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Folate-mediated targeting of polymeric conjugates of gemcitabine

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Abstract

The synthesis of two new macromolecular prodrugs for active tumor targeting was set up. Gemcitabine (2′-deoxy-2′,2′-difluorocytidine) was conjugated to α,β-poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) through succinyl or diglycolyl hydrolysable spacers. The targeting agent folic acid was attached to the macromolecular backbone through the aminocaproic spacer. The two conjugates [PHEA-(5′-succinylgemcitabine)-1′-carboxypentyl-folamide and PHEA-(5′-diglycolyl-gemcitabine)-1′-carboxypentyl-folamide], were purified and extensively characterised by spectroscopic (UV, IR and NMR) and chromatographic analyses to determine the correct chemical structure, the purity degree and the reaction yield. In vitro studies demonstrated that the drug release depends on the spacer arm (diglycolyil or succinyl) and incubation pH. After 30 h incubation at pH 7.4, mimicking the plasma and extracellular compartments, the gemcitabine release from the succinyl and diglycolyl derivatives was 28 and 31%, respectively. After 30 h incubation at pH 5.5, mimicking the lisosomial compartment, the drug released from both bioconjugates was lower than 13%. In plasma, the polymer conjugation increased the drug stability and provided for a sustained drug release. In vitro citotoxicity studies performed using human nasopharyngeal epidermal carcinoma KB cells demonstrated that PHEA-(5′-succinylgemcitabine)-1′-carboxypentyl-folamide displays an higher dose dependent cytotoxic effect with respect to PHEA-(5′-diglycolyl-gemcitabine)-1′-carboxypentyl-folamide.

Keywords: Polymeric conjugates; Gemcitabine; Polyaspartamide; Folic acid; Folate-mediated targeting

1. Introduction

The therapeutic performance of anti-cancer drugs is often compromised by their low selectivity for cancer cells and unacceptable toxicity to normal tissues. Targeted drug delivery systems can optimise the therapeutic index of anti-tumour drugs by increasing the disease tissue/normal tissue drug concentration ratio.

In the recent years, the interest for the identification of ligands able to recognise specific cancer cells as target sites has grown enormously. Among targeting agents directed to membrane bound tumour associated antigens or receptors there are vitamins, hormones and antibody derivatives.

Folic acid is a typical example of low molecular weight targeting agent since the folate receptor is over-expressed by a number of human tumours, including cancer of ovary, kidney, uterus, testis, brain, colon, lung and myelocytic blood cells (Lu and Low, 2002; Weitman et al., 1992). Folic acid conjugated drugs or drug carriers has been demonstrated to accumulate into tumour cell by folate receptor-mediated endocytosis (Lu and Low, 2002; Wang and Low, 1998).

Since its targeting properties, folic acid was exploited for selective delivery of imaging agents (Wang et al., 1996, 1997), genes (Reddy et al., 2001 Benns et al., 2002), therapeutic agents (Lee et al., 2002; Li et al., 1998; Ladino et al., 1997), proteins (Leamon and Low, 1994; Leamon et al., 1993), liposomes (Lee and Low, 1994), nano-particles and other macromolecular or supramolecular systems to tumour tissues. Soluble polymers, in particular, have been successfully used to increase the drug payload, promote the tumour accumulation by passive mechanisms (Matthews et al., 1996; Garnett, 2001), enhance the stability and solubility of active agents, modify their pharmacokinetic profiles and allow for drug release in selected biological environments (Matthews et al., 1996; Garnett, 2001).

α,β-Poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) (Giammoma et al., 1987) is a water-soluble, non-toxic,

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non-antigenic and non-immunogenic, protein-like structured polymer already proposed as plasma substitute. Since its multifunctional character, PHEA was demonstrated to be a suitable carrier for anti-inflammatory (Giammona et al., 1991), antiviral (Giammona et al., 1992, 1995) and anticancer drugs (Cavallaro et al., 2001, 2004). In order to exploit the carrier properties of PHEA, new bioconjugates were synthesised by attachment of gemcitabine (2′,2′-difluorodeoxycytidine) (Hertel et al., 1988) and folic acid as targeting agent to cancer cells to the polymeric backbone. Gemcitabine was used because of its activity spectrum against human solid tumors (Hertel et al., 1990; Braakhuis et al., 1991; Csoka et al., 1995) and tumour cell lines which overexpress folate receptor (Weitman et al., 1992). Nevertheless, gemcitabine displays adverse properties such as short half-life and haematological and renal toxicity (Abratt et al., 1994). The new derivatives were obtained by drug attachment through two different hydrolysable spacers, diglycolyl and succinyl moieties, which allow for the drug release. The solubility and stability of the two derivatives as well as their selectivity towards folate receptor over-expressing cells was evaluated.

2. Materials and methods

2.1. Apparatus

Infrared spectra were obtained using a Perkin-Elmer 1720 IR Fourier Transform Spectrophotometer in potassium bromide disks.

Ultraviolet (UV) spectra were recorded by using a Perkin-Elmer 330 Instrument equipped with a 3600 Station.

Elemental analysis (C, H, N) was carried out on a Carlo Erba model 1106 analyzer.

The mass spectra were obtained using a Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (manufactured by Perkin-Elmer Sciex Instruments) equipped with an Apple™ Macintosh System 8.6 Hardware operating with TOFTune 1.5b37 software by using the FAB Technique.

The ^{1}H NMR spectra were obtained by a Bruker AC-250 instrument operating at 250.13 MHz. A size exclusion chromatography (SEC) system was used for determination of weight average of molecular weight, using two Ultrahydrogel (1000 and 250 Å of pores size) columns from Waters (Mildford, MA, USA) eluted with 0.15 M NaCl at a flow of 0.8 ml/min. The chromatographic system was equipped with a 410 differential refractometer (DRI) from Waters.

Centrifugations were performed using a Centra MP4R IEC centrifuge.

Purification by silica-gel chromatography was carried out using a Chromatotron model 7924T equipped with a 1 mm silica gel $60_{PF\text{-}254}$ rotor layer.

TLC analyses were performed on silica gel Plate 60 F_{254} (Merck) and on RP-18 silica gel plate F^S_{254} (Merck).

HPLC analyses were carried out on a system consisting of a Agilent Liquid Chromatography 1100 Series (with a 10 μl loop), a spectrophotometric Detector 1100 on line with a computerized HP workstation. In the HPLC method, a reversed phase C_{18} column (μBondapak; 10 μm of 250 mm × 4.6 mm i.d., obtained from Waters) equipped with a direct-connect guard column C_{18} (Waters) was used. The HPLC method was used to evaluate the purity of all synthesized compounds and to assess the drug released amount during in vitro incubation studies.

Molecular weights of conjugates were determinated by SEC chromatographic system equipped with a 410 differential refractometer (DRI) from Waters (Milford, MA, USA) as concentration detector. A standard SEC method was used to determine molecular weight of conjugates consisting of DMF +0.01 M LiBr as mobile phase, 50 °C, 0.6 ml/min and two Phenogel columns from Phenomenex (5 μ particle sizes 10^3 and 10^4 of pores size). The molecular weights were estimated based on PEO/PEG standards (range 318.000–4.000 Da).

2.2. Materials

Gemcitabine HCl (2′-deoxy-2′,2′-difluorocytidine monohydrochloride) (GEM·HCl) was a gift of Eli Lilly. Folic acid, DL-aspartic acid, succinic anhydride, diglycolic anhydride, ε-aminocaproic acid, ethanolamine, 1,1′-carbonyldiimidazole (CDI), triethylamine (TEA) and acetone were purchased from Fluka (Buchs, Switzerland). Anhydrous *N*,*N*-dimethylformamide (DMF), anhydrous dimethylacetamide (DMA), diethylether, buthanol and Sephadex G-25 were from Aldrich (Steinheim, Germany). Methanol and chloroform were from Merck (Darmstadt, Germany). All other chemicals were reagent grade. Foetal calf serum was obtained from GIBCO (Grand Island, NY). Folate-depleted Dulbecco's modified Eagle's medium (DMEM), glutamine solution, penicillin–streptomycin–amphotericin B solution, trypsin solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sterile PBS and sodium dodecyl sulphate (SDS) were purchased from Sigma (St. Louis MO, USA).

PHEA was prepared and purified according to a previously reported procedure (Giammoma et al., 1987). The weight-average molecular weight of PHEA, measured by SEC, was 52,200 Da (M_w/M_n = 1.72).

Human plasma was obtained from voluntary healthy blood donors.

2.3. Synthesis of 5′-succinyl-2′-deoxy-2′,2′-difluorocytidine monohydrochloride (5′-succinylgemcitabine) (**1**)

Succinic anhydride (66.3 mg, 0.66 mmol) and TEA (31 μl) were added to a solution of 132 mg (0.44 mmol) of GEM·HCl in 8 ml of an 85:15 (v/v) anhydrous DMA/DMF mixture. The reaction mixture was kept under stirring for 24 h and then added of further 22.1 mg (0.22 mmol) of succinic anhydride and 31 μl of TEA. After 24 h stirring the mixture was dried under vacuum and the oily residue was treated with 8 ml of chloroform/diethylether 3:1 (v/v) and the fine precipitate dried. The reaction yield was 90% based on gemcitabine HCl. The final product was analysed by HPLC by using sodium acetate 0.4 M pH 5.6/MeOH, 90:10 (v/v) as mobile phase at a flow rate of 1 ml/min and monitoring the elute at λ = 269 nm. The product was characterised by mass spectrometry, elemental analysis, UV, IR and ^{1}H NMR spectroscopy.

The mass spectrum obtained by FAB technique showed the $M + H^+$ at *m/e* 399 corresponding to succinyl derivative of GEM·HCl.

Elemental analysis for C_{13} H_{16} F_2 N_3 O_7 Cl. Calculated: C, 39.09; H, 4.01; N, 10.52. Found: C, 43.11; H, 4.71; N, 11.89.

UV spectrum of **1** (methanol) showed an absorption in the range between 230 and 350 nm with a maximum at 269 nm.

IR [KBr]: 3300–3500 cm^{-1} (ν –OH, NH_2), 1734 cm^{-1} (ν C=O ester), 1710 cm^{-1} (ν C=O carboxylic), 1683 cm^{-1} (ν =N–C=O–N=).

^{1}H NMR (DMF-d_7) δ (ppm): 8.67 (s, 2H, ^{4}C–NH_2), 8.04 (d, 1H, H-6), 6.60 (s, 1H, $^{3'}$C–OH), 6.31 (d, 1H, H-1′), 6.15 (d, 1H, H-5), 4.45 (m, 1H, H-3′), 3.95 (m, 1H, H-4′), 3.88 (m, 2H, H-5′a, H-5′b), 2.66 (m, 4H, *protons of succinic group* –CO–CH2–CH2–CO–).

*2.4. Synthesis of 5'-diglycolyl-2′-deoxy-2′,2′-difluorocytidine monohydrochloride (5′-diglycolyl-gemcitabine) (**2**)*

Diglycolic anhydride (87.6 mg, 0.75 mmol) and 35 μl of TEA were added to a solution of 150 mg (0.50 mmol) of GEM·HCl in 9.5 ml of an 85:15 (v/v) anhydrous DMA/DMF mixture. The reaction mixture was kept under stirring for 22 h and then was added of further 29.2 mg (0.25 mmol) of diglycolic anhydride and 17 μl of TEA. After 22 h stirring the solvents were eliminated under vacuum and the oily residue was treated with 8 ml of a 3:1 chloroform/diethylether (v/v) mixture. The fine precipitate was dried under vacuum. The yield was 85% based on gemcitabine–HCl. The final product was analysed by HPLC by using sodium acetate 0.4 M pH 5.6/MeOH, 90:10 (v/v) with 1 ml/min flow rate and monitoring the eluate at λ = 269 nm. The purified product was characterised by mass spectrum, elemental analysis, UV, IR and ^{1}H NMR spectroscopies.

The mass spectrum obtained by FAB Technique shows the $M + H^+$ at *m/e* 416 corresponding to the diglycolyl derivative of GEM·HCl.

Elemental analysis for C_{13} H_{16} F_2 N_3 O_8 Cl. Calculated: C, 41.16; H, 3.85; N, 11.07. Found: C, 42.05; H, 4.15; N, 11.79.

UV spectrum of **2** (methanol) showed an absorption in the range between 230 and 350 nm with a maximum at 269 nm.

IR [KBr]: 3300–3500 cm^{-1} (ν –OH,–NH_2), 1730 cm^{-1} (ν C=O ester), 1717 cm^{-1} (ν C=O carboxylic), 1684 cm^{-1} (ν =N–C=O–N=).

^{1}H NMR (DMSO-d_6) δ (ppm): 7.71 (s, 2H, ^{4}C–NH_2), 7.53 (d, 1H, H-6), 6.56 (s, 1H, $^{3'}$C–OH), 6.15 (d, 1H, H-1′), 5.86 (d, 1H, H-5), 4.38 (m, 1H, H-3′), 4.24 (m, 1H, H-4′), 4.00 (m, 2H, H-5′a, H-5′b), 2.51-2.42 (m, 4H, *protons of diglycolic group*, –CO–CH_2–O–CH_2–CO–).

*2.5. Synthesis of PHEA-(5′-succinylgemcitabine) (**3**) and PHEA-(5′-diglycolyl-gemcitabine) (**4**) conjugates*

One milliliter of 1,1′-carbonyldiimidazole (CDI) solution in anhydrous DMF (66.2 mg/ml, 0.40 mol L^{-1} for the synthesis of conjugate **3** and 56.4 mg/ml, 0.113 mol L^{-1} for the synthesis of conjugate **4**) was added at 0 °C to 3 ml solution of **1** (45 mg/ml, 0.113 mol L^{-1}) or **2** (45 mg/ml, 0.097 mol L^{-1}), respectively in anhydrous DMF. The mixture was maintained under stirring at 0 °C for 30 min and then was added drop-wise to 2 ml of anhydrous DMF containing PHEA (135.2 mg, 0.0026 mmol, corresponding to 0.85 mmol of repeating units, for the synthesis of conjugate **3** and 114.6 mg, 0.0022 mmol, corresponding to 0.72 mmol of repeating units, for the synthesis of conjugate **4**). The reaction mixture was kept under inert atmosphere and stirring at 0 °C for 10 min, at 9 °C for 1 h and then was maintained at room temperature under inert atmosphere and stirring for 4 days. After this time the reaction mixture was added drop-wise to 100 ml of butanol. The obtained suspension was centrifuged at 4 °C for 3 min at 10,000 rpm, then treated several times with methanol, centrifuged and dried under low pressure. The obtained residue (135 mg of **3** and 120 mg of **4**) was dissolved in distilled water, purified by gel permeation on a Sephadex G-25 column (1 cm diameter, 80 cm length) and finally lyophilised. The final product was analysed by HPLC. The amount of unlinked drug in the conjugates was evaluated less than 0.2% (w/w).

Conjugates **3** and **4** were characterised by UV, IR and ^{1}H NMR spectroscopy.

UV spectrum of conjugate **3** in the range between 230 and 400 nm shows an absorption maximum at 267 nm, absent in the spectrum of PHEA. The UV spectrum of conjugate **4** in the range between 230 and 400 nm shows an absorption maximum at 269 nm, absent in the spectrum of PHEA.

IR spectrum (KBr) of conjugate **3** showed a broad band centred at 3300–3500 cm^{-1} (OH; –NH–; –NH_2) and bands at 1739 cm^{-1} (ν C=O ester group), 1660 cm^{-1} (amide I, PHEA) and 1545 cm^{-1} (amide II, PHEA). IR spectrum (KBr) of conjugate **4** showed a broad band centred at 3300–3500 cm^{-1} (OH; –NH–; –NH_2) and bands at 1739 cm^{-1} (ν C=O ester group), 1660 cm^{-1} (amide I, PHEA) and 1549 cm^{-1} (amide II, PHEA).

^{1}H NMR (DMF-d_7) of conjugate **3**, δ (ppm): 8.04 (d, 1H, H-6), 6.01 (d, 1H, H-1′), 5.68 (d, 1H, H-5), 4.75 (1H –NH–*CH*(CO)CH_2–), 4.32–3.99 (m, 4H, H-3′, H-4′, H-5′a, H-5′b), 3.60 (m, 2H, CH_2–*CH_2*–OH), 3.36 (m, 2H –NH–*CH_2*–CH_2–OH), 2.69 (m, 4H, *protons of succinic group*, –CO–CH2–CH2–CO–). ^{1}H NMR (DMF-d_7) of conjugate **4**, δ (ppm): 7.75 (d, 1H, H-6), 6.38 (d, 1H, H-1′), 5.50 (d, 1H, H-5), 4.75 (1H, *proton of PHEA* –NH–*CH*(CO)CH_2–), 4.16–3.99 (m, 4H, H-3′, H-4′, H-5′a, H-5′b), 3.52 (m, 2H, *protons of PHEA* –CH_2–*CH_2*–OH), δ 3.25 (m, 2H, *proton of PHEA* –NH–*CH_2*–CH_2–OH), 2.61–2.52 (m, 4H, *protons of diglycolic group*, –CO–*CH2*–O–*CH2*–CO–).

The content of linked drug in the conjugates was evaluated by alkaline hydrolysis, followed by HPLC and confirmed by UV analysis in water.

By alkaline hydrolysis, performed in KOH 0.1N, without degradation of GEM·HCl, the amount of linked drug was estimated equal to 9.4 molar percent for conjugate **3** and 9.1 molar percent for conjugate **4**; by UV analysis, comparing $E^{1\%}_{269}$ value of gemcitabine linked to the polymer ($E^{1\%}_{269} = 33.1 \pm 0.9$, in water) with that of GEM·HCl ($E^{1\%}_{269} = 245.0$, in the same medium), a value of linked drug equal to 13.5 and 13.1% (w/w), respectively were found corresponding to 9.6 molar percent for

conjugate **3** and 9.2 molar percent for conjugate **4** of linked drug.

2.6. Synthesis of the ε-aminocaproic acid-derivatized folic acid [(1′-carboxypentyl)-folamide] (**5**)

Folic acid (100 mg, 0.23 mmol) in 6 ml of 2:1 anhydrous DMSO/DMF mixture was reacted at 0 °C with 74 mg (0.45 mmol) of CDI dissolved in 2 of anhydrous DMF. To the mixture, kept under stirring at 0 °C for 1.5 h, a solution of 60 mg (0.49 mmol) of ε-aminocaproic acid in 4 ml of anhydrous DMSO was added drop-wise. The reaction mixture was maintained at room temperature under inert atmosphere and stirring for 17 h. The reaction time course was checked by TLC analysis (silica gel plate, methanol/chloroform 7/3 (v/v), being Rf values for folic acid and the capro-folate derivative of 0.1 and 0.4, respectively). After this time the reaction mixture was added drop-wise to 150 ml of a diethylether/acetone, 1:1 (v/v) mixture. The obtained suspension was centrifuged at 4 °C for 3 min at 10,000 rpm, washed twice with 40 ml of acetone, twice with 40 ml of acidified water (at pH equal to 3), once with 40 ml of distilled water and finally once with 40 ml of acetone. The suspension was finally centrifuged and dried under low pressure. The unreacted folic acid was separated by Chromatotron on silica Gel-60, using methanol as eluent. The obtained product (90 mg), was analysed by HPLC using as mobile phase H_3PO_4 0.1%/MeOH 60:40 (v/v), at a flow rate of 1 ml/min and monotoring the elute at λ = 364 nm.

The obtained mixture of α and γ (ε-aminocaproic acid)-derivatized folic acid was characterised by mass spectrum, elemental analysis, UV, IR and ^{1}H NMR spectroscopy.

The mass spectrum obtained by FAB Technique shows the $M + H^+$ at *m/e* 555.

Elemental analysis for $C_{25}H_{31}N_8O_7$. Calculated: C, 54.09; H, 5.58; N, 20.20; Found: C, 54.12; H, 5.61; N, 20.25.

UV analysis (water): the UV spectrum showed absorption in the range between 230 and 400 nm shows absorption with three maximum peaks at 256 nm (ε = 26,500), 276 nm (ε = 25,000) and 364 nm (ε = 8500).

IR [KBr]: 3300–3500 cm^{-1} (ν –OH, –NH–, NH_2), 1710 cm^{-1} (ν C=O carboxylic), 1650 cm^{-1} (ν C=O amidic).

^{1}H NMR (DMSO-d_6) δ (ppm): 8.64 (s, 1H, C7–H), 7.62 (d, 2H, *aromatic protons of 4-aminobenzoyl moiety*), 6.62 (d, 2H, *aromatic protons of 4-aminobenzoyl moiety*), 4.49 (d, 2H, C9–H_2), 4.31 (dd, 1H, C19–H), 3.01 (t, 2H, –CH_2ε), 2.28 (m, 2H, C22–H_2), 2.17 (t, 2H, –CH_2α), 2.08–1.85 (m, 2H, C21–H_2), 1.69–1.49 (m, 4H, –CH_2β, –CH_2δ), 1.34 (m, 2H, –CH_2γ).

2.7. Synthesis of PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide (**6**) and PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide (**7**) conjugates

A solution of **5** (mixture of α and γ isomers) (20.7 mg, 0.037 mmol, for the synthesis of conjugate **6** and 21.8 mg, 0.039 mmol, for the synthesis of conjugate **7**) in 3 ml of an anhydrous 2:1 (v/v) DMSO/DMF mixture was added at 0 °C to 1 ml solution of CDI (7.3 mg, 0.045 mmol, for the synthesis of conjugate **6** and 7.7 mg, 0.047 mmol, for the synthesis of conjugate **7**) in anhydrous DMF. The mixture was maintained under stirring at 0 °C for 30 min and then added drop-wise to a solution of **3** or **4** (66 mg, corresponding to 0.35 and 0.37 mmol of free repeating units of PHEA for **3** and **4**, respectively) in 3 ml of the same anhydrous mixture. The reaction mixture was maintained at room temperature under inert atmosphere and stirring for three days. After this time the reaction mixture was added drop-wise to 50 ml of diethylether and the obtained suspension was collected by centrifugation, then treated twice with 30 ml of methanol and twice with 30 ml of acetone, centrifuged and dried under low pressure. The obtained residue was dissolved in distilled water and purified by gel permeation on a Sephadex G-25 column and then lyophilised.

The amount of unlinked carboxypenthyl-folamide in the conjugates **6** and **7**, evaluated by HPLC, was less than 0.4% (w/w). The yield was about 88% (w/w) based on starting conjugate.

The final product was characterised by UV (water), IR (KBr) and ^{1}H-NMR spectroscopy.

The UV spectrum of conjugate **6** shows absorption in the range between 230 and 400 nm with two maximum peaks at 276 and 364 nm, that are absent in the spectrum of PHEA. The UV spectrum of conjugate **7** shows absorption in the range between 230 and 400 nm with two maximum peaks at 280 and 364 nm.

IR spectrum of conjugate **6** showed a broad band centred at 3300–3500 cm^{-1} (OH; –NH–; $-NH_2$) and bands at 1735 cm^{-1} (ν C=O ester group), 1717 cm^{-1} (ν C=O carboxylic group), 1656 cm^{-1} (amide I, PHEA) and 1549 cm^{-1} (amide II, PHEA). IR spectrum of conjugate **7** showed a broad band centered at 3300–3500 cm^{-1} (OH; –NH–; $-NH_2$) and bands at 1739 cm^{-1} (ν C=O ester group), 1721 cm^{-1} (ν C=O carboxylic group), 1660 cm^{-1} (amide I, PHEA) and 1549 cm^{-1} (amide II, PHEA).

^{1}H NMR (DMSO-d_6) of conjugate **6** δ (ppm): 8.64 (s, 1H, *proton of folic acid*, C7–H), 7.61 (d, 2H, *aromatic protons of folic acid*), 6.62 (d, 2H, *aromatic protons of folic acid*), 4.57 (1H, *proton of PHEA* –NH–*CH*(CO)CH_2–), 4.46 (d, 2H, *protons of folic acid* C9–H_2), 4.05 (m, 1H, *proton of gemcitabine*, H-3′), 3.75 (m, 1H, *proton of gemcitabine* H-4′), 3.61 (m, 2H, *protons of gemcitabine* H-5′a, H-5′b), 3.39 (m, 2H, *protons of PHEA* –CH_2–*CH_2*–OH), 3.12 (m, 2H, *protons of PHEA* –NH–*CH_2*–CH_2–OH), 2.65–2.52 (m, 4H, *protons of succinic group* –CO–*CH2*–*CH2*–CO–), 2.29 (m, 2H, *protons of folic acid* C22–H_2), 2.03–1.90 (m, 2H, *protons of folic acid* C21–H_2). ^{1}H NMR (DMSO-d_6) of conjugate **7** δ (ppm): 8.62 (s, 1H, *proton of folic acid* C7–H), 7.59 (d, 2H, *aromatic protons of folic acid*), 6.60 (d, 2H, *aromatic protons of folic acid*), 4.50 (1H, *proton of PHEA* –NH–*CH*(CO)CH_2–), 4.41 (d, 2H, *protons of folic acid* C9–H_2), 4.0 (m, 1H, *proton of gemcitabine* H-3′), 3.82–3.65 (m, 1H, *proton of gemcitabine* H-4′ *and* 2H, *protons of gemcitabine* H-5′a, H-5′b), 3.12 (m, 2H, *protons of PHEA* CH_2–*CH_2*–OH), 2.85 (m, 2H, *protons of PHEA* –NH–*CH_2*–CH_2–OH), 2.51–2.46 (m, 4H, *protons of diglycolic group* CO–*CH2*–O–*CH2*–CO–), 2.29 (m, 2H, *protons of folic acid* C22–H_2), 2.03–1.90 (m, 2H, *protons of folic acid* C21–H_2).

The content of linked GEM·HCl in the conjugates, estimated by alkaline hydrolysis in KOH 0.1N, followed by HPLC, was

estimated equal to 9.4 molar percent for conjugate **6** and 9.2 molar percent for conjugate **7**.

The content of linked folamide in this conjugates, evaluated by UV analysis, by comparing $E^{1\%}_{364}$ of folic acid linked to the polymer in the conjugate ($E^{1\%}_{364} = 15.2 \pm 0.2$ in water) with that of folic acid in the free form in the same medium ($E^{1\%}_{364} = 169.0$), was equal to about 9.0% (w/w).

Molecular weights of the two conjugates, determined by organic SEC analysis, as previously reported, were estimated equal to 34.7 kDa (polydispersity 1.8) for conjugate **6** and 31.8 kDa (polydispersity 1.9) for conjugate **7**, respectively.

2.8. Synthesis of PHEA-(1′-carboxypentyl)-folamide conjugate (8)

A solution of 20 mg (0.0357 mmol) of **5** in 3 ml of 2:1 (v/v) anhydrous DMSO/DMF mixture was added under stirring at 0 °C to a solution composed by 7.0 mg (0.043 mmol) of CDI in 1 ml of anhydrous DMF. The mixture was maintained under stirring at 0 °C for 30 min and then added drop-wise to a solution of 56 mg (corresponding to 0.35 mmol of repeating units) of PHEA in 3 ml of the same anhydrous mixture and thermostated at 0 °C. The reaction was then processed as reported above for conjugate **6**. The yield was 80% based on PHEA. The amount of free (1′-carboxypentyl)-folamide in the conjugate, evaluated by HPLC, was equal to 0.1% (w/w).

The final product was characterised by UV, IR and ^{1}H NMR spectroscopy.

UV analysis (water): the UV spectrum shows absorption in the range between 230 and 400 nm with three maximum peaks at 253, 278 and 364 nm, that are absent in the spectrum of PHEA.

IR spectrum (KBr) showed: a broad band centered at 3300–3500 cm^{-1} (OH; –NH–; $–NH_2$) and bands at 1736 cm^{-1} (ν C=O ester group), 1718 cm^{-1} (ν C=O carboxylic group), 1658 cm^{-1} (amide I, PHEA) and 1550 cm^{-1} (amide II, PHEA).

^{1}H NMR (DMSO-d_6) δ (ppm): 8.62 (s, 1H, *proton of folic acid* C7–H), 7.59 (d, 2H, *aromatic protons of folic acid*), 6.60 (d, 2H, *aromatic protons of folic acid*), 4.50 (1H, *proton of PHEA* –NH–*CH*(CO)CH_2–), 4.41 (d, 2H, *protons of folic acid* C9–H_2), 3.12 (m, 2H, *protons of PHEA* CH_2–*CH_2*–OH), 2.85 (m, 2H, *protons of PHEA* –NH–*CH_2*–CH_2–OH), 2.29 (m, 2H, *protons of folic acid* C22–H_2), 2.03–1.90 (m, 2H, *protons of folic acid* C21–H_2).

The content of linked folamide in the conjugate **8**, evaluated by UV analysis, by comparing $E^{1\%}_{364}$ of folic acid linked to the polymer in the conjugate **8** ($E^{1\%}_{364} = 20.3$ in water) with that of folic acid in the free form in the same medium ($E^{1\%}_{364} = 169.0$), was equal to about 12% (w/w) corresponding to about 4.1% (mol/mol).

2.9. Hydrolysis studies in buffer solutions at pH 5.5 and 7.4

The gemcitabine release from conjugates **3**, **4**, **6** and **7** was studied using 3.3 mg/ml conjugate solutions in 20 mM phosphate, 0.15 M NaCl buffer, pH 7.4 and 5.5.

The solutions were maintained at 37 ± 0.1 °C and analysed at scheduled intervals by HPLC for gemcitabine and folic acid release estimation. Each experiment was repeated in triplicate. The analysis was carried out using a C_{18} column eluted with sodium acetate 0.4 M pH 5.6/MeOH 90:10 (v/v), at 1 ml/min flow rate of and monitoring the eluate at 269 nm to evaluate the release of GEM·HCl and eluting with H_3PO_4 0.1%/MeOH 60:40 monitoring the elute at 364 nm to evaluate the release of folic acid. Quantification of the species was done by referring the peak area to a standard calibration curve.

2.10. Hydrolysis of conjugates (6) and (7) in plasma

The hydrolysis of conjugates **6** and **7** was studied also in human plasma.

Sample series of 0.5 ml phosphate buffered solutions at pH 7.4 (see above) containing PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide (**6**) (1 mg/ml) or PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide (**7**) (1 mg/ml) or free GEM·HCl (as control) (0.1 mg/ml) were added to 1.5 ml volumes of human plasma. The samples were kept at 37 ± 0.1 °C by a water bath, under continuous stirring and at suitable time intervals, added of 3 ml of 10% trifluoroacetic acid (v/v). The samples were stirred and then centrifuged for 5 min at 10,000 rpm at 4 °C. The supernatant, was filtered with a 0.45 μm filter membrane and analysed by HPLC, as above reported, to determine the amount of released GEM·HCl and folic acid.

All experiments were repeated in triplicate. The method was preliminarily validated by addition of known amounts of GEM·HCl or folic acid (or their derivatives **1**, **2**, or **5**) dissolved in 0.5 ml of phosphate buffered solutions at pH 7.4 (see above) to 1.5 ml of plasma which was processed and analysed according to the above reported procedure. The recovery of all compounds was more than 96% (w/w).

After 30 h of incubation, samples were added of 1 ml of 0.1N NaOH and analysed by HPLC to evaluate the complete drug release.

2.11. Cytotoxic assays

Human nasopharyngeal epidermal carcinoma KB cells were cultured as a monolayer in 75 cm^2 tissue culture treated flasks at 37 °C in a humidified atmosphere containing 5% CO_2 using folate-depleted Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 4 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml of amphotericin B. Folic acid concentration in folic acid-free medium was in the physiological range (∼2 nM). Cells were routinely treated with a 500 μg/ml trypsin in 0.02 M phosphate buffer, 0.15 M NaCl, 200 μg/ml EDTA (PBS), pH 7.4. Cells were cultured for 14 days in folic acid-depleted medium before any viability test was carried out. KB cells were seeded in 96-wells tissue culture treated plates at a density of 5×10^3 cells/well. After 24 h, the culture medium was replaced with 200 μl of foetal calf serum-free medium containing increasing concentrations of conjugates **3**, **4**, **6**, **7**, **8** (as negative control) and free GEM·HCl. The concentration of polymer bounded folic

acid in the test solutions ranged from 0 to 160 μM, while the concentration of polymer bounded GEM·HCl or free gemcitabine in the test solutions ranged from 0 to 505 μM. After 3.5 h incubation, the medium was removed and the cells were washed twice with 200 μl of PBS, pH 7.4. Fresh medium (200 μl) containing 10% foetal calf medium was added to each well and cells were cultured for further 48 h. MTT (5 mg/ml) in 20 μl of PBS was added to each well and the plates were incubated for 4 h at 37° C. Medium was then carefully removed from each well and replaced with 200 μl of 20 g/l SDS, 50% (v/v) DMF, pH adjusted to 4.7 by adding acetic acid. The plates were gently stirred overnight in order to dissolve the formazan crystals and absorbance was measured by a Bio-Tek Instruments microplate reader at 570 nm. The cell viability was expressed as percent of the samples/reference absorbance ratio. The reference was constituted by plain medium.

3. Results and discussion

The two macromolecular prodrugs, PHEA-(5′-succinyl-gemcitabine)-(1′-carboxypentyl)-folamide (**6**) and PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide (**7**) were prepared by covalent linkage of GEM·HCl and folic acid to the PHEA backbone.

GEM·HCl was used as anticancer drug model because of the analogy between its spectrum of activity against human solid tumours and the folate receptor over-expressing tumour cell lines (Hertel et al., 1990; Braakhuis et al., 1991; Csoka et al., 1995; Weitman et al., 1992). Despite its short half-life and haematological and renal toxicity, GEM·HCl is a promising antineoplastic drug approved for treatment of pancreatic and non-small cell lung cancers. Moreover this drug is active against a wide number of human solid tumours including colon, breast, bladder, ovarian, head and neck, cervical and hepatocellular cancers (Hertel et al., 1990; Braakhuis et al., 1991; Csoka et al., 1995).

Folic acid was conjugated to obtain bioconjugates with targeting properties to human cancer cells. Folic acid was attached to the polymer backbone through ε-aminocaproic acid as spacer to improve its interaction with the cell membrane receptor. GEM·HCl was linked to the polymer through two different hydrolysable spacers: succinic group in the case of PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide and diglycolic group in the case of PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide.

3.1. Synthesis of polymeric conjugates

The gemcitabine conjugation to PHEA carrier has been carried out by preparation of the two derivatives such as succinyl-gemcitabine and diglycolyl-gemcitabine followed by the conjugation of these GEM·HCl derivatives to the polymer using CDI as condensing agent. The chemical identification of the gemcitabine derivatives, degree of conjugation to PHEA, reaction yield and purity degree was performed by spectroscopic and chromatographic analysis.

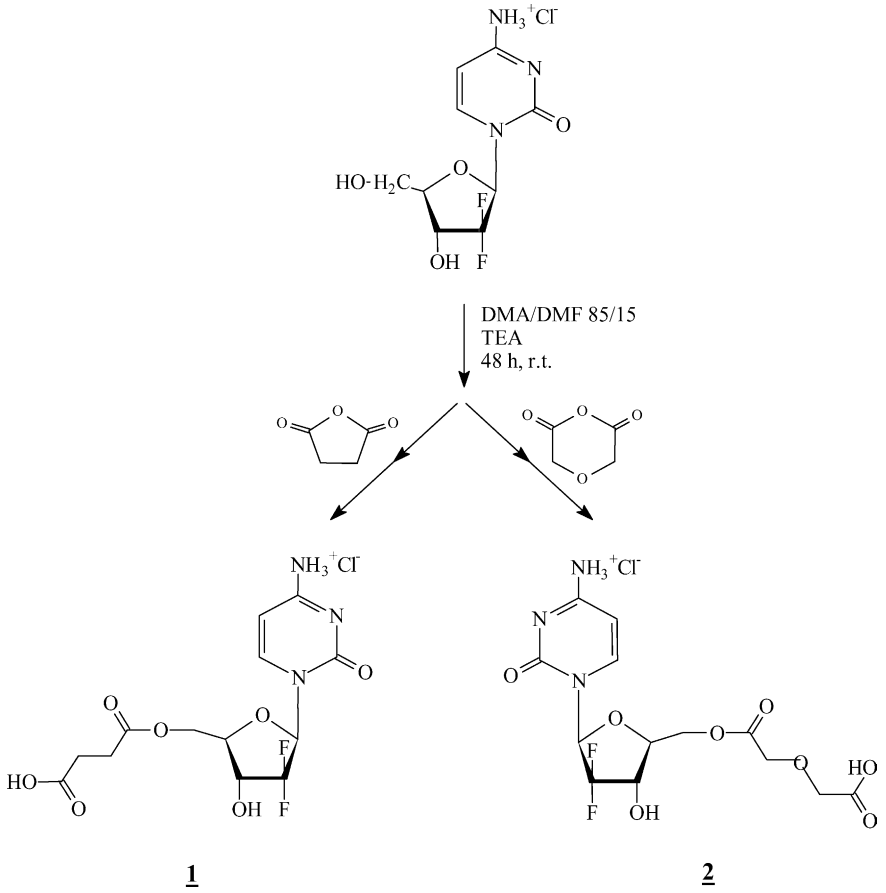
The GEM·HCl reaction with succinyl- or diglycolyl-anhydride was found to yield 95 and 94% of 5′-succinyl- and 5′-diglycolyl-gemcitabine derivatives, respectively. The higher reactivity of the anhydrides for the 5′ hydroxyl group of GEM·HCl as compared to the 3′ hydroxyl group limited the 3′-succinyl- and 3′-diglycolyl-gemcitabine isomers at the level of 5 and 6%, respectively (Guo and Gallo, 1999). Besides, the use of gemcitabine hydrochloride avoided the partial derivatization of amino group and the mild experimental conditions used in this synthesis exclude the formation of a difunctional derivative (two molecules of anhydrides and one of GEM·HCl) (Guo and Gallo, 1999).

The GEM·HCl derivatives conjugation to the polymer was efficient because about the 25% of used gemcitabine derivative was covalently linked to PHEA; besides the purification of the macromolecular prodrugs (PHEA-spacer-drug) was effective being the free GEM·HCl content about 0.1% (w/w) and 0.2% (w/w) in the case of the succinyl and diglycolyl derivative, respectively, with a yield over than 85% respect to starting PHEA. The content of linked drug in the former was calculated to be 9.4% (mol/mol) by alkaline hydrolysis and 9.6% (mol/mol) by UV analysis, corresponding to 13.5% (w/w). In the latter, the content of linked drug was evaluated to be 9.1% (mol/mol) by alkaline hydrolysis and 9.2% (mol/mol) by UV analysis, corresponding to 13.0% (w/w).

Folic acid was first functionalised with ε-aminocaproic and then conjugated to the polymer through the aminocaproic spacer. The conjugation of ε-aminocaproic acid to folic acid was carried out under proper reaction conditions to modify selectively γ-carboxylic group of folic acid. The α carboxyl group is, in fact, involved in the interaction with the folate receptor and its modification is detrimental for the biological recognition properties of this vitamin. The HPLC analysis of the folic acid-ε-aminocaproic derivative demonstrated the absence of free folic acid and that the reaction yielded a mixture of two derivatives: (1′-carboxypentyl)-α-folamide and (1′-carboxypentyl)-γ-folamide isomers in the ratio 80:20, respectively. This result is in agreement with what reported in the literature which indicates that, despite the selective reaction conditions, about 10–20% of the α derivative can be formed and that the mild experimental conditions used in this synthesis exclude the formation of a difunctional derivative (two molecules of 6-aminohexanoic acid and one of folic acid) (Wang et al., 1996). Since the difficulty of separation of the two folic acid isomers, the mixture of the two derivatives was used for conjugation to the PHEA backbone.

The conjugation of the folic acid-ε-aminocaproic derivative to the PHEA-gemcitabine derivatives was found to yield 8.9 and 9.1% (w/w) of linked folate in the succinyl and diglycolyl conjugate, respectively. The content of linked GEM·HCl in the final bioconjugate was confirmed to be 9.4 and 9.6% (mol/mol) in the succinyl and diglycolyl conjugate, respectively. After purification the PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide (**6**) and PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide (**7**) yield was over 65% based on starting PHEA and the amount of free (1′-carboxypentyl)-folamide was in the range of 0.3–0.4% (w/w) (Schemes 1–3).

Considering that the 5γ folammide derivative was the most abundant species in the mixture used for conjugation to PHEA



Scheme 1. Synthesis of 5′-succinylgemcitabine monohydrochloride (**1**) and 5′-diglycolyl-gemcitabine monohydrochloride (**2**).

and that the minor steric hindrance on carboxyl group of aminohexanoic residue makes more likely its involving in the conjugation reaction (in comparison with the unreacted carboxyl group of the folic acid), just the covalent attachment of this carboxyl group to PHEA is represented in Scheme 4.

The obtained polymeric derivatives: PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide, PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide and PHEA-(1′-carboxypentyl)-folamide (**8**) were highly water-soluble. Therefore, despite the extensive modification with the low soluble folic acid and GEM·HCl, the polymer maintains its high water solubility, which is a requisite for the use of the macromolecular prodrug.

3.2. *In vitro hydrolysis*

Hydrolysis studies were carried out to evaluate the drug and folic acid release from the macromolecular conjugates under different environmental conditions. The study was performed by incubation of the macromolecular PHEA derivatives in phosphate buffer solutions at pH 5.5 and 7.4. The two buffers were chosen to mimic the lisosomial and extracellular compartments, respectively.

Fig. 1 shows the GEM·HCl release profiles from conjugates **6** and **7** in buffer at pH 5.5 and 7.4. In order to evaluate whether gemcitabine hydrolysis rate in conjugates **6** and **7** was influenced by the folamide residue, hydrolysis studies were carried out also using derivatives **3** and **4**.

The profiles reported in Fig. 1 shows that the GEM·HCl release depends on the conjugation spacer and incubation pH. The diglycolyl derivative (**7**) underwent to slower gemcitabine release as compared to the succinyl derivative (**6**) and the hydrolysis rate was significantly lower at pH 5.5 than at pH 7.4.

The percentage of released gemcitabine after 30 h incubation was about 9% (w/w) from **7** and 12% (w/w) from **6** at pH 5.5, and 28% (w/w) from **7** and 31% (w/w) from **6** at pH 7.4. These

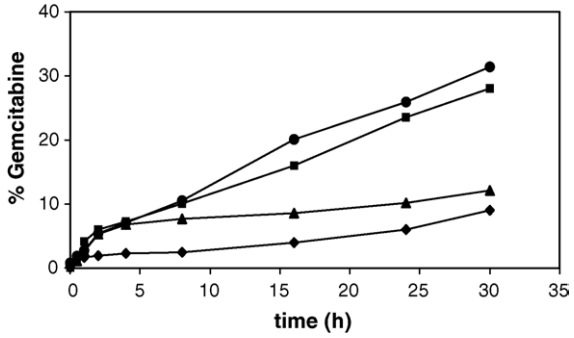
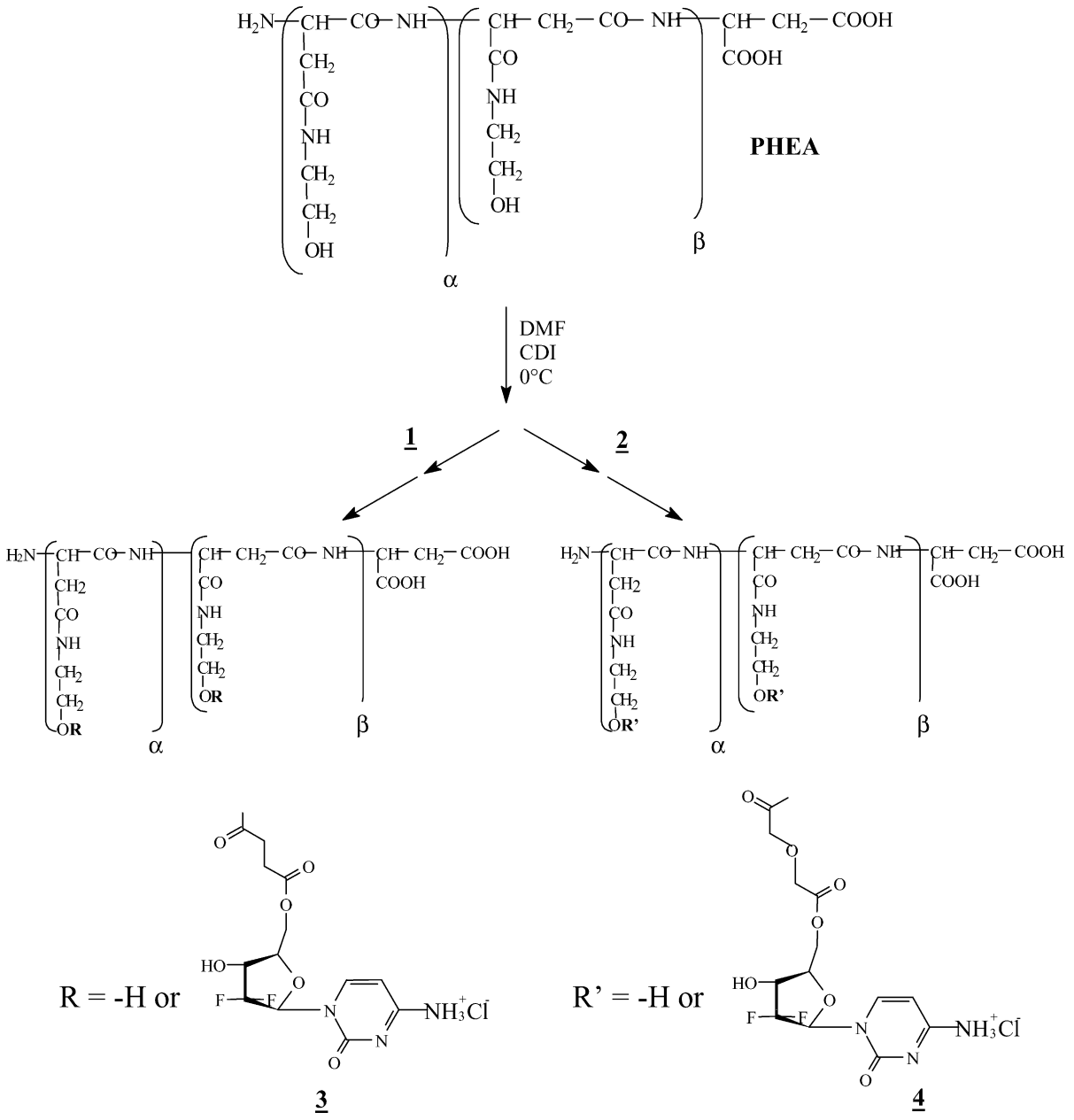


Fig. 1. Release of gemcitabine from conjugate PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide (**6**) at pH 7.4 (●) and at pH 5.5 (▲) and from conjugate PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide (**7**) at pH 7.4 (■) and 5.5 (♦).



Scheme 2. Synthesis of PHEA-(5′-succinylgemcitabine) (**3**) and PHEA-(5′-diglycolyl-gemcitabine) (**4**).

results were in agreement with that obtained with conjugates without the folate group (conjugates **3** and **4**) reported in Fig. 2.

After 30 h incubation the GEM·HCl release was 3% (w/w) from **4** and 8% (w/w) from **3** at pH 5.5 and 39% (w/w) from **4** and 56% (w/w) from **3** at pH 7.4. However, by comparing the data obtained with the conjugates with or without folic acid, it is possible to conclude that the presence of the folate group in the macromolecular structure influences the chemical stability of the ester bond between GEM·HCl and spacer. Probably, the hydrophobic folic acid confers to the macromolecule a different conformational arrangement as function of the incubation pH which can influence the drug release. At this regard it is important to stress that no release of folic acid and its folammide derivatives 5α and 5γ from conjugates **6** and **7** was detected after 30 h of incubation in plasma at 37 °C (data not shown).

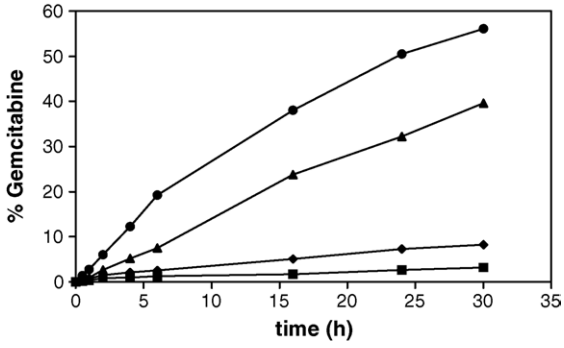
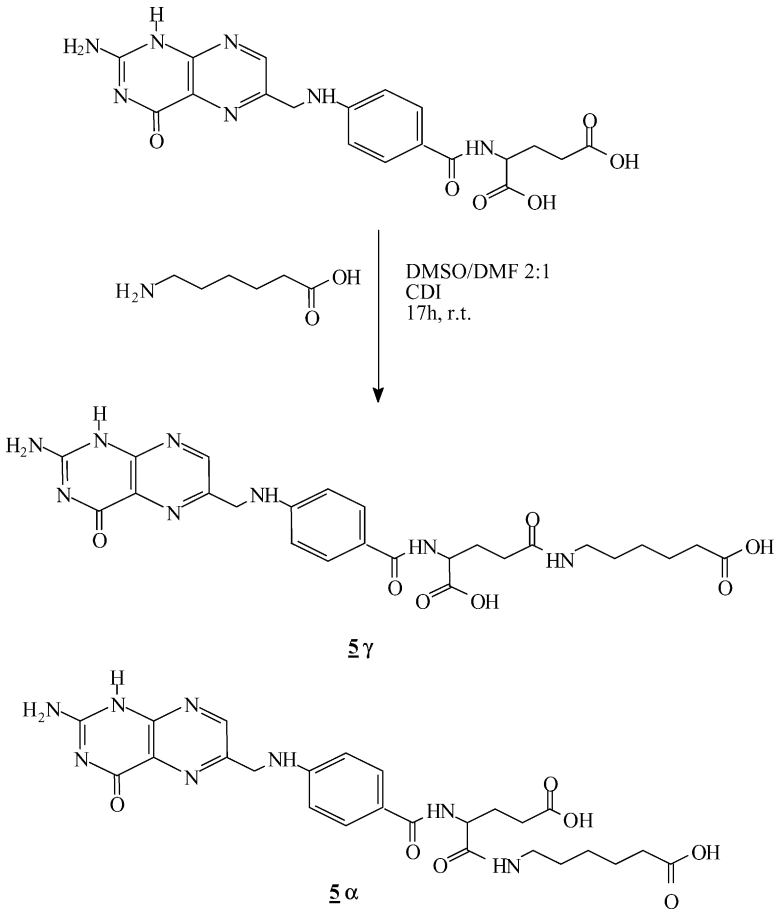


Fig. 2. Release of gemcitabine from conjugate PHEA-(5′-succinylgemcitabine) (**3**) at pH 7.4 (●) and at pH 5.5 (♦) and from conjugate PHEA-(5′-diglycolyl-gemcitabine) (**4**) at pH 7.4 (▲) and 5.5 (■).



Scheme 3. Synthesis of (1′-carboxypentyl)-folamide (**5**).

Fig. 3 reports the GEM release from conjugates **6** and **7** in plasma at 37 °C. According with the results discussed above, the release rate was significantly higher in the case of PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide conjugate. In particular, the amount of released drug within 30 h was about 16% (w/w) from conjugate **6**, but only 1% (w/w) from conjugate **7**. In addition the release of gemcitabine from conjugate PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide in plasma is faster than in aqueous buffer and this can be explained evoking the action of esterases in this medium.

However, after about 2 h, the GEM concentration reached a plateau which was maintained for about 28 h. To get more information about this behaviour, a stability study was carried out by GEM·HCl incubation in plasma. The results reported in Fig. 4 shows that GEM·HCl undergoes complete degradation in 7 h.

Therefore, in the case of our conjugates, it can be noted that the drug release kinetics reaches in a short time the plateau due to the rapid degradation of drug in plasma and subsequent establishment of GEM release/degradation dynamic equilibrium. Moreover in order to have more information about the

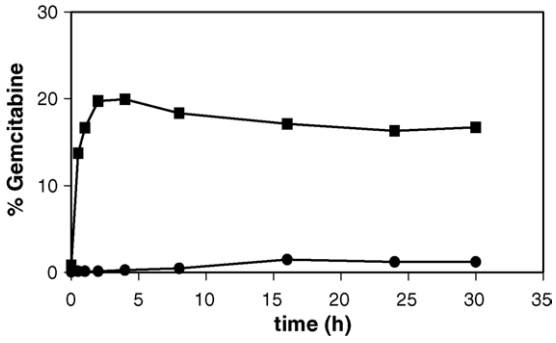


Fig. 3. Release of gemcitabine from conjugates PHEA-(5′-succinyl-gemcitabine)-(1′-carboxypentil)-folamide (**6**) (■) and PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentil)-folamide (**7**) (●) in plasma.

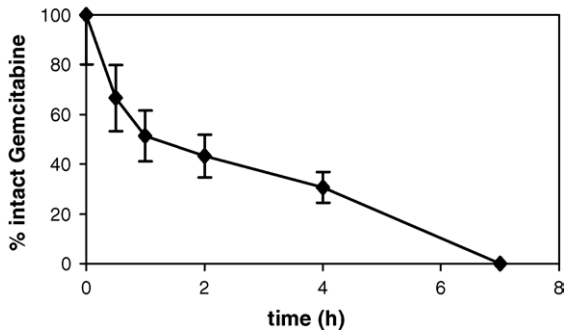
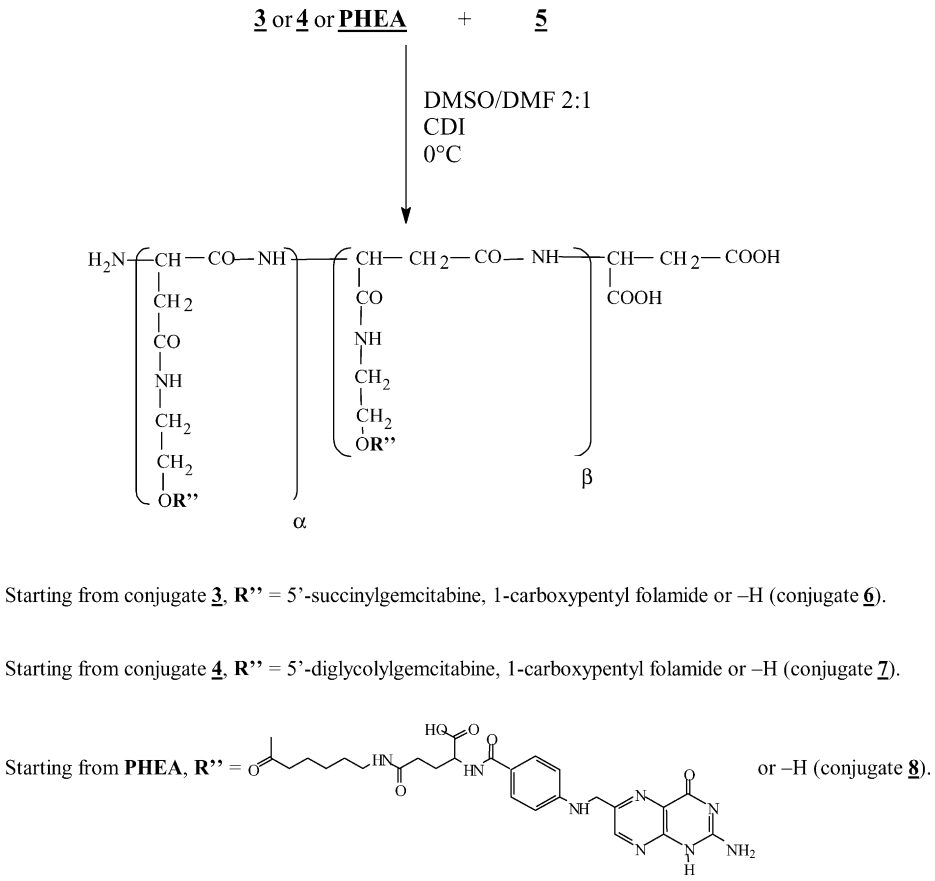


Fig. 4. Stability profile of gemcitabine in plasma.



Starting from conjugate **3**, **R''** = 5'-succinylgemcitabine, 1-carboxypentyl folamide or –H (conjugate **6**).

Starting from conjugate **4**, **R''** = 5'-diglycolylgemcitabine, 1-carboxypentyl folamide or –H (conjugate **7**).

Starting from **PHEA**, **R''** = or –H (conjugate **8**).

Scheme 4. Synthesis of PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide (**6**), PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide (**7**) and PHEA-(1′-carboxypentyl)-folamide (**8**) conjugates.

ability of the chosen polymeric carrier to protect remained linked drug, the amount of GEM still unreleased in the conjugates after 30 h of incubation was evaluated. This amount (estimated by HPLC, after alkaline hydrolysis, in not degrading drug conditions, i.e. KOH 0.1N for 30 min at 25 °C) was about 7.5% of the total linked drug for both conjugates. Therefore, it is possible to conclude that the macromolecular system provides for the drug stabilization and extensive release which can partially compensate the drug degradation.

Hydrolysis studies also demonstrated that only free GEM is released from the synthesized polymeric conjugates and no release of the gemcitabine derivatives containing the spacer (derivatives **1** and **2**) was observed in all incubation media.

3.3. Cytotoxic studies

The cytotoxicity of PHEA-gemcitabine conjugates was studied using human nasopharyngeal epidermal carcinoma KB cells, a cell line which over-expresses the folate receptor (Saikawa et al., 1995). The use of KB cells allowed for the evaluation of the targeting properties of the PHEA conjugates and their biological effect. According to the protocol reported in the literature for the BSA-folic acid uptake, the cell proliferation was evaluated after 3.5 h time exposure to different concentrations of free GEM·HCl and polymeric prodrugs PHEA-(5′-succinylgemcitabine) (**3**), PHEA-(5′-diglycolyl-gemcitabine) (**4**), PHEA-(5′-succinylgemcitabine)-(1′-carboxypentyl)-folamide (**6**), PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentyl)-folamide (**7**). The cell viability was estimated after further 48 h incubation in medium containing 10% foetal calf serum by the MTT assay (Hansen et al., 1989). PHEA-(1′-carboxypentyl)-folamide (**8**) was used as negative control at the same folic acid equivalent concentrations used with conjugates **6** and **7**. Actually, the literature reports that after about 30 min BSA-folic acid exposure the folate receptor saturation is obtained (Leamon and Low, 1991).

The effects of conjugates **6** and **7** on the KB cell viability are depicted in Fig. 5. For comparison, the results obtained with free GEM·HCl have been reported in the same figure. Free GEM·HCl as well as **7** and **8** did not display any activity in the concentration range used in the study. On the contrary conjugate **6** displayed a dose dependent cytotoxic effect. In particular, when the conjugate **6** concentration equivalent to 505 μM GEM·HCl concentration was used the cell viability decreased of about 35% as compared to the untreated cell. On the contrary, free GEM·HCl and conjugate **7** had only a negligible effect on the cell viability. These results can be explained on the basis of the gemcitabine stability and on the drug release rate. Actually, the lack of GEM·HCl activity on cell viability is attributable to the low stability of this drug which rapidly degrade under the experimental conditions. Also, the low incubation time adopted in the present study may limit the GEM·HCl efficiency as it has little

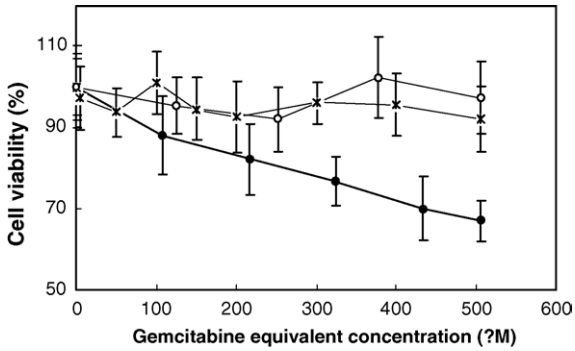


Fig. 5. Evaluation of viability of human nasopharyngeal epidermal carcinoma KB cells after incubation with gemcitabine (◯), PHEA-(5′-succinylgemcitabine)-(1′-carboxypentil)-folamide (●) and PHEA-(5′-diglycolyl-gemcitabine)-(1′-carboxypentil)-folamide (×).

permeability for cell membrane. The literature reports IC_{50} of 16.9 nM for KB cells over 72 h exposure (Miuraa et al., 1999) while in the present study the IC_{50} is higher than 500 μM.

The GEM·HCl conjugation to the macromolecular carrier was found to enhance the drug stability which improves its availability. The drug release studies discussed above demonstrated that when GEM·HCl is conjugated to PHEA via the succinic spacer its susceptibility to chemical and enzymatic hydrolisis is strongly increased. Therefore, the faster and efficient drug release from the succinic derivative can provide for a more significant cytotoxic effect than the diglicolyl derivative. Furthermore, the active bioconjugate up-take induced by the folic acid moiety and drug release through the succinyl-gemcitabine bond cleavage into the cell can be the reason of the high pharmacological activity. On the contrary, the diglycolic derivative was devoid of cytotoxic effect indicating that, though the carrier can be actively taken up by the cell, the drug is not released efficiently into the cell.

The relevance of the cell up-take mechanism in the biological performance of the pro-drug is supported by the results obtained with conjugates **3** and **4** (data not showed). Both conjugates did not display, in fact, cytotoxic activity, indicating that the cell-up-take of the macromolecule is a fundamental step to achieve a pharmacological response.

4. Conclusions

The covalent linkage of GEM·HCl and folic acid molecules to the polymeric carrier PHEA was found to endow macromolecular pro-drugs with interesting physico-chemical and pharmacological properties. Actually, the drug conjugation through an hydrolysable ester bond succeded in increasing the drug stability and availability providing for a sustained drug delivery. However, the therapeutic performance of the macrolomecular pro-drug depends on a combination of parameters: the chemical nature of the hydrolysable bond between drug and polymer and the introduction of targeting moieties into the macromolecular structure. The choice of the drug conjugation chemistry was found to play a fundamental role in dictating the drug release rate which determines the drug availability and, consequently, the pharmacological performance of the system. The folic acid conjugation was also essential for the biological activity of the therapeutic system. This targeting agent can in fact promotes the cell up-take of the macromolecule allowing for the cell feeding with the drug. Interestingly, the absence of targeting moieties which promote the target cell recognition yielded derivatives devoid of biological activity indicating that the pharmacological activity of the therapeutic system is strictly related to its cell-up-take.

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