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A polyphosphoester conjugate of melphalan as antitumoral agent

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ABSTRACT

The low molecular weight of many chemotherapeutics causes their untargeted distribution in the body and fast renal clearance, which leads to a loss of therapeutic activity and to unspecific toxic side effects. Therefore, there is a growing interest in conjugating anticancer drugs to water soluble polymers and thus, take advantage of the 'enhanced permeability and retention' (EPR) effect in tumors. In this study, water soluble polyphosphoesters were used as polymer carriers of melphalan hydrochloride (hydrochloride of *p*-bis(2-chloroethyl)amino-L-phenylalanine), which is a multifunctional alkylating agent. Melphalan was chemically immobilized by covalent bonding to poly(oxyethylene *H*-phosphonate) under Atherton-Todd reaction conditions. Novel polymer-melphalan complexes with ionic and hydrogen bonds were designed as controls, basing on two other biodegradable polyphosphoesters: poly(hydroxyoxyethylene phosphate) and poly(methyloxyethylene phosphate). The structure of the formed products was elucidated by ^{1}H, ^{13}C, ^{31}P NMR and FT-IR spectroscopy. The cytotoxic effect of the melphalan formulations was evaluated on different tumor cell lines. The novel polymer formulations showed a concentration dependent antitumoral activity, comparable to the effect of unmodified melphalan. The polymer-melphalan conjugate was also evaluated *in vivo* in the human hepatocellular carcinoma HuH7 xenograft mouse model. It improved the therapeutic efficacy of pure melphalan without causing side effects.

1. Introduction

Chemotherapy plays an important role in the treatment of cancer, but its clinical use is still limited. Conventional cytostatic drugs, being low-molecular-weight substances, distribute randomly in the body and have poor tumor selectivity. This leads to a low therapeutic efficacy and to strong toxic side effects, restricting the successful application of anticancer drugs (Gabor Miklos, 2005; Leaf, 2004). For a safe and more effective treatment, chemotherapy needs to be guided to the tumor tissue. Besides active targeting, cytostatic drugs can be targeted passively (Gianasi et al., 1999; Seymour et al., 1994) through the attachment to a polymeric carrier.

Polymer-anticancer drug conjugates were first described by Ringsdorf (1975). These conjugates usually include a water soluble polymeric carrier, a biodegradable linker and an antitumor drug (Duncan et al., 2005). The polymeric conjugation enhances the solubility of hydrophobic drugs (Meerum Terwogt et al., 2001) and improves their pharmacokinetic characteristics (Vasey et al., 1999). Thus, the circulation time is prolonged and the clearance hampered. Depending on a minimum molecular weight of the carrier, the drug is passively targeted to the tumor tissue through the EPR effect. Additionally, the polymer can protect the drug against degradation (Yurkovetskiy and Fram, 2009) and can overcome multidrug resistance (MDR) (Duncan, 2005b) by avoiding P-glycoprotein triggered export out of the cells.

The EPR effect was first reported by Matsumura and Maeda (1986). This effect is based on a special morphology of tumor vessels. To ensure a sufficient supply with nutrients, tumors possess several pathophysiological characteristics like intense angiogenesis (Folkman, 1971, 1995), hypervasculature with defective, leaky vessels and a reduced lymphatic drainage (Fang et al., 2003). These characteristics enable macromolecules, like polymer-drug conjugates, to reach the tumor cells through leaky vessels and to accumulate in the tumor tissue due to the impaired lymphatic drainage. Small molecules in contrast, pass through the endothelium of normal blood vessels into any tissue. This leads to an unspecific body distribution and a short plasma half-life due to fast renal clearance. Since its first description, the EPR effect is basis for modifications of diverse anticancer therapeutics (Greish et al., 2004; Harrington et al., 2001), including several drugs that are already evaluated in clinical trials (Duncan, 2003; Matsumura and Kataoka, 2009).

The nitrogen mustard melphalan, also known as 4-[bis(2-chloroethyl)amino]-L-phenylalanine, L-phenylalanine mustard, L-PAM,

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or L-sarcolysin, is a bifunctional alkylating agent with activity against several human neoplastic diseases, including multiple myeloma, ovarian cancer and malignant melanoma (Dollery, 1991; Gornati et al., 1997). The structure of melphalan incorporates an active nitrogen mustard moiety linked to L-phenylalanine. The drug induces cross-linking between bases in the complementary strands of a DNA molecule, as well as DNA–protein cross-links (Rothbarth et al., 2004; Sarosy et al., 1988). However, the therapeutic efficacy of melphalan is limited by toxic side effects and a low bioavailability and short half-life through reactions with nucleophiles such as water, proteins and thiols.

The current study refers to the incorporation and conjugation of melphalan to biodegradable, water soluble polyphosphoesters. Polymers with repeating phosphoester bonds (—CR_2—O—P—O—CR_2—) in the backbone are attractive candidates for biomedical and pharmaceutical applications because of their biocompatibility and structural similarity to bio-macromolecules, such as nucleic acids (Zhang et al., 2005; Zhao et al., 2003). Polyphosphoesters are structurally versatile and biodegradable under physiological conditions through hydrolysis and enzymatic cleavage at the phosphoester linkages (Kato et al., 2006; Koseva et al., 2008; Pencheva et al., 2008; Wen et al., 2004). They are especially attractive materials due to the relatively easiness of their preparation from commercially available low cost building blocks, and the variety of molecular weights attainable. These polymers possess reactive functional groups in their backbone, which allows conjugation of bioactive molecules to the chains and gives many opportunities for the preparation of new drug delivery systems with improved therapeutic indexes (Kato et al., 2006; Kraicheva et al., 2010; Leong et al., 1995; Koseva et al., 2008; Pencheva et al., 2008; Troev, 2006; Troev et al., 2007; Wang et al., 2001; Wen et al., 2003; Wen et al., 2004; Xu et al., 2003; Zhang et al., 2005; Zhao et al., 2003).

In this work, we report the synthesis and spectroscopic characterization of three novel melphalan phosphopolymer formulations (two complexes and one conjugate), and their *in vitro* antitumor evaluation. The noncovalent complexes served as well soluble melphalan formulation controls in these studies. The covalent conjugated melphalan (conjugate **4**) was also investigated in a human hepatocellular tumor xenograft model in mice to analyze its cytostatic effect *in vivo*.

2. Materials and methods

2.1. Starting materials

Because of the hydrolysis-sentivie nature of the investigated substances, special care was taken that the immobilization of melphalan (MPh) was performed under strictly controlled conditions – dry acetonitrile, room temperature and inert atmosphere, avoiding possible side reactions which may lead to inactivation of melphalan, or cleavage of the polymer chains and reduction in the molecular weight. Poly(oxyethylene H-phosphonate) **1** (PEGP-H), poly(hydroxyoxyethylene phosphate) **2**, and poly (methyloxyethylene phosphate) **3** were synthesized analogous to published procedures (Troev, 2006; Troev et al., 2007). Dimethyl H-phosphonate (Sigma Aldrich, Steinheim, Germany) was initially stirred over CaH_2 powder, then purified *via* vacuum distillation prior to use. Poly(ethylene glycol) with an average molecular weight of 600 g mol^{-1} (PEG 600) was purchased from Fluka and dried just before use by two-stage process: an azeotropic distillation with toluene and a subsequent 5 h heating at 120 °C under dynamic vacuum ($p < 0.1$ mmHg) to remove any traces of the solvent, while simultaneously bubbling a stream of dry argon through the heated liquid. Methanol and ethanol were dried by magnesium methoxide and stored over molecular sieves. Carbon tetrachloride, dichloromethane and acetonitrile were Sigma Aldrich products, and dried following standard procedures and distilled before use. Triethylamine (Fluka) were dried and distilled prior to use and stored over molecular sieves under argon atmosphere in round-bottomed flasks, equipped with pressure/vacuum glass stopcocks. Melphalan hydrochloride (MPh-HCl) was purchased from Sigma–Aldrich.

2.2. Spectroscopic characterization

^{1}H NMR, ^{31}P NMR, ^{13}C NMR, ^{1}H—^{1}H COSY45 NMR, ^{1}H—^{13}C HMQC NMR, ^{1}H—^{13}C HMBC spectra were recorded on JEOL Eclipse 400 MHz spectrometer operating at 400.18 MHz (^{1}H), 100.63 MHz (^{13}C) and 161.997 MHz (^{31}P) in $CDCl_3$ or d^6-DMSO solutions. Infrared spectra were recorded in KBr pellets on a Perkin Elmer Spectrum One FT-IR spectrophotometer.

2.3. Synthesis of poly(oxyethylene H-phosphonate) **1**

The synthesis of poly(oxyethylene H-phosphonate) **1**, was performed analogously as it has been described previously (Troev, 2006; Troev et al., 2007). The product was obtained as a waxy solid.

^{1}H NMR ($CDCl_3$), δ (ppm): 6.92 (d, $^1J_{PH}$ = 716.3 Hz, 1H, P-*H* of repeating unit), 6.85 (d, $^1J_{PH}$ = 709.0 Hz, 1H, P(*H*)OCH_3 end group), 6.78 (d, $^1J_{PH}$ = 700.5 Hz, 1H, P(*H*)OH end group), 4.12–4.26 (m, 4H, $CH_2OP(O)OCH_2$), 3.64–3.69 (m, 50H, CH_2OCH_2); ^{13}C{^{1}H}NMR ($CDCl_3$), δ (ppm): 70.6 (CH_2OCH_2), 70.2 (d, $^3J_{PC}$ = 5.4 Hz, $POCH_2CH_2$), 64.4 (d, $^2J_{PC}$ = 6.2 Hz, $POCH_2CH_2$); ^{31}P NMR ($CDCl_3$), δ (ppm) 10.6 (d of sextets, $^1J_{PH}$ = 709.0 Hz, $^3J_{PH}$ = 10.5 Hz, P(*H*)OCH_3 end group), 9.9 (d of quintets, $^1J_{PH}$ = 716.3 Hz, $^3J_{PH}$ = 9.9 Hz, P of repeating unit), 7.2 (dt, $^1J_{PH}$ = 700.5 Hz, $^3J_{PH}$ = 11.0 Hz, P(*H*)OH end group). IR (KBr) (cm^{-1}): 2439 P–H, 1251 P═O, 1106 CH_2OCH_2, P—O—CH_2.

2.4. Synthesis of poly(hydroxyoxyethylene phosphate) **2**

Poly(hydroxyoxyethylene phosphate) was synthesized as described in the literature (Troev, 2006; Troev et al., 2007). Quantitative conversion of **1** into **2** was achieved via Atherton-Todd reaction using water as nucleophile reagent. A solution of precursor **1** (2.39 g, 3.7×10^{-3} mol of repeating units) in dichloromethane (15 ml) was added dropwise within 3 h at ambient temperature under continuous stirring to a solution of carbon tetrachloride (15 ml), triethylamine (0.52 ml, 3.7×10^{-3} mol) and bidistilled water (0.066 ml, 3.7×10^{-3} mol) in a three-necked flask equipped with a magnetic stirrer, a thermometer, an inlet for inert gas and a reflux condenser. The reaction was allowed to proceed for 24 h. The reaction mixture was cooled to 4 °C to precipitate the triethylamine hydrochloride. After filtration of the precipitated triethylamine hydrochloride, the filtrate was concentrated and the polymer product **2** was precipitated by addition of diethyl ether. **2** was purified by dissolution in *N,N*-dimethylformamide and reprecipitation in diethyl ether. The isolated product was dried at 30–40 °C under reduced pressure (1 mmHg). The product obtained as a waxy solid. The product is very soluble in dichloromethane and ethanol, soluble in water, THF and N,N-dimethylformamide, but insoluble in diethyl ether. Yield 96%.

^{1}H NMR (d^6-DMSO), δ (ppm): 3.92–3.83 (m, 4H, CH_2OPOCH_2), 3.61–3.50 (m, 50H, CH_2OCH_2). ^{13}C{H} (d^6-DMSO), δ (ppm): 70.4 (CH_2OCH_2); 70.0 (d, $^3J_{PC}$ = .6 Hz, $POCH_2CH_2$), 64.6 (d, $^2J_{PC}$ = 6.2 Hz, $POCH_2CH_2$); ^{31}P{H} NMR (d^6-DMSO): δ = 1.2 ppm. IR (KBr) ν(cm^{-1}): 2675 P—OH, 1253 P═O, 1105 CH_2OCH_2, 1034 P—O—CH_2.

2.5. Synthesis of poly(methyloxyethylene phosphate) **3**

A typical procedure is described by Troev and co-authors (Troev, 2006; Troev et al., 2007). Quantitative conversion of **1** into