No aggregation was observed under these conditions. The Q(0,0) band around 625 nm (into the therapeutic window) has characteristic absorption coefficients *ca.* 10^3 $M^{-1}.cm^{-1}$, values very similar to Photofrin® (λ = 630 nm, ε = 1170 $M^{-1}.cm^{-1}$) [2]. The fluorescence spectra of the new sensitizers are very similar to the Photofrin spectrum [18], showing two bands of similar relative intensity at *ca.* 625 and 700 nm corresponding to Q(0-0) and Q(0-1) emissions respectively. The last fluorescence band is much more intense than the corresponding band in Photofrin® or *meso*-phenylporphyrins, indicating a high contribution of the emission to the first vibrational level. The fluorescence excitation spectra at the maxima of the fluorescence emission bands fit well with the absorption spectra of each sensitizer. In Fig. 1, the normalized spectra of absorption, fluorescence and fluorescence excitation of porphyrin **12c** are shown.

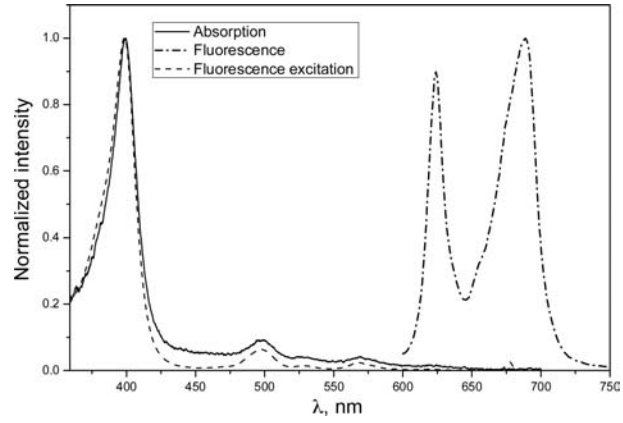


Fig. 1. Absorption, fluorescence and fluorescence excitation normalized spectra of sensitizer **12c**

Evaluation of the photodynamic activity of porphyrins

The activity of the synthesized porphyrins as photosensitizers in photodynamic therapy was evaluated against WiDr colon adenocarcinoma cells. For the photodynamic experiments, cancer cells were plated in 48 multiwell plates (Corning Costar Corp), in a concentration of 40,000 cells/mL (well) and kept in the incubator overnight, in order to allow the attachment of the cells. The sensitizers **10**, **12a–c** were administered to cells dissolved in a ternary mixture of H_2O/PEG_{400}/ethanol (50:30:20, v/v/v) in several concentrations and incubated for 24 h. In the control experiments cells were incubated only with the ternary solvent mixture. Subsequently, cells were washed with PBS and new drug-free medium was added. Each plate was then irradiated with a fluence rate of 7.5 mW/cm^2 until a total of 10 J of energy was reached. Cell viability was evaluated after 24 h using the MTT test. Figure 2 shows the concentration-dependent cell survival curves for **10**, **12a–c** and for Photofrin® as reference compound.

The photodynamic activity of this series of porphyrins is relatively lower than that observed for our tetrahydroxyphenyl porphyrins for the same cancer cell type under the same conditions [9]. Clearly, from Fig. 2, the

Table 1. Absorption and fluorescence data of porphyrins **10** and **12a–c** in methanolic solutions

Compound	Absorption λ_{max}, nm (ε, $M^{-1}.cm^{-1}$)					Fluorescence λ_{max}, nm	
	B(0-0)	Qy(1-0)	Qx(0-0)	Qy(0-0)	Qx(1-0)	Q(0-0)	Q(0-1)
10	406.5	507.4	541.0	575.0	629.0	630	694
	2.85×10^5	2.16×10^4	8.76×10^3	1.05×10^4	3.57×10^3		
12a	399.0	498.8	529.0	568.0	627.2	627	688
	1.68×10^5	1.05×10^4	7.80×10^3	8.40×10^3	1.26×10^3		
12b	400.5	497.3	535.6	569.8	623.2	627	691
	8.23×10^4	5.37×10^3	3.33×10^3	2.71×10^3	8.27×10^2		
12c	399.2	498.4	528.8	620.5	627.2	627	688
	9.50×10^4	7.19×10^3	2.58×10^3	3.57×10^3	1.26×10^3		

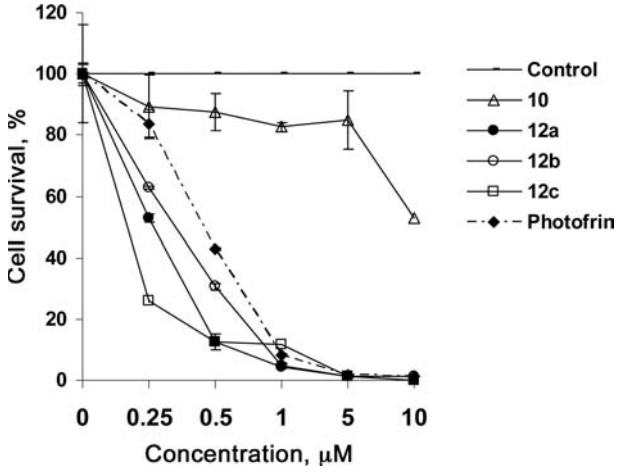


Fig. 2. Cell viability (%) for WiDr human colon adenocarcinoma cells using photosensitizers **10**, **12a–c** and Photofrin®

porphyrins with two propionate groups are more active than the porphyrin with four propionate groups (**10**). Photofrin®, which is a mixture of oligomers of hematoporphyrin (two propionate chains) appears to behave like monomeric porphyrins with two propionate groups. Sensitizers **12a–c** and Photofrin® present high photodynamic activity for concentrations above 1 μM, with cellular viabilities below 15%; dipropionate porphyrins present higher activity than Photofrin®. The photodynamic activity order is **12c** > **12a** > **12b** > Photofrin® > **10** with IC_{50} (sensitizer dose (μM) necessary to observe a 50% cellular viability) of 0.14, 0.27, 0.34 and 0.46, respectively. Among dipropionate porphyrins, non-halogenated porphyrin **12c** is the most active sensitizer.

Activity of these compounds is related to the ability to enter the cells and the presence of carboxylic acid groups may have a decisive role [19]. Cellular uptake was carried out for porphyrins **10** with four carboxylic groups and **12c** with two carboxylic groups. For the cellular uptake measurements, 1×10^5 cells were incubated with solutions of the sensitizer of 5 μM concentration for 24 hours, released by trypsinization and washed with cold PBS. To ensure full disaggregation and cell rupture, 5 mL of methanol were added. The intracellular concentration was determined from the fluorescence intensity ($\lambda_{ex} = \lambda_{max}$ of the Soret band *ca.* 400 nm) directly measured from these solutions using a calibration curve obtained from the fluorescence intensity in methanol for concentrations between 10^{-6} and 10^{-8} M. The results showed that the cellular uptake of sensitizer **10** (0.06 ± 0.02 μM) is three times lower than the cellular uptake of sensitizer **12c** (0.18 ± 0.02 μM). Also, a report with *meso*-phenylcarboxyl groups showed the same behavior [19]. The higher hydrophilicity (logP = -1.9) of porphyrin **10** relative to the value obtained for **12c** (logP = 1.8) can explain the more difficult penetration in cells and the less favorable results for porphyrin **10**. The photodynamic activity of photosensitizers of different structures has been related to the amphilicity of the molecule, which is a balance between hydrophilic and hydrophobic regions. The porphyrin with two propionate chains may correspond to the best balance of these two factors and can also have a better distribution inside cells for photodynamic action [8, 20].

In summary, a series of porphyrins with propionate chains at the β-positions have been synthesized and their *in vitro* photocytotoxicity evaluated on human colon tumor WiDr cells. Photosensitizer **10** with four propionate chains showed lower cellular uptake and much lower activity than photosensitizer **12c** with only two propionic chains. Photodynamic activity of these sensitizers depends on the number of propionic acid chains and the kind of substitution. Brominated porphyrins **12a** and **12b** and Photofrin® demonstrated similar photocytotoxicities. Non-halogenated porphyrin **12c** showed the highest photocytotoxicity on cells.

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