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Macromolecular prodrugs V. Polymer-broxuridine conjugates

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Abstract

Broxuridine (BrdU) was covalently bound to α,β-poly[(2-hydroxyethyl)-DL-aspartamide] (PHEA) and α,β-poly[(2-aminoethyl)-DL-aspartamide]-α,β-poly[(2-hydroxyethyl)-DL-aspartamide] (PAHA). BrdU was first chemically modified to 3′-*O*-acetyl-5′-*O*-chloroformyl-5-bromo-2′-deoxyuridine (AcCBrdU) and 3′-*O*-acetyl-5′-*O*-phosphooxydichloride-5-bromo-2′-deoxyuridine (AcPBrdU). These compounds were bound to PHEA by carbonate and phosphodiester linkages, respectively. 5-Bromo-2′-deoxyuridine 5′-monophosphate (PBrdU) was linked to PAHA by an amide type bond. Neuroepithelial cells were used as a model system to assess the suitability of the conjugated BrdU for cell proliferation. Parallel experiments were performed with unconjugated BrdU and the extent of incorporation into DNA was determined by immunocytochemistry using an BrdU antibody. The results from these studies suggest that conjugated BrdU can be used as an alternative to currently used means of BrdU delivery.

Keywords: Polymeric prodrug; Polymer-drug conjugate; 5-Bromo-2′-deoxyuridine; α,β-Poly[(2-hydroxyethyl)-DL-aspartamide]; α,β-Poly[(2-aminoethyl)-DL-aspartamide]-α,β-poly[(2-hydroxyethyl)-DL-aspartamide]; Cell proliferation; Immunocytochemistry

1. Introduction

The use of macromolecular prodrugs in which the drugs are covalently linked to polymeric matrixes has many advantages compared with other drug delivery systems. The main advantage is their targetability. Using targetable polymer-drug conjugates it is possible to accumulate the drug at the site of the pathological process and to minimize its toxicity on normal tissue. One can also prolong drug release, decrease the required dose and increase drug solubility (Kopeček, 1982; Duncan and Kopeček, 1984; Kopeček and Duncan, 1987).

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; TrBrdU, 5′-*O*-trityl-5-bromo-2′-deoxyuridine; AcBrdU, 3′-*O*-acetyl-5-bromo-2′-deoxyuridine; PBrdU, 5-bromo-2′-deoxyuridine 5′-monophosphate; AcCBrdU, 3′-*O*-acetyl-5′-*O*-chloroformyl-5-bromo-2′-deoxyuridine; AcPBrdU, 3′-*O*-acetyl-5′-*O*-phosphooxydichloride-5-bromo-2′-deoxyuridine; L-Asp, L-aspartic acid; PSI, poly(2,5-dioxo-1,3-pyrrolidinediyl); PHEA, α,β-poly[(2-hydroxyethyl)-DL-aspartamide]; PAHA, α,β-poly[(2-aminoethyl)-DL-aspartamide]-α,β-poly[(2-hydroxyethyl)-DL-aspartamide]; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; TEA, triethylamine; DMF, *N,N*-dimethylformamide; DMEM, Dulbecco's modified essential medium

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In our previous papers, the use of α,β-poly[(2-hydroxyethyl)-DL-aspartamide (PHEA) as a drug carrier for carboxylic and amino acid drugs was described (Zorc et al., 1993a,b; Zorc and Butula, 1994). The drugs were linked to PHEA by ester bonds which readily underwent chemical and enzymatic hydrolysis, releasing the active compounds.

In this paper, we describe the attachment of broxuridine to PHEA and a newly synthesized copolymer α,β-poly[(2-aminoethyl)-DL-aspartamide]-α,β-poly[(2-hydroxyethyl)-DL-aspartamide (PAHA). Broxuridine (5′-bromo-2-deoxyuridine; BrdU) is a widely used antineoplastic agent and a marker for cell cycle flow studies (Gratzner, 1982; Dolbeare et al., 1983; Vanderlaan and Thomas, 1985). The most common way to detect cell replication in in vivo and in vitro studies involves the incorporation of tritiated thymidine, followed by autoradiography. Although this is a very useful method, its negative aspects are radioactive contamination, waste disposal and long exposure times. In an effort to avoid these problems, we have prepared a conjugated compound with BrdU which can be incorporated into DNA synthesizing cells and subsequently be detected using immunocytochemical techniques. The main reason for the development of a conjugated compound is to avoid multiple injections of BrdU in in vivo experiments and to minimize surgical manipulations (e.g., by implanting permanently installed cannulae) or to reduce the amount of polymer simultaneously administered with BrdU (e.g., in the case of local administration of microencapsulated BrdU).

2. Materials and methods

2.1. Materials

IR spectra were recorded on a Perkin-Elmer 457 and UV spectra on a Pye Unicam SP-100 spectrophotometer. Viscosity measurements were carried out at 25° C using an Ostwald viscosimeter, with a flow time for water from 100 to 200 s. Polymer solutions were dialyzed against several changes of deionized water using 18/22 inch Visking dialysis tubing (Serva, Heidelberg) with a molecular weight cut-off of 12000–14000 and lyophilized. For thin-layer chromatography (TLC) silica gel sheets (Kieselgel 60 F_{254}, Merck, Darmstadt) were used. Solvent systems were chloroform/methanol 9:1, 1:1 and 1:3. AcBrdU (**1**) was purified by column chromatography (silica gel 0.063–0.200 mm and ethyl acetate/benzene 6:4). L-Aspartic acid was purchased from Kemika (Zagreb), 5-bromo-2′-deoxyuridine, 5-bromo-2′-deoxyuridine 5′-monophosphate sodium salt and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) being obtained from Sigma (St. Louis, MO). All solvents were of analytical grade quality and were dried and distilled prior to use.

The cell line from the embryonic rat ventral mesencephalic area was used. Cell immortalization and BrdU labelling of cells were conducted as described by Almazan and McKay (1992). The cells were grown in DMEM (high glucose), 10% fetal bovine serum, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin and 1 mmol l^{-1} sodium pyruvate (Gibco, Toronto). Cells were grown in plastic wells (24 wells per plate). Cells were exposed to BrdU (307 ng ml^{-1}) or conjugates **4** and **7** (30 mg ml^{-1} corresponding to 1.9 mg ml^{-1} BrdU for **4** and 2.4 mg ml^{-1} BrdU for **7**). Cells were exposed to these compounds for 1, 24 and 48 h, respectively, before fixation. The immunocytochemical procedure was carried out using anti-BrdU (Boehringer, Mannheim) as primary antibody, followed by treatment with bispecific monoclonal antibody MoC10 (anti mouse IgG/anti-HRP) (Cuello and Cote, 1993). Cells in five fields were counted under a microscope (Leiz, Germany) and BrdU-labelled nuclei expressed as % of total number of cells (at 40 × magnification).

2.2. Chemistry

2.2.1. 3′-O-Acetyl-5-bromo-2′-deoxyuridine (AcBrdU) (1)

Compound **1** was prepared following the procedure described by Montgomery and Thomas (1967). The yield of 5′-*O*-trityl-5-bromo-2′-de-

oxyuridine (TrBrdU) was 69% and for AcBrdU, 43%.

2.2.2. 3'-O-Acetyl-5'-O-chloroformyl-5-bromo-2'-deoxyuridine (AcCBrdU) (2)

A solution of 0.105 g (0.0003 mol) AcBrdU (**1**) in 3 ml dry dioxane was added dropwise to 3 ml cold 20% phosgene/toluene solution. The reaction mixture was stirred for 30 min at 0° C and 2 h at room temperature. The solvent was evaporated in vacuo. A small amount of toluene was added twice and evaporated in order to remove traces of phosgene. The crude product was used in further reactions without purification. IR(KBr): ν_{max} 3510, 3195, 3040, 1710, 1670, 1620 cm^{-1}.

2.2.3. 3'-O-Acetyl-5'-O-phosphooxydichloride-5-bromo-2'-deoxyuridine (AcPBrdU) (3)

A mixture of 0.305 g (0.00085 mol) AcBrdU (**1**) and 0.347 g (0.0034 mol) TEA in 3 ml dry ether was added to a solution of 0.456 g (0.00297 mol) phosphorus oxychloride in 2 ml cold ether (−5° C). A small amount of sodium dithionite (10 mg) was added in order to prevent oxidation. The reaction mixture was kept for 40 min at −5° C and 19 h at room temperature. Solvent was evaporated and the crude product was immediately used in further reactions.

2.2.4. PHEA-AcCBrdU conjugate (4)

A solution of **2** (0.123 g, 0.0003 mol) in 2 ml dry DMF was added dropwise to a suspension of 0.142 g PHEA (**8**) in 20 ml pyridine at 0° C. The reaction mixture was stirred for 18 h at room temperature and evaporated under reduced pressure. The crude product was washed several times with acetone to give 0.084 g (33%) of **4**. UV: λ_{max} 277 nm ($A = 0.312$; $\gamma = 165$ $\mu g\ ml^{-1}$, H_2O).

2.2.5. PHEA-AcPBrdU conjugate (5)

A solution of **3** (0.396 g, 0.00085 mol) and 0.694 g (0.00687 mol) TEA in 10 ml dry DMF was added dropwise to a solution of 0.410 g PHEA in 20 ml dry DMF at 0° C. The reaction mixture was stirred at room temperature for 22 h and evaporated to give 0.52 g (72%) of product **5**. UV: λ_{max} 277 nm ($A = 0.258$; $\gamma = 211$ $\mu g\ ml^{-1}$, H_2O).

2.2.6. α,β-Poly[(2-aminoethyl)-DL-aspartamide]-α,β-poly[(2-hydroxyethyl)-DL-aspartamide (PAHA) (6)

To an ice-cold solution of 2.000 g PSI in 20 ml dry DMF 0.830 g (0.014 mol) 2-aminoethanol was added dropwise. The reaction mixture was stirred for 17 h at room temperature and added dropwise to a solution of 2.460 g (0.041 mol) 1,2-diaminoethane in 12 ml dry DMF. The solution was left at room temperature for 24 h and evaporated under reduced pressure. The crude product was washed several times with a small amount of acetone in order to remove excess amine. Yield: 1.130 g (35%).

2.2.7. PAHA-PBrdU conjugate (7)

A solution of 0.100 g (0.00024 mol) of PBrdU sodium salt and 0.231 g PAHA (**6**) in 2 ml 0.1 mol l^{-1} HCl was neutralized with NaOH (pH 7.5). EDAC (0.095 g) was added and the reaction mixture was kept in the dark for 24 h. Solvent was removed in vacuo. The crude product was dissolved in a small amount of glacial acetic acid and precipitated with acetone. The sticky product was dissolved in water and lyophilized to give a white, solid product **7** (0.165 g, 42%). UV: λ_{max} 281 nm ($A = 0.521$; $\gamma = 203$ $\mu g\ ml^{-1}$, H_2O).

3. Results and discussion

We report the synthesis of several polyaspartamide derivatives carrying BrdU through different kinds of spacers via carbonate, phosphodiester or amide type bonds. The first polyaspartamide used in our experiments was PHEA with a weight-average molecular weight of 54 850. The second was the newly synthesized copolymer PAHA (Fig. 1). One can consider PAHA as a PHEA derivative bearing both hydroxy and amino groups which widen the possibilities for use of the parental polymer as a drug carrier. PAHA was prepared by aminolysis of poly(2,5-dioxo-1,3-pyrrolidinediyl) (polysuccinimide; PSI) with 2-aminoethanol and subsequently with 1,2-diaminoethane. PSI was prepared by thermal polycondensation of L-aspartic acid in the presence of phosphoric acid under reduced pressure at 160° C

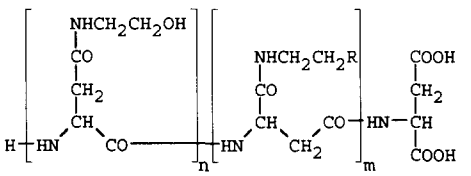
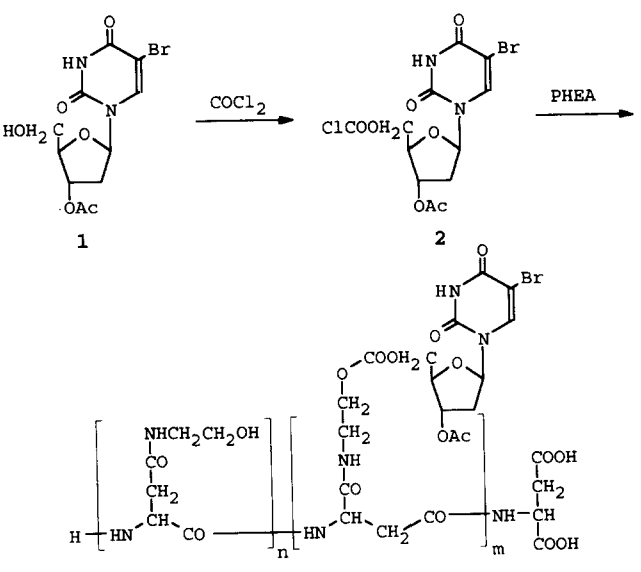


Fig. 1. Structure of PHEA and PAHA. R = OH, PHEA = α,β-poly[(2-hydroxyethyl)-DL-aspartamide]; R = NH_2, PAHA = α,β-poly[(2-aminoethyl)-DL-aspartamide]-α,β-poly[(2-hydroxyethyl-DL-aspartamide].

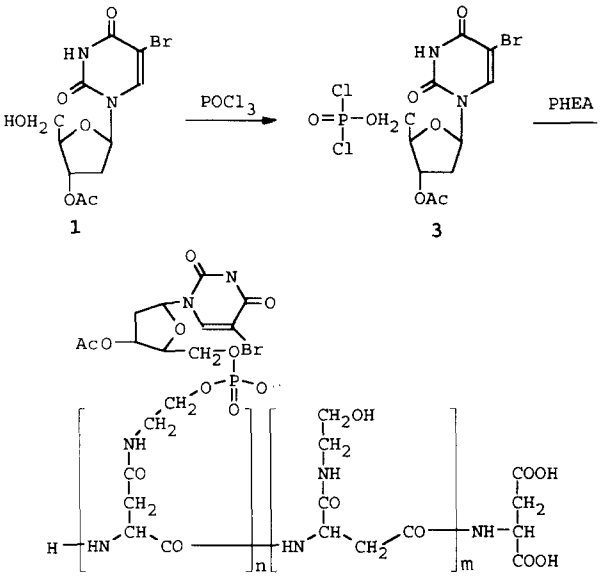
(Zorc et al., 1993b). The molar ratio of PSI monomer and 2-aminoethanol was 3:1 and partial ring opening occurred. 1,2-Diaminoethane was used to finish succinimide ring opening. Excess amine was necessary in order to prevent crosslinking. The aminolysis of PSI with 1,2-diaminoethane has previously been described (Kovacs et al., 1961).

The structure of BrdU offers the possibility of different chemical modifications. BrdU was firstly modified to 3′-*O*-acetyl-5-bromo-2′-deoxyuridine (AcBrdU) (**1**) via 5′-*O*-trityl derivative (TrBrdU) following the procedure of Montgomery and Thomas (1967). Compound **1** reacted with phosgene or phosphorus oxychloride and gave 3′-*O*-acetyl-5′-*O*-chloroformyl-5-bromo-2′-deoxyuridine (AcCBrdU) (**2**) and 3′-*O*-acetyl-5′-*O*-phosphooxydichloride-5-bromo-2′-deoxyuridine (AcPBrdU) (**3**), respectively. These products were allowed to react with PHEA in DMF/pyridine or DMF/TEA solutions affording polymer-drug conjugates PHEA-AcCBrdU (**4**) and PHEA-AcPBrdU (**5**) (Schemes 1 and 2). In conjugates **4** and **5** BrdU was bonded through carbonate and intermolecular or intramolecular phosphodiester linkages. On the other hand, BrdU was linked to PAHA by an amide type bond, actually an N-P bond (conjugate **7**). 5-Bromo-2′-deoxyuridine 5′-monophosphate (PBrdU) was coupled with PAHA by means of EDAC as the coupling agent (Scheme 3) (Halloran and Parker, 1966; Sheehan and Ledis, 1973).

The proof that BrdU was covalently bound to the polymeric backbone in compounds **4**, **5** and **7** could be found in the UV spectra. Compounds **4** and **5** had an absorption maximum at 277 nm as did BrdU itself, while the maximum of **7** was shifted to 281 nm. PHEA and PAHA had no UV absorption at those wavelengths. On the other hand, the TLC control demonstrated the absence of the free drugs. The drug loading in the conju-



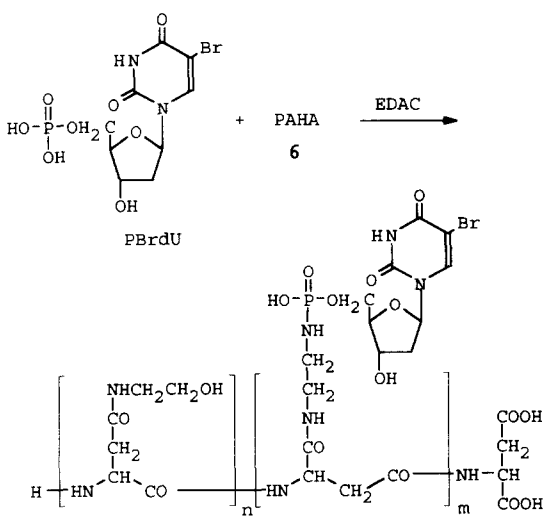
Scheme 1.



Scheme 2.

gates was also estimated by UV spectroscopy, using the molar absorption coefficient for BrdU of $\varepsilon_{277} = 10350$ in H_2O ($c = 4.57 \times 10^{-5}$ mol ml^{-1}). The loading of BrdU was 6.3% in **4**, 3.9% in **5** and 8.3% in **7**.

The suitability of conjugated BrdU for cell proliferation was tested using neuroepithelial cells



Scheme 3.

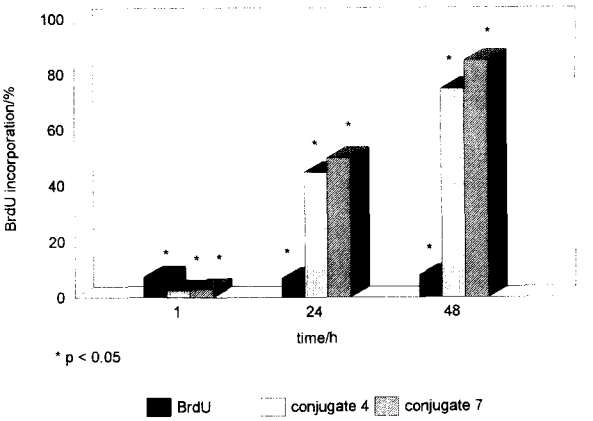


Fig. 2. BrdU incorporation into DNA in proliferating cells.

as a model system. Parallel experiments were performed with unconjugated BrdU. The incorporation of BrdU into DNA was determined by immunocytochemistry using an anti-BrdU antibody. The results are presented in Fig. 2. The total amount of conjugated BrdU in **4** and **7** was 6- and 8-fold higher, respectively, than in parallel experiments with free BrdU and the incorporation into cells after 48 h was 9.3- and 10.6-fold greater. One could conclude that there was a significant increase in the number of labelled cells in the presence of conjugated BrdU in the medium in comparison with labeling with BrdU alone. Furthermore, there were no significant differences between the proliferation indices between adducts **4** and **7**. The greatest extent of incorporation of BrdU with free BrdU was reached after 1 h and with conjugated BrdU after 2 days due to gradual drug release from the conjugates.

The use of BrdU for cell proliferation studies is increasing, mainly due to its advantages over [^{3}H]thymidine autoradiography which is time consuming and involves the use of radioactive material. However, in some cases, a single pulse of BrdU is not adequate and either repeated doses should be applied or osmotic mini-pumps should be installed. The results from these studies suggest that BrdU conjugated to water-soluble polymers can be used for chronic treatment in vivo as an alternative to currently used means of BrdU delivery.

References

Almazan, G. and McKay, R., An oligodendrocyte precursor cell line from rat optic nerve. *Brain Res.* 579 (1992) 234–245.

Cuello, A.C. and Cote, A., Preparation and application of conventional and nonconventional monoclonal antibodies. In Cuello, A.C. (Ed.), *Immunohistochemistry II*, IBRO, Wiley, Chichester, 1993, p. 136.

Dolbeare, F., Gratzner, H., Pallavicini and Gray, J.W., Flow cytometric measurement of total RNA content and incorporated bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA*, 80 (1983) 5573–5579.

Duncan, R. and Kopeček, J., Soluble synthetic polymers as potential drug carriers. In Dušek, K. (Ed.), *Polymers in Medicine*, Springer, Berlin, 1984, pp. 53–101.

Gratzner, H.G., Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science*, 218 (1982) 474–475.

Halloran, M.J. and Parker, C.W., The preparation of nucleotide-protein conjugates: carbodiimides as coupling agents. *J. Immunol.*, 96 (1966) 373–378.

Kopeček, J., Biodegradation of polymers for biomedical use. In Benoit, H. and Rempp, P. (Eds), *IUPAC Macromolecules*, Pergamon, Oxford, 1982, pp. 305–320.

Kopeček, J. and Duncan, R., Targetable polymeric prodrugs. *J. Controlled Release*, 6 (1987) 315–327.

Kovacs, J., Nagy Kovacs, H., Könyves, I., Császár, J., Vajda. T. and Mix, H., Chemical studies of polyaspartic acids. *J. Org. Chem.*, 26 (1961) 1084–1091.

Montgomery, J.A. and Thomas, H.J., Bis(pyrimidine nucleoside) phosphates. *J. Med. Chem.*, 10 (1967) 1163–1165.

Sheehan, J.C. and Ledis, S.L., Total synthesis of a monocyclic peptide lactone antibiotic, etamycin. *J. Am. Chem. Soc.*, 95 (1973) 875–879.

Vanderlaan, M. and Thomas, C.B., Characterization of monoclonal antibodies to bromodeoxyuridine. *Cytometry*, 6 (1985) 501–504.

Zorc, B. and Butula, I., Macromolecular prodrugs: III. Esters of fenoprofen and probenecid. *Acta Pharm.*, 44 (1994) 103–108.

Zorc, B., Antolić, S. and Butula, I., Macromolecular prodrugs: I. Synthesis of nonsteroidal anti-inflammatory drug esters. *Acta Pharm.*, 43 (1993a) 127–133.

Zorc, B., Ljubić, M., Antolić, S., Filipović-Grčić, J., Maysinger, D., Alebić-Kolbah T. and Jalšenjak, I., Macromolecular prodrugs: II. Esters of L-dopa and α-methyldopa. *Int. J. Pharm.*, 99 (1993b) 135–143.