Synthesis and Biological In Vitro Evaluation of Novel PEG−Psoralen Conjugates

F. Bettio,[†] M. Canevari,[†] C. Marzano,[†] F. Bordin,[†] A. Guiotto,[†] F. Greco,[‡,§] R. Duncan,[‡] and F. M. Veronese*,[†]

Department of Pharmaceutical Sciences, University of Padua, Via Francesco Marzolo 5, 35131 Padova, Italy, and Centre for Polymer Therapeutics, Welsh School of Pharmacy, Cardiff University, King Edward VII Ave., Redwood Building, CF10 3XF Cardiff, UK

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Psoralens are well-known photosensitizers, and 8-methoxypsoralen and 4,5′,8-trimethylpsoralen are widely used in photomedicine as "psoralens plus UVA therapy" (PUVA), in photopheresis, and in sterilization of blood preparations. In an attempt to improve the therapeutic efficiency of PUVA therapy and photopheresis, four poly(ethylene glycol) (PEG)−psoralen conjugates were synthesized to promote tumor targeting by the enhanced permeability and retention (EPR) effect. Peptide linkers were used to exploit specific enzymatic cleavage by lysosomal proteases. A new psoralen, 4-hydroxymethyl-4′,8-dimethylpsoralen (**6**), suitable for polymer conjugation was synthesized. The hydroxy group allowed exploring different strategies for PEG conjugation, and linkages with different stability such ester or urethanes were obtained. PEG (5 kDa) was covalently conjugated to the new psoralen derivative using four different linkages, namely, (i) direct ester bond (**7**), (ii) ester linkage with a peptide spacer (**8**), (iii) a carbamic linker (**9**), and (iv) a carbamic linker with a peptide spacer (**12**). The stability of these new conjugates was assessed at different pHs, in plasma and following incubation with cathepsin B. Conjugates **7** and **8** were rapidly hydrolyzed in plasma, while **9** was stable in buffer and in the presence of cathepsin B. As expected, only the conjugates containing the peptide linker released the drug in presence of cathepsin B. In vitro evaluation of the cytotoxic activity in the presence and absence of light was carried out in two cell lines (MCF-7 and A375 cells). Conjugates **7** and **8** displayed a similar activity to the free drug (probably due to the low stability of the ester linkage). Interestingly, the conjugates containing the carbamate linkage (**9** and **12**) were completely inactive in the dark (IC_{50} > 100 μM in both cell lines). However, antiproliferative activity become apparent after UV irradiation. Conjugate **12** appears to be the most promising for future in vivo evaluation, since it was relatively stable in plasma, which should allow tumor targeting and drug release to occur by cathepsin B-mediated hydrolysis.

Introduction

Psoralens are well-known photosensitizing drugs. 8-Methoxypsoralen (8-MOP) and 4,5′,8-trimethylpsoralen (4,5′,8-TMP) are currently used in PUVA (psoralens plus UVA) therapy to treat several skin diseases (Figure 1),[1] in photopheresis to treat various autoimmune disorders, and to prevent rejection in organ transplantation.[2] Recently, furocumarins have been also proposed as sterilizing agents for blood preparations.[3]

Photochemotherapy represents one of the usual treatments of cutaneous T-cell lymphoma (CTCL), which mainly affects the skin. PUVA therapy is mediated by the ability of such derivatives to react, in the presence of light, with DNA, leading to the formation of both mono- and bifunctional covalent adducts with pyrimidine bases.[4,5] This effect, however, lacks tissue specificity. In an attempt to improve the therapeutic index, we decided to design polymer−psoralens to increase the drug concentration inside the tumor cells. On the basis of our previous experience,[6] the drug was conjugated with poly(ethylene glycol) (PEG) by means of a linkage designed to be selectively cleaved by lysosomal enzymes.

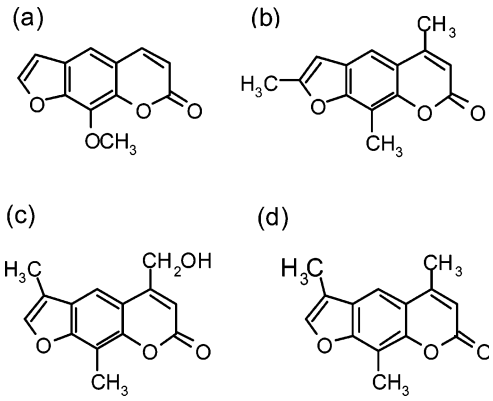

Figure 1. Psoralen structures: (a) 8-methoxypsoralen (8-MOP), (b) 4,5′,8-trimethylpsoralen (4,5′,8-TMP), (c) 4-hydroxymethyl-4′,8-dimethylpsoralen (4-OHMeP), and (d) 4,4′,8-trimethylpsoralen (4,4′,8-TMP).

Several studies have shown that the covalent conjugation of a low molecular weight anticancer agent to a water-soluble, biocompatible polymer alters the drug biodistribution, thus improving its pharmacokinetic and pharmacodynamic properties.[7−10] This leads to a decreased toxicity and also, for poorly water-soluble compounds, to increased solubility. Due to the hyperpermeabilty of the endothelium of tumor vessels,[11] polymers of appropriate size (ideally, between 5 and 40 kDa) are able to selectively accumulate in the tumor matrix. Furthermore,

* Corresponding author. Phone: +390498275694. Fax: +390498275366. E-mail: francesco.veronese@unipd.it.
[†] University of Padua.
[‡] Cardiff University.
[§] Current address: School of Pharmacy, University of Reading Whiteknights, P.O. Box 224, RG6 6AD Reading, Berkshire, UK.

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the poor lymphatic drainage ensures that the polymer is retained longer in the tumor tissue. This passive tumor targeting has been described as the "enhanced permeability and retention (EPR) effect". The carried drug payload can then be released either externally, in the extracellular compartment, or inside the tumor cell after endocytic uptake. Tumor targeting by polymeric prodrug strategy has already been successfully applied to photoactive drugs. However, porphyrin derivatives were mainly employed.[12−15]

On the basis of these considerations, polymer conjugates of a new biologically active psoralen were synthesized and chemically and biologically characterized in this study. First, a novel psoralen derivative was synthesized that contained a reactive hydroxy group suitable for conjugation. Considering the strong photosensitizing activity and the wide spectrum of mono- and bifunctional lesions induced by methylated psoralens,[4,16] in particular of 4,4′,8-trimethylpsoralen, a derivative bearing a hydroxyl group in the pyronic ring, namely, 4-hydroxymethyl-4′,8-dimethylpsoralen (4-OHMeP), was synthesized (Scheme 1). The presence of a hydroxyl group could allow the linkage to PEG through ester or carbamate moieties. PEG was selected as polymeric carrier, due to its well-documented biocompatibility, solubility in aqueous as well organic media, low immunogenicity and antigenicity, good kidney excretion, and its FDA approval for internal administration.[6,17] Moreover, PEG does not exhibit specific affinity for organs, reducing the risk of toxicity. For this study, linear monomethoxy-PEG (mPEG) (5 kDa) was selected as polymeric carrier, as the presence of a single reactive group minimizes conjugate heterogeneity.[18]

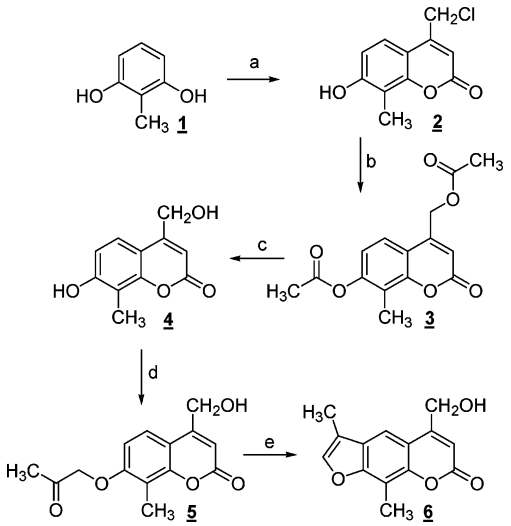
The release of anticancer agents from polymeric carriers outside or inside the target cell is a critical issue in a prodrug approach.[19] The conjugate must be sufficiently stable to survive storage administration conditions and residence in plasma and to cross blood vessels unaltered, while able to release the active moiety selectively inside the target tissue by hydrolytic or enzymatic cleavage. In this perspective, a rational design of the linker is a key factor to ensure the overall efficacy of the system. Four polymer conjugates were synthesized that differed only with respect to the linker used. 4-OHMeP was bound to $mPEG_{5\ kDa}$ by either an ester or an ester with the tetrapeptide spacer Gly-Leu-Phe-Gly or a carbamic linkage or a carbamic linkage with the tetrapeptide spacer Gly-Leu-Phe-Gly. This tetrapeptide spacer was chosen as it is selectively cleaved by the lysosomal proteases cathepsin B,[20,21] present and overexpressed in tumors while absent in blood.

To summarize, first a novel psoralen derivative was synthesized. Then, novel PEG conjugates containing such derivative were prepared and characterized. Finally, the biological profile of these conjugates was assessed with respect to (i) drug release in different environments (mimicking those encountered in the body) and (ii) the cytotoxic activity of the conjugates and the free drug in MCF-7 and A375 cells.

Experimental Section

Materials and Equipment. Solvents and reagents were from Aldrich Chemical Co. and Carlo Erba Reagenti (Milano, Italy). The PEG was from Shearwater Polymer (Nektar, AL). The tetrapeptide H-Gly-Leu-Phe-Gly-OH was synthesized by the University of Ferrara (Italy). Bovine cathepsin B was from Sigma Chemical Co. (St. Louis, MO) (1 mg of enzyme powder containing 11.36 units). 4,4′,8-Trimethylpsoralen was a kind gift from Prof. Sergio Caffieri (Department of Pharmaceutical Science, Padova, Italy). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Norit A charcoal, dextran T-70, and

Scheme 1. Synthesis of 4-OHMeP (**6**)[a]



[a] (a) Ethyl 4-chloroacetoacetate, H_2SO_4 36 N, 100 °C, (51%). (b) Ac_2O, AcONa, reflux, (69%). (c) Aqueus KOH, reflux, (84%). (d) K_2CO_3 anhydrous, chloroacetone, DMF, 40 °C, (71%). (e) Ethanolic KOH 5%, reflux, (30%).

DMSO were obtained from Sigma Chemical Co. (St. Louis, MO). MCF-7 human estrogen dependent breast carcinoma cell line was kindly donated by Tenovus, Centre for Cancer Research at Cardiff University. The human malignant melanoma (A375) cell line was obtained from ATCC (Rockville, MD). Culture media, fetal bovine serum, and L-glutamine were purchased from GIBCO BRL Life Technologies (Paiseley, UK).

The melting point determination was made using a Gallenkamp MFB-595-010M melting point apparatus, and the values given are uncorrected. Analytical TLC was performed on precoated 60 F254 silica gel plates (0.25 mm; Merck); they were developed with a $CHCl_3$/MeOH mixture (9:1) unless otherwise indicated. Preparative column chromatography was performed using silica gel 60 (0.063−0.100 mm; Merck), eluting with $CHCl_3$. ^1H NMR spectra were recorded at 200 MHz on a Varian Gemini-200 spectrometer or at 300 MHz on a Bruker Biospin spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS used as internal standard, and the coupling constants (J values) are given in hertz (Hz). Elemental analyses were obtained on all intermediates, and they are within ±0.4% of theoretical values. RP-HPLC analysis were performed with Jasco HPLC system (Japan) equipped with an Alltima C18 analytical column (200 × 4.6 mm) (Alltech, Italy) and when necessary with a preparative RP Jupiter C18 10 μm, 300 Å column, 250 × 21.2 mm (Phenomenex), eluted with MilliQ grade water with 0.05% TFA (eluent A) or acetonitrile with 0.05% TFA (eluent B) (see methods). UVA exposures were performed with a Philips HPW 125 lamp, equipped with a built-in Philips filter; emission was in the 320−400-nm range, with a maximum, over 90% of the total, at 365 nm. Irradiation intensity, determined by a radiometer (model 97503, Cole-Parmer Instrument Co.), was 0.9×10^{-6} W·m^{-2}.

Synthesis of 4-Hydroxymethyl-4′,8-dimethylpsoralen (4-OHMeP) (6). The synthesis is described in Scheme 1. Each step is described below.

4-Chloromethyl-7-hydroxy-8-methylcoumarin (**2**). A mixture of 2-methylresorcin (**1**) (30.00 g, 241.7 mmol), concentrated sulfuric acid (30.0 mL), and ethyl 4-chloroacetoacetate (32.7 mL, 241.7 mmol) was heated at 100 °C under dry nitrogen. After the disappearance of the starting material (2-methylresorcin) (4 h, TLC), the mixture was poured into an ice−water mixture (500 mL), and the solid was collected by filtration. It was washed with a large amount of water and crystallized from methanol to give 4-chloromethyl-7-hydroxy-8-methylcoumarin (**2**). Yield: 51%. Mp: 246 °C. ^1H NMR (DMSO): $\delta = 10.54$ (s, 1H, 7-OH), 7.54 (d, $J = 8.8$ Hz, 1H, 5-H), 6.89 (d, $J = 8.8$ Hz, 1H, 6-H), 6.42 (s, 1H, 3-H), 4.94 (s, 2H, CH_2Cl), 2.16 (s, 3H, 8-Me).

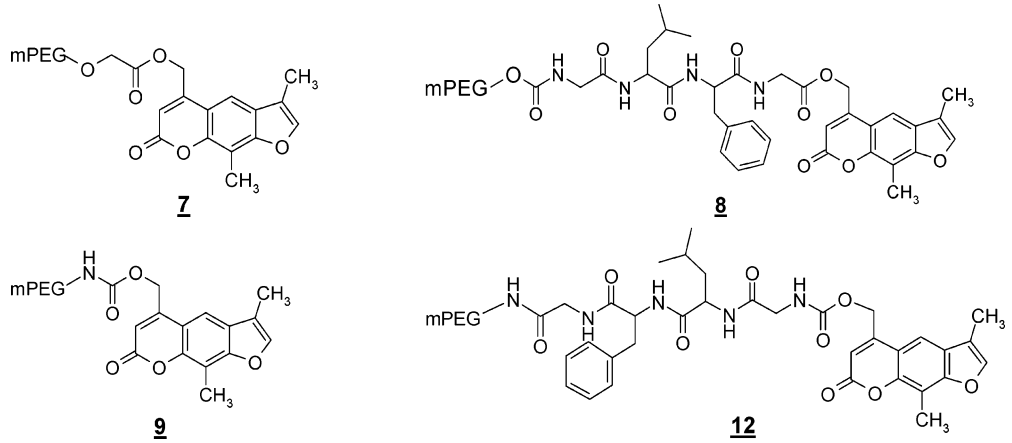


Figure 2. Structures of mPEG−4-OHMeP conjugates: mPEG−$OCH_2COOMeP$ (*7*), mPEG−OCONH-Gly-Leu-Phe-Gly-CO-OMeP, (8) mPEG−NHCOOMeP (*9*), mPEG−NHCO-Gly-Phe-Leu-GlyNH-COO-4-OHMeP (*12*).

4-Acethoxymethyl-7-hydroxy-8-methylcoumarin (**3**). A mixture of **2** (27.69 g, 123.3 mmol), acetic anhydride (100.0 mL), and anhydrous AcONa (30.00 g) was refluxed for 3 h until the starting material disappeared (TLC). Water (100.0 mL) was added dropwise to the previous mixture and then it was stirred overnight at room temperature. The solid was filtered, washed with water, and crystallized from methanol to obtain 4-acethoxymethyl-7-hydroxy-8-methylcoumarin (**3**) Yield: 69.4%. Mp: 203 °C. ^{1}H NMR (DMSO): $\delta = 7.65$ (d, $J = 8.8$ Hz, 1H, 5-H), 7.17 (d, $J = 8.8$ Hz, 1H, 6-H), 6.46 (t, $J = 1.4$ Hz, 1H, 3-H), 5.37 (d, $J = 1.4$ Hz, 2H, CH_2OCOCH_3), 2.36 (s, 3H, 8-Me), 2.18 (s, 6H, 2 $OCOCH_3$).

4-Hydroxymethyl-7-hydroxy-8-methylcoumarin (**4**). A solution of 20% aqueous KOH (10.0 mL, 37.9 mmol) was slowly added to a solution of **3** (5.0 g, 17.2 mmol) in ethanol (300 mL). The mixture was refluxed in the dark until the starting material disappeared (20 min; TLC). It was then cooled and acidified with concentrated HCl to obtain a white precipitate which was collected and washed with a large amount of water to give 4-hydroxymethyl-7-hydroxy-8-methylcoumarin (**4**). Yield: 84.5%. Mp: >300 °C. ^{1}H NMR (DMSO): $\delta = 10.38$ (s, 1H, 7-OH), 7.37 (d, $J = 8.8$ Hz, 1H, 5-H), 6.83 (d, $J = 8.8$ Hz, 1H, 6-H), 6.24 (t, $J = 1.4$ Hz, 1H, 3-H), 5.55 (t, $J = 5.4$ Hz, 1H, CH_2OH), 4.69 (dd, $J = 5.4$ Hz, $J = 1.4$ Hz, 1H, CH_2OH), 2.16 (s, 3H, 8-Me).

4-Hydroxymethyl-7-acetonyloxy-8-methylcoumarin (**5**). Chloroacetone (10 mL, 13.1 mmol) and anhydrous K_2CO_3 (2.40 g, 17.4 mmol) were added to a solution of **4** (1.35 g, 6.6 mmol) in *N*,*N*-dimethylformamide (120.0 mL). The mixture was heated at 40 °C for 6 h, the starting materials had then disappeared (TLC), and the reaction mixture was poured into an ice−water mixture (600 mL) with stirring. The precipitate was collected and washed with water to obtain 4-hydroxymethyl-7-acetonyloxy-8-methylcoumarin (**5**). Yield: 71.6%. Mp: 200 °C. ^{1}H NMR (DMSO): $\delta = 7.47$ (d, $J = 8.8$ Hz, 1H, 5-H), 6.88 (d, $J = 8.8$ Hz, 1H, 6-H), 6.31 (t, $J = 1.4$ Hz, 1H, 3-H), 5.60 (t, $J = 4.96$ Hz, 1H, CH_2OH), 4.98 (s, 2H, OCH_2COCH_3), 4.72 (bd, $J = 4.96$ Hz, 2H, CH_2OH), 2.25 (s, 3H, 8-Me), 2.18 (s, 3H, OCH_2COCH_3).

4-Hydroxymethyl-4′,8-dimethylpsoralen (4-OHMeP) (**6**). To a solution of **5** (4.6 g, 17.4 mmol) in ethanol (250.0 mL) was added slowly a 5% KOH ethanolic solution (85.6 mL, 76.3 mmol). The mixture was refluxed for 30 min in the dark until the starting material disappeared (TLC) and then cooled and acidified with HCl. Ethanol was evaporated from the mixture under reduced pressure and the residue extracted several times with EtOAc. The solvent was evaporated from the organic phase, and the residue was purified by LC eluting with $CHCl_3$ to yield 4-hydroxymethyl-4′,8-dimethylpsoralen (**6**). Yield: 26.1%. Mp: 232 °C. ^{1}H NMR (DMSO) $\delta = 7.87$ (q, $J = 1.3$ Hz, 1H, 5′-H), 7.74 (s, 1H, 5-H), 6.43 (t, $J = 1.5$ Hz, 1H, 3-H), 5.67 (t, $J = 5.6$ Hz, 1H, CH_2OH), 4.86 (dd, $J = 5.6$ Hz, $J = 1.5$ Hz, 2H, CH_2OH), 2.48 (s, 3H, 8-Me), 2.24 (d, $J = 1.3$ Hz, 3H, 4-Me).

Synthesis of mPEG−$OCH_2COOMeP$ (7). The structures of conjugates are shown in Figure 2.

mPEG−OCH_2COOH. A solution of 5 g (1.0 mmol) of mPEG-OH in 150 mL of toluene was dried by azeotropic distillation with a Dean−Stark tube. After cooling to room temperature, 6 mL (6.0 mmol) of a 1.0 M solution of potassium *tert*-butoxide in *tert*-butyl alcohol was added, the mixture was stirred for 1 h at 25 °C, and 1.17 g of *tert*-butyl bromoacetate (6.0 mmol) was then added. The resulting cloudy mixture was heated to reflux for 1 h and then it was stirred for 18 h at 50 °C, cooled to room temperature, and filtered with Celite to remove KBr, and the volume was finally reduced under vacuum. To the obtained *tert*-butyl-mPEG-carboxylate, 30 mL of a mixture of TFA/$CHCl_3$ 50:50 and 1% of water was added, and the mixture was stirred at room temperature for 3 h. The solvent was removed under vacuum, and the resulting oil rinsed 8−10 times with $CHCl_3$. This was treated with diethyl ether (250 mL) to precipitate the wanted product as a white powder that was filtered and dried under reduced pressure. Yield: 4.2 g, 95%.

mPEG−$OCH_2COOMeP$ (**7**). mPEG−OCH_2COOH (400 mg, 0.0755 mmol) and 37 mg (0.1510 mmol) of 4-OHMeP were dissolved in 50 mL of toluene, and the mixture was distilled azeotropically to remove approximately 45 mL of solvent. The residue was suspended in 15 mL of dry chloroform, TEA (20 μL, 0.1132 mmol) added, and the mixture cooled at 0 °C. HOBt (15.28 mg, 0.1132 mmol) and EDC (19.2 mg, 0.1132 mmol) were also added. The slurry was stirred at room temperature for 15 h. The resulting yellow mixture was washed with 0.1 N HCl (2 × 20 mL) and this was washed in turn with fresh chloroform. The pooled organic phases were dried over Na_2SO_4 and concentrated at reduced pressure. The oily residue was then diluted with 20 mL of 2-propanol and left to crystallize at 4 °C. The pale yellow crystalline precipitate was washed with cold 2-propanol and diethyl ether, yielding the wanted conjugate (**7**) that was dried at reduced pressure. The yield was 461 mg (85%). The 4-OHMeP loading was 2.08% (wt/wt). RP-HPLC: Phenomenex Jupiter C 18, eluents H_2O/ACN + 0.05% of TFA; gradient time (% A) 0 min (90%), 30 min (10%), 36 min (90%); $t_R = 24.3$ min. ^{1}H NMR ($CDCl_3$): $\delta = 2.28$ (d, 3H), 2.58 (s, 3H, CH_3), 3.36−3.87 (m, PEG's CH_2), 4.33 (s, 2H), 5.48 (s, 2H), 6.46 (s, 1H), 7.43 (s, 1H), 7.48 (s, 1H, 5′-H).

Synthesis of mPEG−OCONH-Gly-Leu-Phe-Gly-COOMeP (8). The structures of conjugates are shown in Figure 2.

mPEG−p-Nitrophenyl Carbonate. mPEG−OH (2.5 g, 0.5 mmol), dried by azeotropic distillation, was treated under stirring at room temperature for 18 h with an excess of 4-nitrophenyl chloroformate (302 mg, 1.5 mmol) in dried dichloromethane in the presence of triethylamine (110.5 μL, 1.5 mmol). The triethylammonium salts were removed by filtration with Celite, and the obtained mPEG-*p*-nitrophenyl carbonate was recovered by addition of dried diethyl ether. The precipitate was washed several times with fresh diethyl ether to remove the adsorbed 4-nitrophenyl chloroformate. The degree of polymer activation was determined spectrophotometrically at 400 nm by measuring the released 4-nitrophenate after treating the product (10

mg) with 50 mL of 0.2 N NaOH. Only product with >90% activation was employed.

mPEG−OCONH-Gly-Leu-Phe-Gly-COOH. Tetrapeptide (H-Gly-Leu-Phe-Gly-OH; 0.7044 mmol) in a water/acetonitrile (1:1) solution, made alkaline by the addition of triethylamine (6 equiv), was added portionwise to the activated polymer (mPEG−4-nitrophenyl carbonate) (600 mg, 0.1174 mmol). After 18 h of stirring at room temperature, the pH was adjusted to 3 with 1 N HCl. The acetonitrile was removed under vacuum and MilliQ grade water (30 mL) was added. The aqueous solution was extracted with diethyl ether (3 × 30 mL) to remove the excess of 4-nitrophenyl chloroformate, and the PEG−tetrapeptide was extracted with CH_2Cl_2 (3 × 50 mL) until the aqueous phase was negative to I_2 assay. The combined CH_2Cl_2 extracts were dried over Na_2SO_4 and then evaporated under vacuum to about 3 mL. The resulting oil was dropped into cold ethyl ether (150 mL), and stored for 1 h at 4 °C. The precipitate was collected by filtration and then dried under vacuum to obtain the product as a white powder. Yield: 545 mg, (86%).

mPEG−OCONH-Gly-Leu-Phe-Gly-CO-OMeP (**8**). The mPEG−OCONH-Gly-Leu-Phe-Gly-OH (545 mg, 0.1006 mmol) was dried by azeotropic distillation and rinsed with 10 mL of anhydrous chloroform. To the solution were added 0.1509 mmol of TEA, 49 mg of **6** (0.2012 mmol), and, in sequence, 20 mg (0.1509 mmol) of HOBt and 28.9 mg (0.1509 mmol) of EDC. Then, to the activated mPEG−OCONH-Gly-Leu-Phe-Gly-OH, 49 mg of 4-OHMeP was added. The solution was left stirring at room temperature for 18 h and then extracted with 5% $NaHCO_3$ and 0.1 M HCl. The organic phase was dried over Na_2SO_4, filtered, and concentrated under vacuum. Finally it was dropped into 200 mL of ethyl ether. Yield: 427 g, 76%. The 4OH-MeP loading was 3.16% (wt/wt). RP-HPLC: Phenomenex Jupiter C 18, eluents H_2O/ACN + 0.05% of TFA; gradient time (% A) 0 min (90%), 30 min (10%), 36 min (90%); t_R = 25.1 min. ^{1}H NMR ($CDCl_3$): δ = 0.9 (m, 6H, Leu), 3.38−3.85 (m, PEG's CH_2), 4.3 (s, CH_2 Gly), 6,45 (s, 1H, 3-H).

Synthesis of mPEG−NHCOOMeP (9). The structures of conjugates are shown in Figure 2.

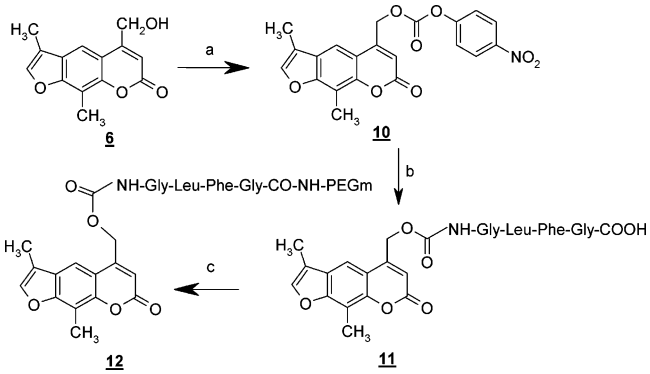
4-O-p-Nitrophenyl-oxycarbonyl 4-OHMeP (**10**). 4-Nitrophenyl chloroformate (693 mg, 3.4392 mmol) was added to 140 mg (0.5732 mmol) of **6** dissolved in 10 mL of anhydrous chloroform, while the pH of the solution was made alkaline with 3.4392 mmol of TEA. The solution was stirred under reflux for 18 h. Then the solvent was evaporated and the oily product dissolved in 2 mL of chloroform. It was purified by flash chromatography (mobile phase chloroform/ethyl acetate 85:15). Yield: 196 mg, 84%. RP-HPLC: Phenomenex Jupiter C 18, eluents H_2O/ACN + 0.05% of TFA; gradient time (% A) 0 min (90%), 30 min (10%), 36 min (90%); t_R of 4-nitrophenyl chloroformate was 13 min. ^{1}H NMR (DMSO): δ = 8.5−7.65 (dd, 4H, CH aa′bb′ system), 7.9 (s, 2H, 5′-H, 3-H), 6.5 (s, 1 H 5-H), 5.8 (s, 2H, $-CH_2O-$), 2.3 (s, 3 H, 4′-CH_3).

mPEG−NHCOOMeP (**9**). mPEG-NH_2 (5 kDa, 300 mg) (0.0537 mmol) was dried by azeotropic distillation and the resulting oil rinsed with 20 mL of CH_2Cl_2. The pH of the solution was adjusted to 8 with TEA. **10** (0.1074 mmol) was added to the PEG solution and the reaction was stirred at room temperature for 24 h. The solvent was removed under reduced pressure, and the remaining oil was then dropped into diethyl ether. Yield: 264 mg, 84%. The 4OH-MeP loading was 3.68% (wt/wt). RP-HPLC: Phenomenex Jupiter C 18, eluents H_2O/ACN + 0.05% of TFA; gradient time (% A) 0 min (90%), 30 min (10%), 36 min (90%); t_R of 4-nitrophenyl chloroformate (1%) = 13 min, t_R of **9** (91%) = 18 min. ^{1}H NMR ($CDCl_3$) δ = 2.3 (s, 3H, CH_3 furan), 5.8 (s, 2H, $-CH_2O-$), 6.5 (s, 1H, 5-H), 7.9 (s, 2H, 5-H, 5′-H), 7.65−8.5 (dd, 4H, CH aa′bb′ system).

Synthesis of mPEG−NHCO-Gly-Phe-Leu-Gly-NHCOOMeP (12). The structures of the conjugates are shown in Figure 2. The synthesis of conjugate **12** is described in Scheme 2. Each step is described below.

4-Hydroxymethyl-4′,8-dimethylpsoralen-COONH Gly-Leu-Phe-Gly (**11**). H-Gly-Leu-Phe-Gly-OH (76.7 mg, 0.2931 mmol) was dissolved in anhydrous DMF (5 mL). After alkalinization with TEA, **10** (0.2931 mmol) was added and then the solution was stirred overnight. The solvent was removed under vacuum, and the product was washed with diethyl ether. Yield: 122 mg, 63%. RP-HPLC: Phenomenex Jupiter C 18, eluents H_2O/ACN + 0.05% of TFA; gradient time (% A) 0 min (90%), 30 min (10%), 36 min (90%). t_R of 4-Hydroxymethyl-4′,8-dimethylpsoralen-COONH Gly-Leu-Phe-Gly = 21 min. A further purification of the product was carried out by preparative HPLC C18: Jupiter column, eluents H_2O/ACN + 0.05% of TFA; gradient time (%A) 0 min (60%), 16 min (45%), 32 min (45%), 36 min (10%), 40 min (60%); t_R of 4-OHMeP-COONH Gly-Leu-Phe-Gly = 23.5 min.

Scheme 2. Synthesis of **12**[a]



[a] (a) 4-Nitrophenyl chloroformate, anhydrous chloroform, TEA, reflux. (b) H-Gly-Leu-Phe-Gly-OH, anhydrous DMF, rt. (c) mPEG-NH_2, anhydrous DMF, TEA, HOBt, EDC, rt.

mPEG−NHCO-Gly-Phe-Leu-Gly-NHCOO-4-OHMeP (**12**). **11** (31.4 mg, 0.0473 mmol) was solubilized in anhydrous DMF (5 mL) and TEA was added to reach alkalinity. To the drug solution were added in sequence: HOBt (8.53 mg, 0.0946 mmol), then EDC (12.10 mg, 0.0946 mmol), and finally azeotropically dried mPEG-NH_2 (158 mg, 0.0316 mmol). The mixture was stirred for 18 h, the DMF was then removed, and the residue was diluted with CH_2Cl_2 (20 mL). This was extracted with KH_2PO_4 solution (10%) and 0.1 M HCl. The organic phase was anhydrified by Na_2SO_4 and precipitated by diethyl ether. Yield of crude product: 143 mg. The powder was further crystallized from 2-propanol and the overall yield was 53 mg (30%). The 4OH-MeP loading was 3.24% (wt/wt). RP-HPLC: Phenomenex Jupiter C 18, eluents H_2O/ACN + 0.05% of TFA; gradient time (% A) 0 min (90%), 30 min (10%), 36 min (90%). t_R = 17.8 min. ^{1}H NMR (DMSO): δ = 8−8.2 (2s, 2H, 5′-H, 5-H), 7.45 (s, 6H, Phe), 6.65 (1s, 1H, 3-H), 5.7 (1s, 2H, $-CH_2O-$), 4.25 (1s, 1H, Gly), 3.6−3.8 (border singlet, PEG' s $-CH_2-$), 3.34 (1s, 3H, CH_3O, PEG) 2.5 (1s, 3H, CH_3 furan), 1 (dd, 6H, CH_3 Leu).

Evaluation of the Total 4-OHMeP Content in the Conjugates. The loading of 4-OHMeP was estimated by UV absorption (at 360 nm). First a calibration curve was prepared using 4-OHMeP. The UV spectrum of PEG derivatives was then evaluated and then compared with the hydrolyzed 4-OHMeP. Since the obtained curves were comparable, we used the free drug molar extinction coefficient to evaluate the 4-hydroxymethyl-4′,8-dimethylpsoralen content in the conjugates (loading).

Measurement of 4-OHMeP Release from Conjugates in Vitro. *Hydrolysis of Conjugates by Cathepsin B at pH 5.5.* A buffer solution was prepared using 0.15 M $KH_2PO_4 \cdot 2H_2O$ and 10^{-3} M EDTA (pH 5.5), and to this was added glutathion (5 μM) just before use. This solution (850 μL) was then added to cathepsin B (50 μL, 0.285 mg/mL) in the same buffer and the mixture incubated for 5 min at 37 °C. Finally, 100 μL of the conjugates (580 μg/mL 4-OHMeP equivalent) was added to the previous solution. Conjugates were separately dissolved in buffer (without enzyme) as a reference blank. At fixed intervals, samples (50 μL) were analyzed by HPLC (RP C18 column) under the same conditions previously reported to evaluate the rate of hydrolysis on the basis of the 4-hydroxymethyl-4′,8-dimethylpsoralen release.

Table 1. Characteristics of mPEG−4-OHMeP Conjugates

compound	structure	4-OHMeP[a] total (wt %)	4-OHMeP[a] free (% total)
Conjugate **7**	mPEG−CO-4-OMeP	2.08	<0.1
Conjugate **8**	mPEG−OCONH-Gly-Leu-Phe-Gly-CO-4-OMeP	3.16	0.5
Conjugate **9**	mPEG−NHCO-4-OMeP	3.68	1.2
Conjugate **12**	mPEG−NHCO-Gly-Phe-Leu-Gly-NHCO-4-OMeP	3.24	1.9

[a] Determined by RP-HPLC

Hydrolysis of Conjugates in Mouse Plasma. Blood (1 mL) was taken from a female BALB mouse. It was centrifuged at 12 000 rpm for 1 min. The plasma (600 μL) was added to different solutions of conjugates (130 μg 4-OHMeP equivalent) dissolved in 75 μL of a PBS buffer pH 7.44 containing $^1/_{15}$ M of KH_2PO_4/Na_2HPO_4 (pH 7.4). They were thoroughly mixed and incubated at 37 °C. At fixed times samples (50 μL) were taken and added to acetonitrile (300 μL) and the mixture was centrifuged for 3 min at 12 000 rpm. The solvent was removed and dried by centrifugation under vacuum. Then the residue was solubilized with MilliQ grade H_2O (100 μL) and analyzed by HPLC using a RP C18 column as descried above. As a reference control a sample was also treated as above, but without plasma.

Evaluation of in Vitro Cytotoxicity (with and without UVA) Using the MTT Assay. The cell lines were maintained in the logarithmic phase at 37 °C in a 5% CO_2 atmosphere. D-MEM (Dulbecco's Modified Eagle's) medium supplemented with L-glutamine containing fetal bovine serum (5%), 50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin was used for A375 cells. As phenol red is mitogenic for MCF-7 cells,[22] all the experiments with MCF-7 were performed in RPMI 1640 phenol-red-free medium (WRPMI), supplemented with charcoal-stripped fetal calf serum (SFCS, prepared as described previously[23]) (5%). As the growth of MCF-7 can be deeply affected by the concentration of steroids in the medium, it was decided to use a medium in which the concentration of steroid was constant. For this reason, SFCS supplemented with a known concentration of estradiol (10^{-9} M) was used in all the experiments carried out with this cell line.

Growth inhibition was evaluated using the MTT assay. Free drug or conjugate (dissolved respectively in DMSO or in water, prepared immediately before the experiments) was added to tissue culture medium to give a final solvent concentration of 0.5%;[24] furthermore, to ensure that this was nontoxic, an equivalent amount of DMSO was always present in the medium of the control.

Briefly, $(4-5) \times 10^3$ cells/well were seeded in 96-well microplates in medium (100 μL) and incubated at 37 °C. After 24 h (for A375) or 4 days (for MCF-7) the medium was removed and replaced with a fresh medium containing the test compound (3.125−100 μM 4-OHMeP equivalent) in quadruplicate. This different time profile was chosen to ensure that the incubation was carried out during the cells' exponential growing phase. After 67 h, MTT (10 μL of a sterile-filtered 5 mg/mL solution in PBS) was added, and the cells were incubated for a further 5 h. The medium was then removal carefully and the precipitated formazan crystals were dissolved in optical grade DMSO (100 μL) over 30 min at 37 °C. Absorbance at 540 nm was measured using a microplate reader (Bio-Rad). The mean absorbance for each concentration was expressed as a percentage of the control value obtained for untreated cells. The drug concentrations that reduced growth by 50% with respect to the untreated control (IC_{50} values) were estimated.

To evaluate the growth inhibitory effect of the free or conjugated drug following exposure to UVA activation, the cells were seed in a 96-well plate as described above. After 24 h or 4 days the medium was removed and replaced with fresh medium that contained the free drug or the PEG-conjugated drug. In all cases, the cells were incubated with the highest concentration that had shown no discernible effect on growth in the cytotoxicity studies performed in the absence of light (i.e., 5 μM for 4-OHMeP and 5 μM 4-OHMeP equivalent for the conjugates in 100 μL of total volume). The cells were incubated with the compounds, in the dark, for 1, 6, or 24 h and then irradiated with a range of UVA doses (1.42, 2.48, and 5.68 kJ/m^2). Following irradiation, the medium was removed, and the cells were washed three times with PBS and incubated with fresh medium for a further 48 h. Finally, cell viability was evaluated by MTT assay as described above.

Results

Synthesis and Characterization of PEG−4-OHMeP Conjugates. A new psoralen derivative with an hydroxy group in the pyrone ring (4-OHMeP, **6**) was successfully synthesized (Scheme 1). NMR analysis confirmed the product identity (see Experimental Section for a detailed description of the characteristics peaks). Furthermore, no impurities were found by HPLC analysis (detection limit 0.1%).

Subsequent conjugation of this derivative with PEG led to the synthesis of a novel family of PEG−4-OHMeP conjugates, which differed for the type of linkage between PEG and 4-OHMeP (Figure 2). Conjugates **7** and **8** contained an ester bond, but the latter also included a tetrapeptide spacer (Gly-Leu-Phe-Gly). To increase the stability of the PEG−psoralen linkage, two more conjugates were synthesized that contained a more stable carbamate bond. These were conjugates **9** and **12** (Figure 2). Conjugate **12** contained also the tetrapeptide spacer. In this case, the carboxy terminal of the tetrapeptide spacer was linked to the amino PEG, while the amino terminal was bound to the drug to form a carbamate linkage. In all cases, covalent binding between 4-OHMeP and PEG was confirmed by HPLC as the compound eluted as a single peak. Furthermore, NMR showed the characteristic peaks of the three main components: psoralen, PEG, and, in the case of **8** and **12**, also the peptide linker (see Experimental Section for details of these peaks). The conjugates were characterized with respect to the total drug content and the residual free drug by UV analysis (these characteristics are summarized in Table 1). In all cases, the total content of 4-OHMeP was approximately 3% wt/wt (range 3.24−3.68). The residual free drug was always below 1.9% wt/wt of the total drug content. The yield of the conjugation was between 30% and 85% (the lowest value was obtained for conjugate **12**, as it required a two-step purification).

Effect of Linker on Rate of 4-OHMeP Release. Conjugates **7** and **8** were both rapidly hydrolyzed in plasma (100% hydrolysis after ~1 h) (Figure 3a,b). While both **7** and **8** were stable at pH 5.5 (<20% drug release after 25 h), the latter was hydrolyzed in presence of cathepsin B ($t_{1/2}$ = 45 min). Significant hydrolysis occurred also after incubation of **9** and **12** in plasma (Figure 3c,d). However, with these conjugates drug release was markedly more gradual than for conjugates **7** and **8** and a complete drug release was reached only after 25 h. Interestingly, drug release in presence of cathepsin B was only observed when the peptidyl linker was present (for **12**, ~40%

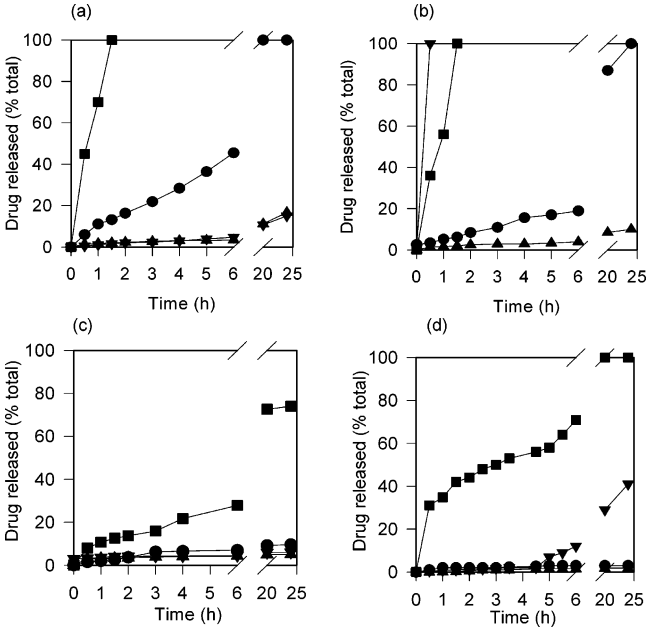


Figure 3. Release of 4-OHMeP from the mPEG−4-OHMeP conjugates at different pH and in the presence of plasma or cathepsin B: (a) conjugate **7**, (b) conjugate **8**, (c) conjugate **9**, and (d) conjugate **12**. In each case the conjugates were incubated in buffer at pH 7.4 or 5.5 and in mouse plasma or cathepsin B, respectively. Release of 4-OHMeP was determined by HPLC. Symbols: ●, pH 7.4; ■, pH 7.4 + plasma; ▲, pH 5.5; ▼ pH 5.5 + cathepsin B.

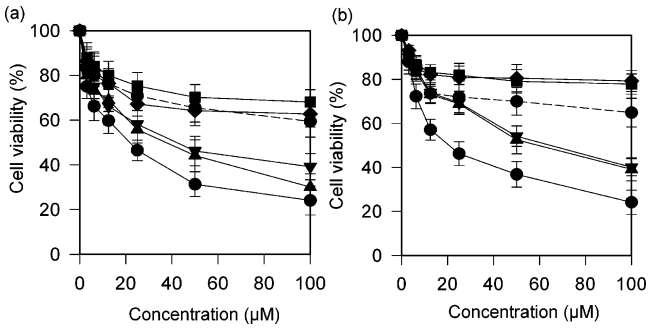


Figure 4. Antiproliferative activity of 4-OHMeP and its conjugates against A375 cells (panel a) and MCF-7 cells (panel b) without UVA activation. Drug exposure was for 72 h. Cell viability was evaluated by the MTT test. Values are the mean (±SD) of three independent experiments. Symbols: ▲, conjugate **7**; ▼, conjugate **8**; ♦, conjugate **9**; ■, conjugate **12**; −●−, 4-OHMeP; - -●- -, 4,4′,8-TMP.

release after 25 h; Figure 3d), while **9** was completely stable under these condition (less than 10% drug release after 25 h; Figure 3c).

In Vitro Cytotoxicity with and without UVA Activation. The cytotoxicity of 4-OHMeP and the PEG−4-OHMeP conjugates against A375 and MCF-7 cell lines was first established in the absence of UVA. Their activity was compared to that of trimethylated psoralen, 4,4′,8-TMP, which was previously studied[16] (Figure 4 and Table 2). 4-OHMeP showed a growth inhibitory potency markedly higher than 4,4′,8-TMP (ineffective, as expected, in these conditions), with IC_{50} values ranging from 34 to 37 μM (for A375, MCF-7 cell lines, respectively). Interestingly, the conjugates that contained the ester linkage showed a very similar toxicity profile to that of the free 4-OHMeP. This was apparent both in MCF-7 and in A375 cells. However, the conjugates containing the carbamate linkage (**9** and **12**) were completely inactive (IC_{50} > 100 μM in both cell lines).

Table 2. Antiproliferative Activity of 4-OHMeP and Its Conjugates against A375 and MCF-7 Cells without UVA Light

	cytotoxicity [IC_{50}[a,b] ± SD[c] (μM)]	
compd	A375	MCF-7
4,4',8-TMP	>100	>100
4-OHMeP (**6**)	34.89 ± 1.91	37.41 ± 1.88
conjugate **7**	48.12 ± 1.97	65.87 ± 2.25
conjugate **8**	59.89 ± 2.15	67.96 ± 2.35
conjugate **9**	>100	>100
conjugate **12**	>100	>100

[a] IC_{50} values were calculated by probit analysis ($P < 0.05$, χ^2-test); cell viability was measured by MTT test. [b] Data are presented as 4-OHMeP equivalent. [c] SD = standard deviation.

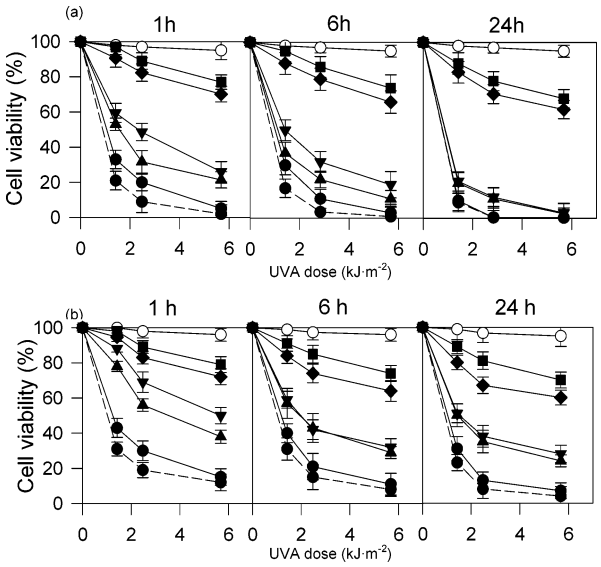


Figure 5. Antiproliferative activity of 4-OHMeP and its conjugates against A375 cells (panel a) and MCF-7 cells (panel b) in presence of UVA irradiation. The tested compounds were added in fresh medium at a final concentration of 5 μM for 1, 6, or 24 h (5% CO_2 at 37 °C). After incubation the cells were irradiated, the medium was removed, and cells were washed three times with PBS and incubated with fresh medium for 48 h. Cell viability was measured by MTT test. Values are the mean (±SD) of three independent experiments. Symbols: ▲, conjugate **7**; ▼, conjugate **8**; ♦, conjugate **9**; ■, conjugate **12**; −●−, 4-OHMeP; - -●- -, 4,4′,8-TMP; ○, UVA light.

Subsequently, the ID_{50} values (the UVA doses that reduced cell growth to 50% when delivered in the presence of a given concentration of tested compound) were also determined in MCF-7 and A375 cells (Figure 5 and Table 3). 4-OHMeP and 4,4′,8-TMP showed similar antiproliferative activity in both cell lines. For example, in A375 cells, the ID_{50} after 24-h incubation was 0.52 and 0.58 kJ/m^2 for 4,4′,8-TMP and for 4-OHMeP, respectively. Once again, the inhibitory effect showed by **7** and **8** was similar to that of 4-OHMeP in both cell lines. Also conjugate **9** and **12** were active after irradiation, although to a lesser extent than conjugates **7** and **8**. In all cases, the antiproliferative activity increased at higher UVA dose.

Discussion

The conjugation of a low molecular weight drug to a polymeric chain to achieve selective tumor targeting is not a novel concept. It was first theoretically suggested more than 30 years ago[25] and since then several conjugates carrying well-known chemotherapeutic agents (e.g., doxorubicin, paclitaxel, and platinates) have undergone clinical evaluation.[26] However,

Table 3. Antiproliferative Activity of 4-OHMeP and Its Conjugates against A375 Cells and MCF-7 Cells in Presence of UVA Irradiation

	cytotoxicity [ID_{50}[a] ± SD[b] (kJ·m^2)]					
	A375 cells			MCF-7 cells		
compd	1 h	6 h	24 h	1 h	6 h	24 h
4,4,8-TMP	1.02 ± 0.3	0.91 ± 0.02	0.52± 0.01	1.72 ± 0.12	1.58± 0.11	0.85± 0.01
4-OHMeP (**6**)	1.48 ± 0.34	1.41 ± 0.07	0.58 ± 0.02	1.92 ± 0.21	1.80 ± 0.12	1.31 ± 0.21
conjugate **7**	2.41 ± 0.72	1.86 ± 0.17	1.17 ± 0.32	3.78 ± 1.34	2.81 ± 0.23	1.90 ± 0.06
conjugate **8**	2.86 ± 0.56	2.49 ± 0.64	1.26 ± 0.45	4.88 ± 1.23	3.93 ± 1.34	2.50 ± 0.66
conjugate **9**	10.40 ± 1.45	9.20 ± 1.95	7.88 ± 1.33	12.48 ± 1.56	8.74 ± 1.76	5.05 ± 1.28
conjugate **12**	14.52 ± 2.15	13.09 ± 1.45	10.20 ± 0.98	14.10 ± 1.45	12.03 ± 2.01	9.45 ± 1.13

[a] ID_{50}: UVA dose that induces a 50% inhibition of cellular growth when delivered in the presence of 5 μM drug concentration; cell viability was measured by the MTT test. [b] SD = standard deviation.

few studies have been reported suggesting the use of polymer−drug conjugate to deliver photodynamic therapy.[13] This approach presents a double advantage. First of all, the use of a polymeric carrier guarantees passive tumor targeting by the EPR effect. Furthermore, as such compounds are inactive in the absence of light, activation selectively occurs only in irradiated areas.

Porphyrins derivatives are the most known phototherapeutic agents. An HPMA conjugate that contained mesochlorin[12] was recently described. Such a conjugate displayed increased activity in vitro and in vivo when administered in combination with chemotherapy.[12] Furthermore, a lyposomal formulation containing a PEG−tetraarylporphyrin conjugate was described.[27] Psoralens are an alternative class of photosensitizer drugs already used in therapy. These derivatives present an advantage over porphyrins. While the latter are activated by irradiation to produce the very reactive and poorly selective singlet oxygen, psoralens[28] directly photobind to DNA (after irradiation UVA $\lambda \sim 365$ nm); hence, they are more specific. Here, the first polymer conjugates that contain psoralens were described.

Polymer−drug conjugates are generally designed to achieve lysosomotropic delivery of the drug. A careful and rational design of the linker is therefore essential. Among the linkages explored in this study it was evident that the ester linker was poorly stable. Indeed, the conjugates that contained the ester linkage released the whole drug content in less than 1 h. This was not totally unexpected, due to the presence of esterases in the blood. Previous studies reporting the use of an ester bond showed contradictory results that depended on the nature of the polymer chain used. For example, the HPMA copolymer−paclitaxel conjugate was poorly stable resulting in a premature drug release and consequently in toxic effects.[29] On the contrary, the polyglutamic acid (PGA)−paclitaxel conjugate was stable in the blood and drug release occurred only after degradation of the main polymeric chain by cathepsin B.[30] It is evident that more complex parameters, most importantly the polymer conformation in solution, should be taken into account. However, for the conjugates described in this study, it was apparent that the presence of the polymeric chain was not sufficient to protect the ester bond from the action of esterases. The conjugates that contained the carbamate linker were, however, more stable in plasma. As expected, drug release following incubation with cathepsin B occurred only when the peptidyl linker was present. Such a linker has been widely described as substrate for this enzyme.[21] It is worth noting that the linkage in conjugate **12** is unusual. In general, the drug is attached to the carboxy terminal of the peptide linker, but in this case, it is linked to the amino terminal. This, however, did not prevent the cleavage of the linker by cathepsin B, as not only does this enzyme have endopeptidase activity but also exopeptidase activity, allowing removal of the remaining amino acid bound to psoralen.

Cytotoxicity studies in the absence of light showed that 4-OHMeP (**6**) was more active than the parent compound 4,4′,8-TMP. This is consistent with previous studies carried out on another group of psoralen derivatives (benzopsoralens). In these derivatives, the addition of an hydroxymethyl group in the position 4 of the pyronic ring led to an increase of the biological activity in the absence of light.[31] The cytotoxicity of the conjugates correlates well with the release profile. The activity found for conjugate **7** and **8** can be attributed to the poor stability of the linker. Interestingly, although completely inactive in the dark, conjugates **9** and **12** were active following UV irradiation. This has important implications. In all cancer treatment, maximum activity is requested at the target site while absence of activity is desirable in all the other tissues. Conjugate **12** was relatively stable in plasma and completely inactive in the dark. However, exposure to the light transformed it to an active compound. Therefore, conjugate **12** may present a double targeting mechanism: EPR and light.

Conclusions

In this study, a novel family of polymer−drug conjugates containing psoralen was described for the first time. The stability of conjugate **12** in plasma, its lack of activity in the dark, and its photoactivation after UVA exposure indicate great potential for this compound. In vivo studies are strongly warranted to verify if tumor targeting is achieved and to confirm its activity.

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