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28 April 2024

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Abstract

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Paclitaxel has been found to be very effective against several human cancers; one of the major problems with its use is its poor solubility, which makes necessary its solubilization with excipients that can determine allergic reactions often severe. The aim of this study is to develop highly water-soluble and less toxic analogues of paclitaxel. For this purpose we prepared a series of new

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paclitaxel-poly(ethylene glycol) (PEG) conjugates that were characterized and evaluated for their *in vitro* stability and cytotoxicity. In particular, in order to modulate the release of paclitaxel from prodrugs, we prepared different compounds introducing PEG in the drug C2' and/or C7 positions via ester or carbamate linkage. The conjugates were obtained in high purity and good yield. The carbamate prodrugs were highly stable in different media, while the compounds obtained linking

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PEG at C2' position through an ester bond showed lower stability. Finally, the cytotoxic activity of the conjugates was evaluated on two cancer cell lines and the results showed that all the derivatives had a reduced cytotoxicity compared to that of paclitaxel.

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1. Introduction

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Paclitaxel (PTX) is a natural product isolated from the bark of *Taxus breviflolia* (Pacific yew tree) and is today considered to be one of the most important drugs in cancer chemotherapy for the clinical treatment of many types of cancers (Kingston and Newman, 2007; Rowinsky, 1997). It is active against a number of cancer types including breast, lung, prostate, ovarian and some

50 leukaemias (Bonomi et al., 1997; Bookman et al., 1996; Nabholtz et al., 1996; Wani et al., 1971). At molecular level, PTX exerts its antitumor activity by interacting with tubulin (Schiff et al., 1979). In contrast to other anti-mitotic agents, such as Vinca alkaloids, which act to inhibit microtubule formation, PTX promotes tubulin polymerization and stabilizes the microtubules. Therefore, cell division is blocked in the late G2 mitotic phase of cell cycle (Kumar, 1981;

55 Manfredi and Horwitz, 1984). However, limited response rates and significant side effects are the major obstacles for more effective cancer therapy. Additionally, PTX's very low water solubility is a real problem in intravenous administration; PTX is currently administered in a vehicle containing Cremophor EL® (polyethoxylated castor oil) and ethanol. Significant side effects associated with hypersensitivity to Cremophor EL® have been observed (Dorr, 1994; Fjallskog et al., 1993), and

60 premedication with corticosteroids and antihistamines is often required (Weiss et al., 1990). In order to overcome these problems, new aqueous-based formulations for PTX, that do not require solubilization by Cremophor EL®, have been developed (Marupudi et al., 2007; Skwarczynski et al., 2006). The prodrug strategy is a promising way in terms of improving the drug solubility and keeping the pharmacological functions unaltered (Stella and Nti-Addae, 2007). Several reports of

65 water-soluble prodrugs of PTX have been reported that are considered to improve water solubility of the parent drug and to avoid the use of toxic detergents during administration (Vyas and Vittorio, 1995).

One of the most used polymers for prodrug delivery is poly(ethylene glycol) (PEG) (Greenwald et al., 2003). PEG is an amphiphilic polymer that is soluble in organic solvents as well as in water,

70 non-toxic and is eliminated from the body by a combination of renal and hepatic pathways; thus, this molecule is ideal to be employed in pharmaceutical applications; moreover, PEG has been approved by the FDA for human intravenous, oral and dermal applications (Hooftman et al., 1996). Some papers describe the different synthetic approaches adopted to covalently attach PEG to PTX: PEG of various molecular weights was linked to the C2' and/or C7 positions of PTX through

75 different bonds either directly or through suitable spacers (i.e. amino acids) or linkers (Feng et al., 2002; Greenwald et al., 1996; Greenwald et al., 1994; Li et al., 1996; Schoenmakers et al., 2004).

However, there are still some problems related to PEG-PTX prodrugs described in literature till now, such as for example poor stability or low improvement of solubility that lead to a limitation in their clinical use (Skwarczynski et al., 2006). Thus, a very attractive challenge in this field is still to obtain new stable water-soluble PTX conjugates with improved activity.

To reach this goal, the aim of this study was to develop highly water-soluble and less toxic analogues of PTX. For this purpose, we prepared a series of new PEG-PTX conjugates that were characterized and evaluated for their *in vitro* stability and cytotoxicity. In particular, in order to modulate the release of paclitaxel from prodrugs, we prepared several conjugates introducing PEG in the drug C2' and/or C7 positions using different synthetic routes and the properties of these derivatives are discussed, together with a preliminary examination of their *in vitro* antitumor activity.

2. Materials and methods

2.1. Materials and instruments

Unless stated otherwise, all reagents and solvents were obtained from commercial sources and were used without further purification. Paclitaxel was a gift from Indena (Milan, Italy). PEG derivatives (alpha-methoxy-omega-amino poly(ethylene glycol), m-PEG-NH$_2$ 5 and 20kDa) were purchased from IRIS Biotech GmbH (Marktredwitz, Germany).

All reactions requiring anhydrous conditions were performed under an Ar or N$_2$ atmosphere.

The reactions were monitored by thin-layer chromatography (TLC) on F$_{254}$ silica gel pre-coated sheets (Merck, Milan, Italy); after development, the sheets were visualized by irradiation by UV light and/or by exposition to iodine vapour. Flash-column chromatography was performed on 230-400 mesh silica gel (Merck).

HPLC analyses were carried out using a LiChroCART C18 column (250x4 mm i.d., 5 μm particle size) equipped with a C18 column guard (Merck) on a Merck-Hitachi HPLC system. The column was eluted using two solvents: water with 0.05% trifluoroacetic acid (TFA) (solvent A) and acetonitrile with 0.05% TFA (solvent B). The flow rate was maintained at 1 mL/min using a gradient protocol as follows: solvent A 90% for 5 min, a linear gradient from A 90% to A 10% for 30 min, A 10% for 10 min, a linear gradient from A 10% to A 90% for 5 min. The eluting fractions were monitored at 227 nm using an L4000UV detector. Peak heights and areas were recorded and processed on a CBM-10A Shimadzu interface (Shimadzu, Milan, Italy).

The ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker 300 Ultrashield instrument (Karlsruhe, Germany) in CDCl₃ solution at room temperature, with SiMe₄ as internal standard. UV-vis spectra were obtained on a Beckman 730 spectrophotometer (Beckman Coulter, Milan, Italy).

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2.2. Chemistry

2.2.1. Preparation of paclitaxel-2'-succinyl-NHS

120   2'-succinyl-paclitaxel (**1**) was prepared as reported elsewhere with modifications (Deutsch et al., 1989). Briefly, PTX (33 mg, 0.0386 mmol) was dissolved in 500 μL of dry pyridine to which 7.7 mg of succinic anhydride (0.0772 mmol) and 0.5 mg (0.00386 mmol) of 4-dimethylaminopyridine were added. The resulting solution was stirred for 3 h at room temperature. The product was purified by flash chromatography with elution in chloroform/methanol (90/10 v/v) to give 34.6 mg

125   of pure product, 94% yield. ^{1}H-NMR (CDCl₃): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 2.6 (m, 4H, COCH₂CH₂CO), 3.34 (d, 1H, C3-H), 4.17 and 4.28 (d, 2H, C20-H), 4.48 (dd, 1H, C7-H), 4.96 (d, 1H, C5-H), 5.51 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 5.80 (d, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-

130   OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

The carboxyl function of 2'-succinyl-paclitaxel (**1**) (20 mg, 0.0210 mmol) was activated in the corresponding *N*-hydroxysuccinimidil derivative (**2**) by reaction with *N*-hydroxysuccinimide (NHS) (3.2 mg, 0.0278 mmol) in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) (5.6 mg, 0.0269 mmol) in dry dichloromethane. The reaction mixture was stirred for 6 h at room temperature. After

135   filtration and evaporation the crude product was dissolved in dichloromethane and washed with brine and did not required any further purification step (19 mg, yield 85%). ^{1}H-NMR (CDCl₃): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 2.63 (m, 2H, 2'-OCOCH₂), 2.66 (m, 4H, NCOCH₂CH₂CO), 2.92 (m, 2H, CH₂CON), 3.34 (d, 1H, C3-H), 4.17 and 4.28 (d, 2H, C20-H), 4.48

140   (dd, 1H, C7-H), 4.96 (d, 1H, C5-H), 5.51 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 5.80 (d, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

2.2.2. Preparation of 4-nitrophenyl-carbonate paclitaxel derivatives

The different carbonate paclitaxel derivatives (**5**, **6** and **7**) were prepared following the method described by de Groot (de Groot et al., 2000) with minor modifications. The reactions were carried out under an argon atmosphere. PTX (50 mg, 0.0585 mmol) was dissolved in dry dichloromethane containing 4 drops of pyridine. For the preparation of 2'-(4-nitrophenyl carbonate)paclitaxel (**5**), 200 mg (1.06 mmol) of 4-nitrophenyl chloroformate in dry dichloromethane was added and the reaction proceeded for 5 h at -35°C. In the case of the synthesis of 2',7-(4-nitrophenyl biscarbonate)paclitaxel (**7**) PTX was reacted with 300 mg (1.59 mmol) of 4-nitrophenyl chloroformate for 24 h at room temperature. Then the reaction mixtures were washed with a solution of potassium bisulfate and dried with anhydrous magnesium sulfate. The solvent was then removed under reduced pressure and the crude products were purified by flash chromatography (hexane/ethyl acetate 55/45 v/v for **5**, 60/40 v/v for **6** and 70/30 v/v for **7**). 7-(4-nitrophenyl carbonate)paclitaxel (**6**) was directly obtained starting from the crude 2',7-(4-nitrophenyl biscarbonate)paclitaxel (**7**) that was left for one night in the column before purification.

Compound **5**, yield 65% (38 mg) ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.34 (d, 1H, C3-H), 4.17 and 4.28 (d, 2H, C20-H), 4.48 (dd, 1H, C7-H), 4.96 (d, 1H, C5-H), 5.51 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 6.10 (dd, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.35 (d, nitrophenyl), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz), 8.26 (d, nitrophenyl).

Compound **6** yield 74% (44 mg) ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.34 (d, 1H, C3-H), 4.17 and 4.28 (d, 2H, C20-H), 4.80 (d, 1H, C2'-H), 4.96 (d, 1H, C5-H), 5.26 (dd, 1H, C7-H), 5.67 (d, 1H, C2-H), 5.78 (d, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.35 (d, nitrophenyl), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz), 8.26 (d, nitrophenyl).

Compound **7** yield 86% (59 mg) ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.34 (d, 1H, C3-H), 4.17 and 4.28 (d, 2H, C20-H), 4.96 (d, 1H, C5-H), 5.28 (dd, 1H, C7-H), 5.53 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 6.15 (dd, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.35 (d, nitrophenyl), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz), 8.26 (d, nitrophenyl).

2.2.3. Preparation of 2'-[methoxypoly(ethylene glycol)amido-N-metyl-glycine carbamate] paclitaxel derivatives

Compound (**8**) was prepared by reaction of 2'-(4-nitrophenyl carbonate)paclitaxel (**5**) (15.5 mg, 0.0152 mmol) dissolved in 2 mL of dry dichloromethane with 8.3 mg (0.0456 mmol) of a solution of sarcosine *tert*-butyl-ester in dry dichloromethane containing triethylamine (85 μL, 0.608 μmol). The reaction proceeded under stirring and a nitrogen atmosphere for 2 h at room temperature. The reaction was washed with 0.1 N HCl, dried with anhydrous magnesium sulphate and evaporated under reduced pressure.

2'-(*N*-methyl-glycine carbamate)paclitaxel (**9**) was obtained by adding 58.5 μL (0.76 μmol) of TFA to a dry dichloromethane solution of **8** (16.2 mg, 0.0152 mmol) and the mixture was allowed to react for 1 h at room temperature. The mixture was then neutralized with 10% aqueous sodium bicarbonate solution and extracted with dichloromethane. The organic layer was dried over anhydrous magnesium sulphate and evaporated under reduced pressure**.** Yield 69% (10.3 mg) ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.2 (s, 3H, 2'-OCONCH$_3$), 3.34 (d, 1H, C3-H), 4.17 and 4.28 (d, 2H, C20-H), 4.32 (s, 2H, 2'-OCONCH$_2$), 4.48 (dd, 1H, C7-H), 4.96 (d, 1H, C5-H), 5.63 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 6.08 (dd, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

A solution of 2-ethoxy-1-(ethoxy-carbonyl)-1,2-dihydroquinoline (EEDQ) (3 mg, 0.0121 mmol) in 100 μL of anhydrous dimethylformamide (DMF) was added dropwise to 10 mg (0.0102 mmol) of **9** in 400 μL of DMF and the reaction was stirred for 30 min at room temperature. Then, 0.0120 mmol of m-PEG-NH$_2$ (5 or 20 kDa) in 100 μL of anhydrous DMF were added and the reaction proceeded for 24 h at room temperature. The course of reaction was followed by HPLC, which showed the presence of the conjugates **10** and **11**. The crude product was purified by HPLC and the collected fractions were dialyzed against water and lyophilized. Yield 63% ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.2 (s, 3H, 2'-OCONCH$_3$), 3.30 (s, PEG OCH$_3$), 3.34 (d, 1H, C3-H), 3.46-3.95 (m, PEG OCH$_2$CH$_2$O), 4.17 and 4.28 (d, 2H, C20-H), 4.39 (s, 2H, 2'-OCONCH$_2$), 4.48 (dd, 1H, C7-H), 4.96 (d, 1H, C5-H), 5.63 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 6.08 (dd, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

2.2.4. General procedure for the preparation of the PEGylated compounds

The previously prepared paclitaxel derivatives (**2**, **5**, **6** and **7**) dissolved in dry DMF were separately reacted with a dry DMF solution of m-PEG-NH$_2$ (5 and 20kDa); the PTX:PEG molar ratio was 1:2 for compound **7** and 1:1 for the other derivatives. The reaction mixtures were stirred for 12 h at room temperature. The course of reactions was followed by HPLC, which showed the presence of the different conjugates. The crude products were purified by HPLC and the collected fractions were dialyzed against water and lyophilized.

Compounds **3** and **4**, yield 68% ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 2.63 (m, 2H, 2'-OCOCH$_2$), 2.95 (m, 2H, CH$_2$CON), 3.30 (s, PEG OCH$_3$), 3.34 (d, 1H, C3-H), 3.45-4.1 (m, PEG OCH$_2$CH$_2$O), 4.17 and 4.28 (d, 2H, C20-H), 4.48 (dd, 1H, C7-H), 4.96 (d, 1H, C5-H), 5.51 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 5.80 (d, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

Compounds **12** and **13,** yield 55% ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.28 (s, PEG OCH$_3$), 3.34 (d, 1H, C3-H), 3.48-4.1 (m, PEG OCH$_2$CH$_2$O), 4.17 and 4.28 (d, 2H, C20-H), 4.48 (dd, 1H, C7-H), 4.96 (d, 1H, C5-H), 5.65 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 6.08 (dd, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

Compounds **14** and **15**, yield 48% ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.30 (s, PEG OCH$_3$), 3.34 (d, 1H, C3-H), 3.42-3.99 (m, PEG OCH$_2$CH$_2$O), 4.17 and 4.28 (d, 2H, C20-H), 4.80 (d, 1H, C2'-H), 4.96 (d, 1H, C5-H), 5.42 (dd, 1H, C7-H), 5.67 (d, 1H, C2-H), 5.78 (d, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

Compounds **16** and **17**, yield 50% ^{1}H-NMR (CDCl$_3$): δ 1.11 (s, 3H, C17-H), 1.19 (s, 3H, C16-H), 1.62 (s, 3H, C19-H), 1.76 (s, 3H, C18-H), 2.2 (m, 2H, C14-H), 2.23 (s, 3H, C10-OAc), 2.43 (s, 3H, C4-OAc), 3.28 (s, PEG OCH$_3$), 3.34 (d, 1H, C3-H), 3.45-4.1 (m, PEG OCH$_2$CH$_2$O), 4.17 and 4.28 (d, 2H, C20-H), 4.96 (d, 1H, C5-H), 5.42 (dd, 1H, C7-H), 5.65 (d, 1H, C2'-H), 5.67 (d, 1H, C2-H), 6.08 (dd, 1H, C3'-H), 6.21 (t, 1H, C13-H), 6.27 (s, 1H, C10-H), 7.07 (d, 1H, NH), 7.3 (m, 3'-Ph), 7.4 (m, 3'-NBz), 7.5 (m, 2-OBz), 7.73 (d, 3'-NBz), 8.1 (d, 2-OBz).

2.3. Water solubility and stability of conjugates

Water solubility was estimated by dissolving appropriate amounts of conjugates in 0.1 mL of water. The hydrolysis rate of conjugates was determined at different pH values, using sodium acetate buffer 0.1 M (pH 5.6), sodium phosphate buffer 0.1 M (pH 7.4), sodium borate buffer 0.1 M (pH 9) or fetal calf serum. Conjugates were dissolved at a concentration of 1 mg/mL. Drug stability was determined by removing portions of samples from the solutions incubated at 37°C for various periods of time. 20 µL of sample were withdrawn from the solution of the different buffers and injected into HPLC. To the samples incubated in serum 200 µL of acetonitrile were added to precipitate proteins, and the resulting solution was vortexed for 30 s and centrifuged at 330 x *g* for 5 min. 150 µL of supernatant were analyzed by HPLC using the conditions already described.

2.4. Tumor cell lines and cell culture

The cell lines used were MCF-7, a human breast cancer, and HT-29, a human colorectal adenocarcinoma. Both cell lines were maintained in RPMI 1640 medium containing 10% fetal calf serum and 1% antibiotics (containing penicillin and streptomycin) in a 5% CO_2 humidified atmosphere at 37°C.

2.5. Cytotoxicity test

MCF-7 and HT-29 cells, maintained as described above, were seeded at 3×10^4 cells/well in microtiter plates and incubated overnight to allow cellular adhesion. Various dilutions of PTX (in dimethylsulfoxide) and conjugates (in water) (expressed as paclitaxel concentration) were added in triplicate, and incubated for 72 h. The supernatants were removed and the cells washed and incubated for 16 h with fresh medium containing 1 mCi of L-[4,5-^{3}H]-leucine (58 Ci/mmol). The cells were harvested using with a Skatron Harvester and the incorporated radioactivity was measured using a Packard-2500 TR Liquid Scintillation Analyzer. The results were expressed as percentages of L-[4,5-^{3}H]-leucine incorporation compared to control cultures, background values being subtracted. Data are means of three separated experiments, in which each individual value is the average of triplicate samples (<7% standard error).

3. Results and discussion

3.1. Chemistry

The schematic structures of the new PEG-PTX prodrugs are reported in Figure 1.

Prodrugs **3** and **4** were prepared starting by the reaction of the 2'-hydroxy function of PTX with succinic anhydride according to the procedure of Deutsch (Deutsch et al., 1989). In order to facilitate the further PEGylation reaction, the carboxyl function of 2'-succinyl-paclitaxel was activated as *N*-hydroxysuccinimidil derivative (PTX-NHS, **2**) using NHS and DCC. PTX-NHS was finally reacted with m-PEG-NH$_2$ (5 and 20 kDa) in DMF to give the desired prodrugs (Scheme 1).

The different PEG-carbamate prodrugs (**12**-**17**) were obtained by preliminary activation of PTX C2' and/or C7 hydroxyl groups with 4-nitrophenyl chloroformate (Scheme 2, compounds **5**, **6** and **7**) and consequent reactions with m-PEG-NH$_2$.

The activated 2'-carbonate (**5**) was obtained reacting PTX with 4-nitrophenyl chloroformate at -35°C, as reported by de Groot (de Groot et al., 2000). The same reaction was performed at room temperature to prepare the 2',7-disubstituted compound (**7**). We were able to obtain the 7-carbonate derivative (**6**) in a single step during purification of the 2',7-disubstituted carbonate by flash chromatography; in fact, during the purification we observed the formation of the 7-(4-nitrophenyl carbonate)paclitaxel obtained by the hydrolysis of the more reactive compound at 2'-position from 2',7-(4-nitrophenyl biscarbonate)paclitaxel. To this aim the crude product was left for about 12 h in the column before purification. This is an easy and less time-consuming procedure for the activation of the hydroxyl group of PTX in 7, since most of the methods reported in literature required the previous protection of 2'-position of PTX (Altstadt et al., 2001; Lee et al., 2005; Ryu et al., 2008; Wang et al., 2006). Other works describe the preliminary preparation of the 2',7-disubstituted derivative and the further removal of the more chemically labile substituent at the 2'-position, but cleavage, reaction work up and purification were necessary to obtain the desired compound (Mathew et al., 1992; Niethammer et al., 2001; Takahashi et al., 1998). Our approach is more flexible and permits to obtain the di- or mono- substituted derivative easily during column purification process.

The preparation of PEG-PTX prodrugs **10** and **11** is reported in Scheme 3. The introduction of a methyl group as steric hindrance on the carbamate nitrogen was obtained by reacting 2'-(4-nitrophenyl carbonate)paclitaxel (**5**) with sarcosine *tert*-butyl-ester, then the protective group was cleaved with TFA and the corresponding derivative **9** was reacted with m-PEG-NH$_2$ (5 and 20 kDa) in DMF in presence of EEDQ to give the corresponding PEGylated compounds. We decided to

insert a steric hindrance in the 2'-carbamate PEG-PTX prodrugs because the unhindered derivatives **12** and **13** were highly unstable in buffer solution at pH 7.4 at 37°C. In fact, in this conditions we observed the rapid formation of the inactive compound baccatin III, due to an addition-elimination sequence as yet reported by de Groot (de Groot et al., 2000). The authors demonstrated that the insertion of a methyl group prevented this unwanted reaction giving more stable derivatives. A quite similar approach was described by Greenwald in the preparation of hindered PEG-camptothecin diesters (Greenwald et al., 1998).

Compounds **14**-**17** were prepared by reacting m-PEG-NH$_2$ with the different carbonate PTX derivatives in order to obtain derivatives characterized by a carbamate linkage and by the presence of PEG in the C2' and/or C7 positions of the drug.

All the purified PEG-PTX conjugates were lyophilized and were stable for several months when stored at -20°C under a nitrogen atmosphere.

3.2. Water solubilty and in vitro stability of PEG-PTX prodrugs

The conjugation of PEG to PTX dramatically increased its aqueous solubility; in fact the prodrugs obtained using PEG of 5kDa of molecular weight showed a solubility of about 340 mg/mL, in the case of monosubstituted compounds, and of 460 mg/mL for the disubstituted ones. The 20 kDa PEG-PTX conjugates showed a decrease in solubility values (150 and 180 mg/mL for mono- and di-substituted, respectively). A similar behavior was also observed for a reported series of PEG-PTX prodrugs, in which the water solubility decreased with the increasing of the polymer's molecular weight (Greenwald et al., 1996).

The *in vitro* stability of the different PEG-PTX conjugates was studied evaluating by HPLC the release of PTX, after incubation at 37°C in buffer at various pH values (5.6, 7.4 and 9) or in serum; the results are shown in Table 1. As previously reported by Greenwald (Greenwald et al., 1996), we also observed that the half-life of the conjugates did not depend on the PEG's molecular weight. The prodrugs **14**-**17** were highly stable and the PTX release after 72 h of incubation was about 3-6% at pH 7.4 and 10% (**14**, **15**) or 3% (**16**, **17**) in serum, indicating that we were able to obtain soluble prodrugs that also provide a slow release of PTX. On the other hand, compounds **10** and **11** released 100% of PTX after 48h, but no baccatin III formation was observed indicating that the introduction of a methyl group as steric hindrance provided a protection of the conjugates. The unhindered conjugates **12** and **13**, on the contrary, gave a rapid release of inactive baccatin III at pH 7.4, that reached 100% after 48h, so these conjugates were not further evaluated for their cytotoxicity.

These experiments clearly demonstrate the higher stability of carbamate linkage over the ester bond (compounds **3** and **4**) and also indicate that the modification of the more hindered C7 position lead to reduction of hydrolysis rate of the conjugates as also reported by other authors (Greenwald et al., 1995; Sugahara et al., 2002). The stability was further improved with the introduction of PEG at both C2' and C7 positions.

3.3. Cytotoxicity

The cytotoxic activity of PEG-PTX conjugates was evaluated on MCF-7 and HT-29 human cancer cell lines after 72 h incubation at 37°C. Free PTX was also tested as control. The results, reported in Table 2, show that PTX was very active and that the prodrugs **3** and **4** display a quite similar toxicity; these data are in accordance with the stability experiments that indicated that PTX was rapidly released from the conjugates **3** and **4**. On the contrary, an important decrease in the cytoxicity (from two to four orders) was observed in the PEG-carbamate prodrugs, in particular for the compounds obtained reacting the C7 position of PTX (**14** and **15**); a reduction in cytotoxic activity of C7 ester or carbamate derivatives was yet reported by other research groups (Greenwald et al., 1996; Greenwald et al., 1995).

Taken together these results show that these PTX derivatives are very stable and less toxic and that they do not need toxic excipients for their formulation as a consequence of the increased aqueous solubility. Thus, these compounds could be of great interest as PTX prodrugs and deserve further investigations in order to evaluate their *in vivo* activity.

4. Conclusions

A series of new PEG-PTX prodrugs characterized by different linkages were prepared using various and sometimes less time-consuming synthetic approaches. The *in vitro* studies showed that these prodrugs are characterized by a good solubility, slow PTX release and low toxicity.

In vivo studies are in progress to deeply investigate the activity and the pharmacokinetic behavior of these compounds.

Acknowledgements

References

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Altstadt, T.J., Fairchild, C.R., Golik, J., Johnston, K.A., Kadow, J.F., Lee, F.Y., Long, B.H., Rose, W.C., Vyas, D.M., Wong, H., Wu, M.J., Wittman, M.D., 2001. Synthesis and antitumor activity of novel C-7 paclitaxel ethers: Discovery of BMS-184476. Journal of Medicinal Chemistry 44, 4577-4583.

390   Bonomi, P., Kim, K., Kusler, J., Johnson, D., 1997. Cisplatin/etoposide vs paclitaxel/cisplatin/G-CSF vs paclitaxel/cisplatin in non-small-cell lung cancer. Oncology (Williston Park, N.Y.) 11, 9-10.

Bookman, M.A., McGuire, W.P., Kilpatrick, D., Keenan, E., Hogan, W.M., Johnson, S.W., Odwyer, P., Rowinsky, E., Gallion, H.H., Ozols, R.F., 1996. Carboplatin and paclitaxel in ovarian carcinoma: A phase I study of the Gynecologic Oncology Group. Journal of Clinical Oncology 14, 395   1895-1902.

de Groot, F.M.H., van Berkom, L.W.A., Scheeren, H.W., 2000. Synthesis and biological evaluation of 2 '-carbamate-linked and 2 '-carbonate-linked prodrugs of paclitaxel: Selective activation by the tumor-associated protease plasmin. Journal of Medicinal Chemistry 43, 3093-3102.

Deutsch, H.M., Glinski, J.A., Hernandez, M., Haugwitz, R.D., Narayanan, V.L., Suffness, M., 400   Zalkow, L.H., 1989. Synthesis of congeners and prodrugs.3. Water-soluble prodrugs of taxol with potent antitumor-acivity. Journal of Medicinal Chemistry 32, 788-792.

Dorr, R.T., 1994. Pharmacology and toxicology of Cremophor EL diluent. The Annals of pharmacotherapy 28, S11-14.

Feng, X., Yuan, Y.J., Wu, J.C., 2002. Synthesis and evaluation of water-soluble paclitaxel 405   prodrugs. Bioorganic & Medicinal Chemistry Letters 12, 3301-3303.

Fjallskog, M.L., Frii, L., Bergh, J., 1993. IS Cremophor EL, solvent for Paclitaxel, cytotoxic? Lancet 342, 873-873.

Greenwald, R.B., Choe, Y.H., McGuire, J., Conover, C.D., 2003. Effective drug delivery by PEGylated drug conjugates. Advanced Drug Delivery Reviews 55, 217-250.

410   Greenwald, R.B., Gilbert, C.W., Pendri, A., Conover, C.D., Xia, J., Martinez, A., 1996. Drug delivery systems: Water soluble taxol 2'-poly(ethylene glycol) ester prodrugs - Design and in vivo effectiveness. Journal of Medicinal Chemistry 39, 424-431.

Greenwald, R.B., Pendri, A., Bolikal, D., 1995. Highly water-soluble taxol derivatives: 7-polyethylene glycol carbamates and carbonates. Journal of Organic Chemistry 60, 331-336.

415   Greenwald, R.B., Pendri, A., Bolikal, D., Gilbert, C.W., 1994. Highly water-soluble taxol derivatives- 2'-polyethyleneglycol esters as potential prodrugs. Bioorganic & Medicinal Chemistry Letters 4, 2465-2470.

Greenwald, R.B., Pendri, A., Conover, C.D., Lee, C., Choe, Y.H., Gilbert, C., Martinez, A., Xia, J., Wu, D.C., Hsue, M., 1998. Camptothecin-20-PEG ester transport forms: the effect of spacer groups 420   on antitumor activity. Bioorganic & Medicinal Chemistry 6, 551-562.

Hooftman, G., Herman, S., Schacht, E., 1996. Review: Poly(Ethylene Glycol)s with Reactive Endgroups. II. Practical Consideration for the Preparation of Protein-PEG Conjugates. Journal of Bioactive and Compatible Polymers 11, 135-159.

Kingston, D.G.I., Newman, D.J., 2007. Taxoids: Cancer-fighting compounds from nature. Current Opinion in Drug Discovery & Development 10, 130-144.

Kumar, N., 1981. Taxol-induced polymerization of purified tubulin. Mechanism of action. The Journal of biological chemistry 256, 10435-10441.

Lee, K.H., Chung, Y.J., Kim, Y.C., Song, S.J., 2005. Anti-tumor activity of paclitaxel prodrug conjugated with polyethylene glycol. Bulletin of the Korean Chemical Society 26, 1079-1082.

Li, C., Yu, D.F., Inoue, T., Yang, D.J., Milas, L., Hunter, N.R., Kim, E.E., Wallace, S., 1996. Synthesis and evaluation of water-soluble polyethylene glycol-paclitaxel conjugate as a paclitaxel prodrug. Anti-Cancer Drugs 7, 642-648.

Manfredi, J.J., Horwitz, S.B., 1984. Taxol: an antimitotic agent with a new mechanism of action. Pharmacology & therapeutics 25, 83-125.

Marupudi, N.I., Han, J.E., Li, K.W., Renard, V.M., Tyler, B.M., Brem, H., 2007. Paclitaxel: a review of adverse toxicities and novel delivery strategies. Expert Opinion on Drug Safety 6, 609-621.

Mathew, A.E., Mejillano, M.R., Nath, J.P., Himes, R.H., Stella, V.J., 1992. Synthesis and evaluation of some water-soluble prodrugs and derivatives of taxol with antitumor-activity. Journal of Medicinal Chemistry 35, 145-151.

Nabholtz, J.M., Gelmon, K., Bontenbal, M., Spielmann, M., Catimel, G., Conte, P., Klaassen, U., Namer, M., Bonneterre, J., Fumoleau, B., Winograd, B., 1996. Multicenter, randomized comparative study of two doses of paclitaxel in patients with metastatic breast cancer. Journal of Clinical Oncology 14, 1858-1867.

Niethammer, A., Gaedicke, G., Lode, H.N., Wrasidlo, W., 2001. Synthesis and preclinical characterization of a paclitaxel prodrug with improved antitumor activity and water solubility. Bioconjugate Chemistry 12, 414-420.

Rowinsky, E.K., 1997. The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annual Review of Medicine 48, 353-374.

Ryu, B.-Y., Sohn, J.-S., Hess, M., Choi, S.-K., Choi, J.-K., Jo, B.-W., 2008. Synthesis and anti-cancer efficacy of rapid hydrolysed water-soluble paclitaxel pro-drugs. Journal of Biomaterials Science-Polymer Edition 19, 311-324.

Schiff, P.B., Fant, J., Horwitz, S.B., 1979. Promotion of microtubule assembly in vitro by taxol. Nature 277, 665-667.

Schoenmakers, R.G., van de Wetering, P., Elbert, D.L., Hubbell, J.A., 2004. The effect of the linker on the hydrolysis rate of drug-linked ester bonds. Journal of Controlled Release 95, 291-300.

Skwarczynski, M., Hayashi, Y., Kiso, Y., 2006. Paclitaxel prodrugs: Toward smarter delivery of anticancer agents. Journal of Medicinal Chemistry 49, 7253-7269.

Stella, V.J., Nti-Addae, K.W., 2007. Prodrug strategies to overcome poor water solubility. Advanced Drug Delivery Reviews 59, 677-694.

Sugahara, S., Kajiki, M., Kuriyama, H., Kobayashi, T.R., 2002. Paclitaxel delivery systems: The use of amino acid linkers in the conjugation of paclitaxel with carboxymethyldextran to create prodrugs. Biological & Pharmaceutical Bulletin 25, 632-641.

Takahashi, T., Tsukamoto, H., Yamada, H., 1998. Design and synthesis of a water-soluble taxol analogue: Taxol-sialyl conjugate. Bioorganic & Medicinal Chemistry Letters 8, 113-116.

Vyas, D.M., Vittorio, F., 1995. 3 Paclitaxel (taxol®) formulation and prodrugs, Pharmacochemistry Library. Elsevier, pp. 103-130.

Wang, S.D., Zhelev, N.Z., Duff, S., Fischer, P.M., 2006. Synthesis and biological activity of conjugates between paclitaxel and the cell delivery vector penetratin. Bioorganic & Medicinal Chemistry Letters 16, 2628-2631.

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., McPhail, A.T., 1971. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. Journal of the American Chemical Society 93, 2325-2327.

Weiss, R.B., Donehower, R.C., Wiernik, P.H., Ohnuma, T., Gralla, R.J., Trump, D.L., Baker, J.R., Vanecho, D.A., Vonhoff, D.D., Leylandjones, B., 1990. Hypersensitivity reactions from taxol. Journal of Clinical Oncology 8, 1263-1268.

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Figure caption

Figure 1. Chemical structures of PEG-PTX prodrugs **3**, **4** and **10**-**17**.