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Preparation of Highly Water Soluble Tacrolimus Derivatives: Poly(Ethylene Glycol) Esters as Potential Prodrugs

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Tacrolimus (FK506), which is isolated from *Streptomyces tsukubaensis*, is a new potent immunosuppressant. Because of poor solubility in water, the conventional intravenous dosage forms of tacrolimus contain surfactants such as cremophor EL (BASF Wyandotte Co.) or hydrogenated polyoxy 60 castor oil (HCO-60) which may cause adverse effects. This study relates to a polymer-tacrolimus conjugate, which can be dissolved in water, formed by chemically binding the sparingly soluble drug, tacrolimus, with the water soluble polymer, methoxypoly(ethylene glycol) (mPEG). Water soluble tacrolimus-mPEG conjugates have been synthesized and shown to be function *in vitro* as prodrugs. These conjugates are in the form of an ester wherein the 24-, 32- or 24,32-positions are esterified. The desired 24-, 32- or 24,32-esterified compounds were obtained by initially acylating of tacrolimus with iodoacetic acid at the 24-, 32-, or 24,32-positions and then reacting the resulting acylated tacrolimus with a mPEG in the presence of a base such as sodium bicarbonate. These conjugates were converted again into tacrolimus by the action of enzymes in human liver homogenate, and the half-lives of the conjugates are approximately 10 min in the homogenate, indicating that the esterified tacrolimus derivatives may be practically applicable as a prodrug for the immunosuppressant.

Key words: Tacrolimus, PEG, Prodrug, Polymer Conjugate

INTRODUCTION

Tacrolimus (Fig. 1), which is a 23-member macrolide with potent immunosuppressive activity, was isolated from *Streptomyces tsukubaensis* in 1984 (Kino *et al.*, 1987a; Kino *et al.*, 1987b). Tacrolimus is a neutral substance and is generally well dissolved in organic solvents but not in water and n-hexane. Due to such low solubility (1-2 μg/mL) in water (Hane *et al.*, 1992), in formulating the composition castor oil derivatives such as cremophor EL (BASF Wyandotte Co.) or hydrogenated polyoxy 60 castor oil (HCO-60) are required to dissolve tacrolimus. For example, in intravenous injections of tacrolimus, 200 mg/mL of HCO-60 and 80% (v/v) absolute alcohol are required as the solubilizing aid for dissolving 5 mg of tacrolimus. However, cremophor EL itself may partly account for the nephrotoxicity of Sandimmune (Besarab *et al.*, 1987; Sokol *et al.*, 1990), and causes anaphylactoid reactions (Howrie *et al.*, 1985; Ennis *et al.*, 1985; Ennis *et al.*, 1986), adverse effects on hemodynamics (Bowers *et al.*, 1991) and cholestasis by transitory hepatotoxicity (Roman *et al.*, 1989). HCO-60 also may cause allergic symptoms such as leukocytosis, hyperpyrexia, eruption (Okabe, 1981; Ohta *et al.*, 1985) and immunological suppression of the growth of erythroid progenitors in humans (Ninomiya *et al.*, 1987).

Although virtually no patient who received tacrolimus injection experienced anaphylaxis, other pharmaceutical compositions containing castor oil derivatives have caused anaphylaxis in a few patients. Therefore, tacrolimus injection is required for patients who cannot take tacrolimus capsules due to a potential risk of such anaphylaxis. In addition, the use of non-aqueous solvents such as ethanol, propylene glycol or polyethylene glycol 400 in parenteral preparations may cause side effects (e.g., hemolysis, and/or local irritation at the site of injection) and needs to be carefully considered.

Poly(ethylene glycol) (PEG) (Harris *et al.*, 2003) is a linear or branched, neutral polymer available in a variety of molecular weights and is well soluble in water and methylene chloride. At molecular weights less than 1000

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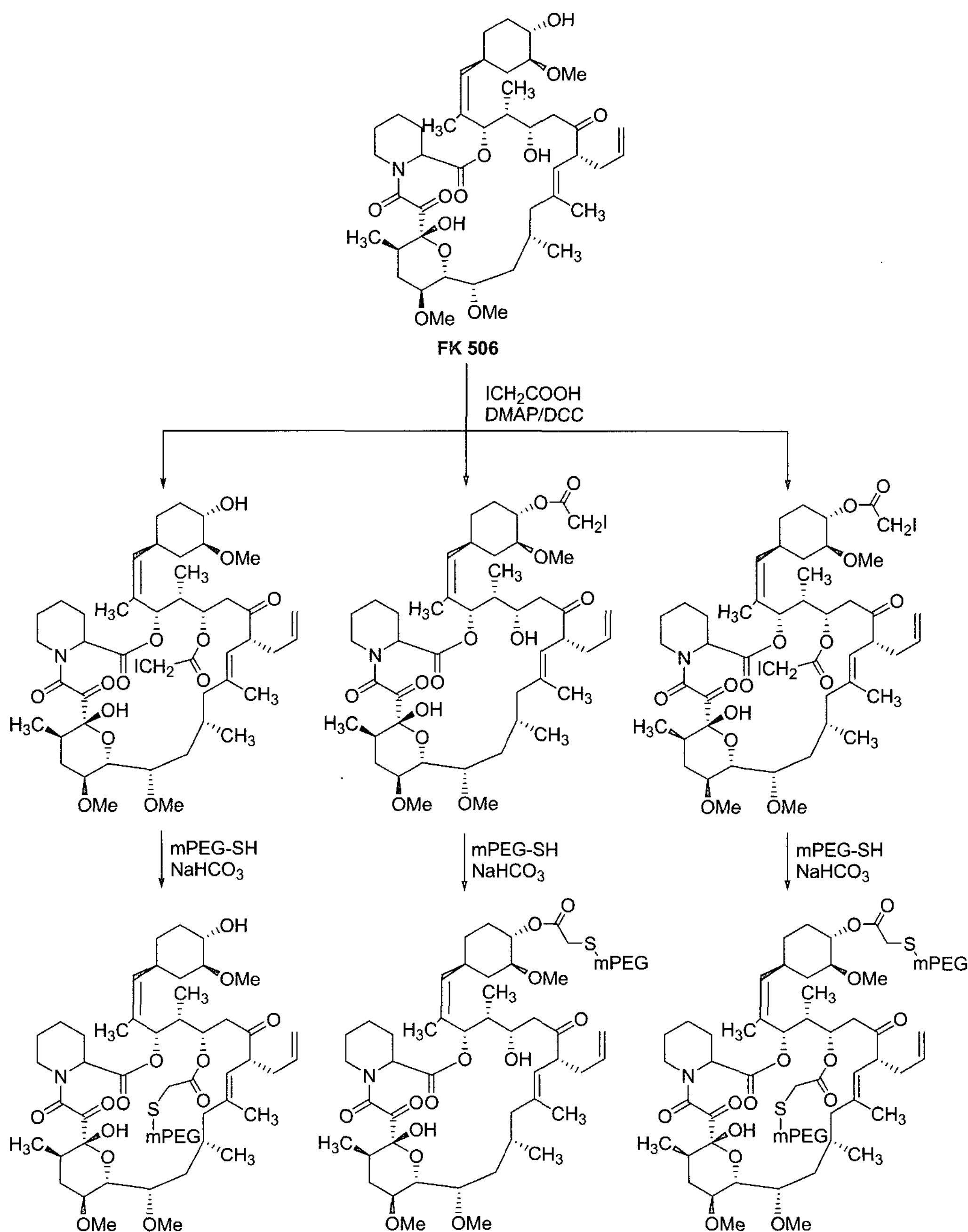
PEGs are viscous, colorless liquids; and higher molecular weight PEGs are waxy, white solids. The melting point of the solid is proportional to the molecular weight. PEGs having molecular weights ranging from a few hundred to approximately 20,000 are commonly used in biological and biotechnological applications.

Of much interest in the biomedical areas is the fact that PEG is nontoxic and was approved by Food and Drug Administration (FDA) for internal consumption. PEG is very widely used in the field of pharmaceuticals, cosmetics and personal sanitary products. One of the most extensively studied drug delivery systems is the covalent binding of methoxy poly(ethylene glycol) (mPEG) to the surface of protein.

Water soluble polymers such as PEG and mPEG are used for binding the non-aqueous drugs. Therefore, the purposes of this study were to develop the novel tacrolimus conjugated compound, which can be dissolved in water, formed by chemically binding the sparingly soluble drug, tacrolimus, with the water soluble polymer, mPEG.

A general approach to the controlled release of drugs based on enzymatic cleavage has been explored exten-



Scheme 1. Derivatives of FK 506

sively by Kopecek *et al.*, (Kopecek, 1990; Kopecek *et al.*, 1987) utilizes *N*-(2-hydroxypropyl)-methacrylamide (HPMA) copolymers containing oligopeptide side chains terminated in anticancer drugs, (Seymour *et al.*, 1990) and has recently been extended to paclitaxel (Mongelli *et al.*, 1994).

We wish to report on the development of an alternate technology, PEG conjugation, for solubilizing. Water soluble prodrugs employing enzymatic cleavage as their mode of activation, have been synthesized and tested *in vitro* in human liver homogenate. This technology can also be applied to other highly insoluble drugs, and we are currently pursuing this course of research in order to further demonstrate the utility of PEG in prodrug strategies.

MATERIALS AND METHODS

Materials

Unless stated otherwise, all reagents and solvents were used without further purification. Analytical HPLCs for mPEG conjugates were performed using Inertsil ODS-2 (4.6×150 mm) reverse-phase column under gradient conditions with a mixture of solution A (95% water-5% acetonitrile) and solution B (95% acetonitrile-5% water) as the mobile phase. Peak elutions were monitored at 214 nm using UV detector. NMR spectra were obtained using a 400 MHz spectrometer. Tacrolimus was extracted using methanol from commercial drug, Prograf. mPEG was purchased from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals not indicated were purchased from Sigma Chemical Co. (St. Louis, MO). All PEG compounds were dried under vacuum prior to use.

General procedure for the synthesis of tacrolimus iodoacetate ester

Tacrolimus (0.390 g, 0.474 mmol), dimethylaminopyridine (3 mg, catalytic amount) and iodoacetic acid (0.110 g, 0.590 mmol) were dissolved in anhydrous methylene chloride (30 mL). To the resulting solution was slowly added dropwise dicyclohexylcarbodiimide (0.167 g, 0.818 mmol)/methylene chloride (15 mL) solution and the mixture was stirred for 4 h at room temperature. The resulting white precipitate was filtered and the filtrate was evaporated and dried. Tacrolimus 32-iodoacetate ester, tacrolimus 24-iodoacetate ester and tacrolimus 24,32-diiodoacetate ester as synthesized were isolated respectively by preparative HPLC on Nova-Pack C_{18} column (19×300 mm, 6 micron) eluting with two mobile phases of 5% acetonitrile (solution A) and 95% acetonitrile (solution B) at an appropriate ratio.

Tacrolimus 24-iodoacetate ester (1)

Peak eluted at about 18 minutes was separated and then distilled under reduced pressure to remove the solvent. MS (FAB) m/z 994 $(M+Na)^+$, m/z 972 $(M+H)^+$

Tacrolimus 32-iodoacetate ester (2)

Peak eluted at about 24.5 minutes was separated and then distilled under reduced pressure to remove the solvent. MS (FAB) m/z 994 $(M+Na)^+$, m/z 972 $(M+H)^+$

Tacrolimus 24,32-diiodoacetate ester (3)

Peak eluted at about 29 minutes was separated and then distilled under reduced pressure to remove the solvent. MS (FAB) m/z 1162 $(M+Na)^+$

Tacrolimus 24-methoxypoly(ethylene glycol) acetate ester (4)

Tacrolimus 24-iodoacetate ester (0.0982 g, 0.119 mmol) was dissolved in 50% acetonitrile-50% sodium hydrogen carbonate (0.1 M) solution (100 mL) and then mPEG-SH (0.747 g, 0.149 mmol) was added thereto. The mixture was stirred for 4 h and extracted with methylene chloride. The methylene chloride layer was dehydrated with anhydrous sodium sulfate and filtered. The filtrate was concentrated to the volume of 5 mL and 250 mL of ether was added thereto to precipitate the product. The precipitated product was filtered to obtain the white amorphous material. The unreacted mPEG was removed by HPLC. According to MS (MALDI/TOF) analysis, the average molecular weight of the product was 5627 and the average molecular weight of mPEG-SH was 4783. The difference in mass (844) exactly matched the tacrolimus acetate moiety. MS (MALDI/TOF) m/z 5627(average molecular weight)

Tacrolimus 32-methoxypoly(ethylene glycol) acetate ester (5)

Tacrolimus 32-iodoacetate ester (0.0982 g, 0.119 mmol) was dissolved in 50% acetonitrile-50% sodium hydrogen carbonate (0.1 M) solution (100 mL) and then mPEG-SH (0.747 g, 0.149 mmol) was added thereto. The mixture was stirred for 4 h and extracted with methylene chloride. The methylene chloride layer was dehydrated with anhydrous sodium sulfate and filtered. The filtrate was concentrated to the volume of 5 mL and 250 mL of ether was added thereto to precipitate the product. The precipitated product was filtered to obtain the white amorphous material. The unreacted methoxypolyethylene glycol was removed by HPLC. According to MS (MALDI/TOF) analysis, the average molecular weight of the product was 5627 and the average molecular weight of mPEG-SH was 4783. The difference in mass (844) exactly matched the tacrolimus acetate moiety. MS (MALDI/TOF) m/z 5627(average molecular weight)

Tacrolimus 24,32-di[methoxypoly(ethylene glycol) acetate ester] (6)

Tacrolimus 24,32-diiodoacetate ester (10 mg, 0.009 mmol)

was dissolved in 50% acetonitrile-50% sodium hydrogen carbonate (0.1 M) solution (50 mL) and then mPEG-SH (0.088 g, 0.018 mmol) was added thereto. The mixture was stirred for one hour at room temperature and extracted with methylene chloride. The methylene chloride layer was dehydrated with anhydrous sodium sulfate and filtered. The filtrate was concentrated to the volume of 15 mL and ether was added thereto to precipitate the product. The precipitated product was filtered to obtain the white amorphous material. According to MS (MALDI/TOF) analysis, the average molecular weight of the product was 10667. MS (MALDI/TOF) m/z 10667(average molecular weight).

Enzymatic hydrolysis test

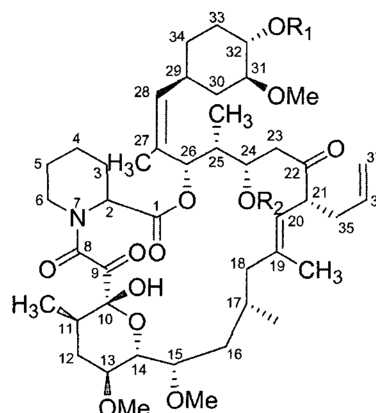
To prove that the conjugated compound as synthesized according to the above method is decomposed in the body to produce tacrolimus, the enzyme hydrolysis test was conducted using human liver homogenate at 37°C. Specifically, 2.5 g of human liver was introduced into 2.5 mL of 0.1 M phosphate buffer (pH 7.4), homogenized on ice and then centrifuged for 10 minutes. The supernatant was transferred to another test tube. The test solution was prepared by dissolving 7.9 mg (20 mg/mL) of the conjugated compound (tacrolimus 32-methoxypolyethylene glycol-thiol acetate ester) in 0.40 mL of 0.1 M phosphate buffer (pH 7.4). 90 μL of the supernatant was introduced into each Eppendorf tube and maintained at 37°C. Then, 10 μL of the test solution which was previously warmed to 30°C, was added thereto. The reaction mixture in each tube was stirred for 5 seconds and 100 μL of acetonitrile was added at the given interval (0, 5, 10, 15, 30, 60, and 120 minutes) and then the mixture in the tube was stirred for one minute. The tube was centrifuged at 13,000 rpm for 10 minutes and then stored on ice. In the tube, the final theoretical concentration of the conjugate was 1 mg/mL. Each 10 μL of the sample solution was analyzed by means of HPLC. For HPLC analysis, a reverse-phase column Inertsil ODS-2 (4.6×150 nm) was used under gradient conditions with a mixture of solution A (95% water-5% acetonitrile) and solution B (95% acetonitrile-5% water) as the mobile phase. Peak elutions were monitored at 214 nm using UV detector.

RESULTS AND DISCUSSION

PEG is a linear or branched, neutral polymer having various molecular weight range and is soluble in water and methylene chloride. PEG having a molecular weight of less than 1000 is viscous and colorless liquid. Higher molecular weight of PEG is a waxy and white solid. The melting point of the solid is proportional to the molecular weight, approaching a plateau at 67°C. The compounds with the molecular weight from a few hundred to about 20,000 are commonly used in biological and biotechnological applications. One of the interests in the biomedical areas is the fact that PEG is nontoxic and was approved by FDA in the United States for internal consumption. PEG is widely used for the synthesis of drug and for a wide variety of cosmetic and personal care products. One of the most extensively studied drug-delivery technologies involves the covalent linkage of the polymer mPEG to the surface of proteins.

Water-soluble polymers such as PEG, and mPEG are utilized to bind to poorly aqueous-soluble drugs and increase a water solubility of the drugs.

Prodrug design comprises an area of drug research that is concerned with the optimization of drug delivery. A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires an enzymatic transformation within the body in order to release the active drug, and has improved delivery properties over the parent molecule (Matthew *et al.*, 1992; Maeda, 1991; Maeda *et al.*, 1992; Noguchi *et al.*, 1998). PEG prodrugs of highly insoluble agents should be especially advantageous since the solubility of the prodrug will exceed that of the original drug, increasing the possibility of more effective drug delivery. Accordingly, we prepared prodrugs based on ester formation (Fig. 1). Esters with PEG as an electron-withdrawing substituent (alkoxy) in the α-position proved to be especially effective linking groups in the design of prodrugs since they aid in the rapid hydrolysis of the ester carbonyl bond, and are thus able to release alcohols



Compound	R_1	R_2
FK506	H	H
1	H	$-COCH_2I$
2	$-COCH_2I$	H
3	$-COCH_2I$	$-COCH_2I$
4	H	$-COCH_2S(CH_2CH_2O)_nCH_3$
5	$-COCH_2S(CH_2CH_2O)_nCH_3$	H
6	$-COCH_2S(CH_2CH_2O)_nCH_3$	$-COCH_2S(CH_2CH_2O)_nCH_3$

Fig. 1. Structures of tacrolimus and polymer-tacrolimus conjugates.

(tacrolimus, 2° alcohols required for activity) in a continuous and effective manner. Therefore, these highly water-soluble mPEG esters of tacrolimus, sparingly soluble drug, was utilized for covalent linking with the carrier polymers to be dissolved in water and shown to function as prodrugs.

The use of esters as prodrugs has been extensively employed for modifying biologically active molecules containing either hydroxyl or carboxyl functionalities (Oliyai *et al.*, 1993). The simplicity of synthesis coupled with facile enzymatic hydrolysis dictates esters as a first choice when considering prodrug strategies.

The esters used in this study (compound **4**, **5**, and **6**) are in the form of an ester wherein the 24-, 32- or 24,32-positions are esterified (Scheme 1 and Fig. 1). These prodrugs were prepared by initially acylating the 24-, 32, or 24- and 32- positions of tacrolimus with an acylating agent, iodoacetic acid, in the presence of a coupling reagent, such as dicyclohexylcarbodiimide (DCC) and a base such as dimethylaminopyridine (DMAP) to provide either a 24-, 32- or 24,32-acylated tacrolimus. Mixtures of 24-, 32-, and 24,32-esters were separated by chromatography. Reaction of the acylated tacrolimus with mPEG-SH in the presence of a base such as sodium bicarbonate provides the desired 24-, 32-, or 24,32-mPEG esters (compound **4**, **5**, and **6**).

As a model, we chose molecular weight 5 kDa mPEG-SH in the reaction with tacrolimus to give water soluble prodrugs (Scheme 1). In addition to all the predicted peaks could clearly be observed in the ^{1}H NMR of compound **4** displays resonances at 2.85 (t, 2H, S-$\underline{CH_2}$), 3.31 (s, 2H, CO-$\underline{CH_2}$-S), 3.39 (s, 3H, -O$\underline{CH_3}$), 3.65 (m, 4H, -O-$\underline{CH_2}$-$\underline{CH_2}$-O), 4.99 (m, 1H, $\underline{H\text{-}24}$). In the case of compound **5**, resonances at 2.85, 3.34, 3.38, 3.65, and 4.67 can also be observed but not as major peaks.

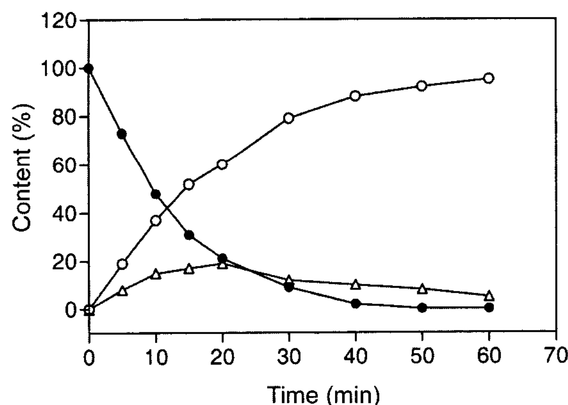


Fig. 2. Concentration-time profile of the hydrolysis of prodrug (tacrolimus 32-methoxy polyethylene glycol-thiol acetate ester) to tacrolimus, as the parent drug, in human liver homogenate at 37°C. Compound **5** (●) was incubated in the presence of human liver homogenate, and the concentration of compound **5**, tacrolimus (○) and (▲) metabolites was determined at the time points indicated using HPLC.

The predicted molecular weights of prodrugs were observed in the MS (MALDI/TOF). MS (MALDI/TOF) showed the average molecular weights of 5627 for prodrugs **4** and **5** and the average molecular weight of mPEG-SH was 4783. The difference in mass of 1260 exactly matched the tacrolimus acetate moiety.

The polymer conjugated compound is decomposed in human liver homogenate to produce the active material, tacrolimus (Fig. 2). Thus, it can be noted that water soluble polymer conjugated prodrug is converted back to tacrolimus by the action of enzymes in human liver homogenate with a short half-life of the hydrolysis reaction. In contrast, the half-life was 20 h in phosphate buffer, pH 7.4, at 37°C, indicating that the derivatives are chemically stable in aqueous solutions. Taken together, these results clearly demonstrate that these derivatives can be formulated prior to injection by a simple dissolution in water for injection without a concern for any significant chemical degradation in the formulation. The pharmaceutical preparation of tacrolimus which is prepared with a surfactant and a cosurfactant, has undesirable side effects (e.g., hemolysis, and/or local irritation at the site of injection). Therefore, this type of water soluble prodrug may greatly reduce such side effects.

In summary, the tacrolimus conjugates with PEGs were designed and synthesized. The derivatives are expected to have comparable pharmacological properties with tacrolimus considering a very short biological half-life in the formation of the active drug. When administered for the treatment of transplantation rejection, the polymer conjugated compounds may be administered various routes (e.g., oral, parenteral, intranasal, intrabronchial, transdermal and topical) considering an acceptable chemical stability of the derivatives. The compounds are particularly advantageous as immunosuppressive, anti-inflammatory, antifungal, antiproliferative and antitumor agents because of their enhanced water solubility. Therefore, these observations indicated that the water soluble derivatives may be practically applicable as a prodrug for tacrolimus.

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