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Preparation of new polymer from podophyllotoxin derivative

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Summary

The new monomer, 4α-acrylpodophyllotoxin(APPT) was synthesized from reaction of acryloyl chloride with the 4α-hydroxy of podophyllotoxin. The homo- and copolymer of new monomer with NIPAAm/AAm have been prepared by free radical polymerization. The new monomer and polymers have been characterized by IR, ^{1}H- and ^{13}C-NMR spectra. The homopolymer have low the molecular weight but it is not oligomer, and the molecular weight of copolymer increased with decreasing new monomer content. The composition of copolymers was close to the original monomer composition. The distinction of antitumor activity among podophyllotoxin, new monomer and homopolymer was smaller. The copolymer with NIPAAm (The PAPPT content (mol-%) =43.7%) did not exhibit any antitumor activity at present condition of experimentation.

Introduction

Podophyllotoxin is a lignan isolated from podophyllum (*podophyllum hexandrum* [1], *podophyllum peltatum* [2] and *diphylleia sinensis* [3]). It has exhibited good antitumor activity but caused toxic effects when used in the body, so podophyllotoxin could not be used directly as antitumor agent. For decreasing toxic effects, it is a suitable way that the C_4-OH of PPT was substituted by suit group such as etoposide [4,5] and teniposid [6,7], which have been used as a new antitumor agent.

A potentially significant aspect of the use of a polymeric drug is the question of a depot effect which might be brought about by either reduced absorption or reduced excretion of the drug [8]. More important is the possibility of varying drastically the pharmacodynamics of drug systems by the use of polymeric substrates. This is due not only to the fact that one may effect variable release of an active component, but also because the toxicity of a drug may be diminished drastically and, in addition, completely different metabolic pathways may result [9,10]. Conceivably, this could influence the amount of drug one has to inject in order to reach the therapeutic does level, and consequently may provide a good opportunity to keep the dosage level very low over a long period of time. Another very important consideration is the possibility of effecting a different body distribution of the polymeric drug relative to its low

molecular weight analog. This might be brought about by varying protein binding of the polymer, the high endocytotic rate of polymers [11] or a possible selective endocytotic uptake by target cells for the achievement of cell-specific effects [12].
To synthesize a new polymeric antitumor agent, we have attempted to prepare new polymer from podophyllotoxin derivative. To the best of our knowledge, there has been no study before about polymer from podophyllotoxin derivative. In this article, we report the synthesis of new monomer, the preparation of its homo- and copolymer and their antitumor activity.

Experimental

Materials
Podophyllotoxin(PPT) was obtained from Lanzhou medical college{It was isolated from podophyllum(*podophyllum emodi* Wall. var. *Chinensis* Sprague)} and recrystallized in benzene-ethanol. N-Isopropylacrylamide (NIPAAm) was synthesized from acrylonitrile and isopropyl alcohol [13]. Acryloyl chloride was always freshly prepared from acrylic acid and phosphorus trichloride [14]. HL60 cells (Human promyelocytic leukemia) were purchased from Shanghai Institute Cell Biology, Chinese Academy of Sciences. Acrylamide (AAm), 2,2' –Azobis(isobutyronitrle) (AIBN) and other reagents were purified by routine methods.

Synthesis of new monomer (4 α -acrylpodophyllotoxin)(APPT)
Podophyllotoxin (4.14 g, 10 mmol) and Triethylamine (1.52 g, 15 mmol) were added to dry chloroform (40 mL) in a three-neck flask. Acryloyl chloride (1.36 g, 15 mmol) was dissolved in dry chloroform (10 mL) in a dropper funnel. The flask was cooled to 0°C with an ice-water bath and the acryloyl chloride solution was added dropwise under constant stirring. As the reaction proceeds, the reactants become completely dissolved. It was stirred at 0°C for 2 h, the temperature was then increased gradually to 25°C and stirring was continued for 24 h. The solvent was removed by vacuum and ethyl acetate was added to precipitate the salt. After removing the salt by filtration, the product was obtained by recrystallization from benzene-ethanol (3.11 g, 66.2 %). M.p.: 179-180°C. $[\alpha]^{20}_{D}$ = -108°(c=7.3mg / mL, $CHCl_3$). Anal. Calcd for $C_{25}H_{24}O_9$ (468.5): C, 64.10%; H, 5.16%; found: C, 64.87%; H, 5.53%. IR (KBr): 1789cm^{-1}(-O-CO-); 1713 and 1631cm^{-1}(-CO-CH=CH_2). ^{1}H-NMR ($DCCl_3$, ppm): δ=4.41(d, 1-H); 2.95(m, 2-H); 2.88(m, 3-H); 4.62(d, 4-H); 6.80(s, 5-H); 6.56(s,8-H); 4.65 and 4.10(m, 11-H_2); 6.35(s, 2' and 6'-H); 3.76 and 3.82(s, 3',4',5' -OCH_3); 5.91(s, -O-CH_2-O-); 6.23(m, 14-H); 5.98 and 6.40(m, 15-H_2). ^{13}C-NMR ($DCCl_3$, ppm): δ=3.70(1-C); 45.54(2-C); 38.68(3-C); 73.70(4-C); 107.00(5-C); 148.13(6-C); 147.59(7-C); 109.67(8-C); 132.46(9-C); 127.66(10-C); 71.20(11-C); 173.55(12-C); 134.74(1'-C); 108.12(2',6'-C); 152.60(3',5'-C) 137.19(4'-C); 56.06 and 60.67(-OCH_3); 101.54(-O-CH_2-O-); 166.33(13-C); 128.22(14-C); 132.20(15-C).

Preparation of homo-and copolymer
Homo- and copolymer of new monomer (APPT) with NIPPAm/AAm were prepared by free-radical polymerization in solution with AIBN as the initiator.
Homopolymerization: APPT (0.468 g, 1 mmol) was dissolved in chloroform (or ethanol) (15 mL) in an ampoule equipped with a magnetic stirrer. The solution was degassed with N_2. AIBN (3.3 mg, 2 mol-%) was added to solution before it was

sealed and placed in an oil bath at room temperature and the temperature was raised slowly during a period of 1 h to 60 °C (or 70 °C in ethanol). The mixture was continuously stirred for 48 h. The mixture in chloroform was completely dissolved during the whole period. The polymer was precipitated in methanol, filtered, washed with methanol and dried. The mixture in ethanol was dissolved at the first period, as the polymerization proceeded, a precipitate formed, which was then filtered, washed with ethanol and dried.

Copolymerization: The copolymer was prepared by a similar procedure. The comonomer were mixed in appropriate molar ratios and dissolved in chloroform. The mixture was continuously stirred at 60 °C for 48 h. Copolymers of APPT with NIPAAm were very soluble in chloroform and can be precipitated from methanol and filtered, washed and dried. For the copolymerization of APPT with AAm, the mixture was dissolved at the first period, as the reaction proceeded, a precipitate formed, which was then filtered, washed and dried.

Cell culture and drug treatment

Cell viability was determined by MTT method [15]. Briefly, 100μL cells ($2x10^8$ cells / L were seeded in each well of 96 well microplate. After incubated in a CO_2 incubator at 37 °C for 24h, the cells were treated with various concentration of podophyllotoxin, APPT, homopolymer and copolymer with NIPAAm (The PAPPT content (mol-%) = 43.7%) for 24h, then the medium was replaced with 100μL MTT (0.4 g / L for 4h. After that, 100μL SDS (10%) was added and A_{570nm} values were measured. $\bar{\chi}\pm s$, n=4.

Characterization

The NMR spectra were recorded on a BRUKER AM 400 spectrometer (400MHz) in deuterated chloroform, which also served as an internal reference (chemical shifts δ_H=7.27 ppm, δ_c=77.00 ppm). IR spectra were measured on a Nicolet AVATAR 360 FT-IR instrument. The chemical composition of the copolymer was determined by analysis of nitrogen element on an element Vario.EL instrument. The optical rotation was measured on a Perkinelmor 341 polarimeter. The molecular weight of the polymers was determined by gel-permeation chromatography (GPC) on a Waters 150Cv system using polystyrene as the standard. The samples were measured at 30°C using THF as the mobile phase (flow rate 1 mL / min), the samples concentration was 5% w/v in THF.

Result and discussion

The APPT was synthesized from reaction of acryloyl chloride with the 4α-hydroxy of podophyllotoxin, and the chemical structure of APPT was established by elemental analysis, analysis of optical rotation, IR spectra and NMR analysis.

On the synthesis of podophyllotoxin derivatives, it was necessary to establish the configuration, because the configuration of H protons at position 2 or 4 on podophyllotoxin will possibly isomerize to that of picropodophyllin (2β-H $\rightarrow$ 2α-H) at the environment of basic capacity and to that of epipodophyllotoxin (4β-H $\rightarrow$ 4α-H) at the environment of acidic capacity [3,16,17], and the antitumor activity will possibly decrease with the change of the configuration [17]. The configuration of H

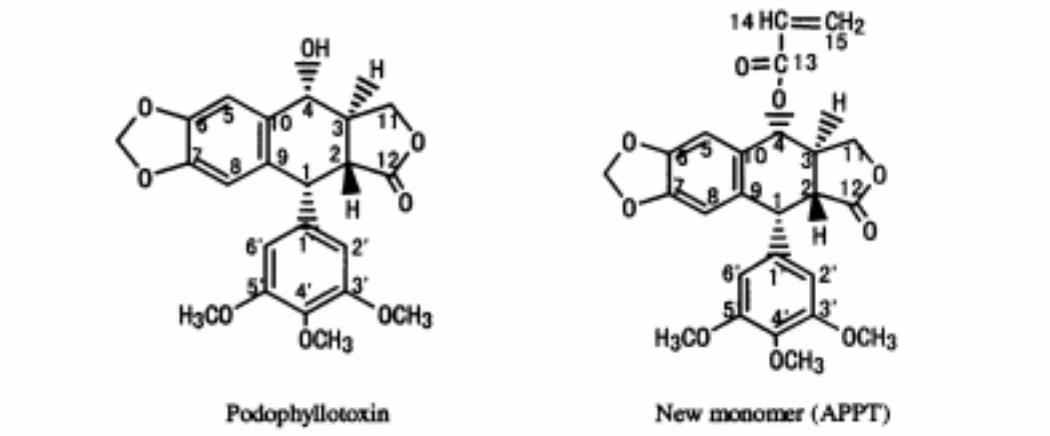

Figure 1. The chemical structure of Podophyllotoxin(1β–H, 2β–H, 3α–H, 4β-H) and APPT

protons on podophyllotoxin skeleton of APPT did not isomerize at our checked condition of synthesis (Figure 1), there are three reasons:

(1) The ismoerization of the configuration of H protons on podophyllotoxin skeleton usually emerged at 60-80°C [3,16,17], but the APPT was synthesized at 0-25°C.

(2) Because triethylamine were added to react with any HCl, the environment of reaction was weak basic capacity. If the ismoerization of the configuration of H protons on podophyllotoxin skeleton had emerged, the podophyllotoxin skeleton of APPT would only have changed into picropodophyllin skeleton (2β-H $\rightarrow$ 2α-H). The optical rotation of APPT indicated that this isomerization did not emerge ($[\alpha]^{20}_{D}$: podophyllotoxin, −132°; picropodophyllin, +9°; acetylpodophyllotoxin, −117°; acetylpicropodophyllin, +19°; APPT, −108°.) [16,17,18,19].

(3) The ^{1}H- and ^{13}C-NMR spectra of APPT were analysed and compared with the ^{1}H- and ^{13}C-NMR spetra of podophyllotoxin, picropodophyllin, epipodophyllotoxin and their 4-acetylate derivatives in previous reports [3,19,20,]. It was found that the ^{1}H- and ^{13}C-NMR spectra of APPT were close to that of acetylpodophyllotoxin. This also indicated that the configuration of H protons on podophyllotoxin skeleton of APPT did not isomerized to that of picropodophyllin and epipodophyllotoxin.

The APPT could readily undergo radically-initiated homopolymerization in chloroform or ethanol. The formation of the polymer was confirmed by solution ^{1}H-NMR, which showed the disappearance of the double bonds of acrylate group and also broadening of the ^{1}H-NMR signals. The GPC results showed that the number- and weight-average molecular weights M_n and M_w of polymer prepared in chloroform were 7500 and 13900, respectively, while the polymer prepared in ethanol had higher molecular weight (M_n =12700, M_w =57800). This indicated that chain transfer reactions of the precipitation polymerization in ethanol was more difficult than that of the homogeneous solution polymerization in chloroform. The ratio of weight- to number-average molecular weight of polymer prepared in chloroform, M_w / M_n =1.85, mach narrower than that of polymer prepared in ethanol (4.55). The molecular weights of homopolymer accorded with the demands of polymeric drug but, for all that it was lower [21,22]. In addition, homopolymer processed better film forming property.

The homopolymer was more hydrophobicity and did not posses amphiphilic properties, there is an obvious way to improve the hydrophilicity of the polymer: the

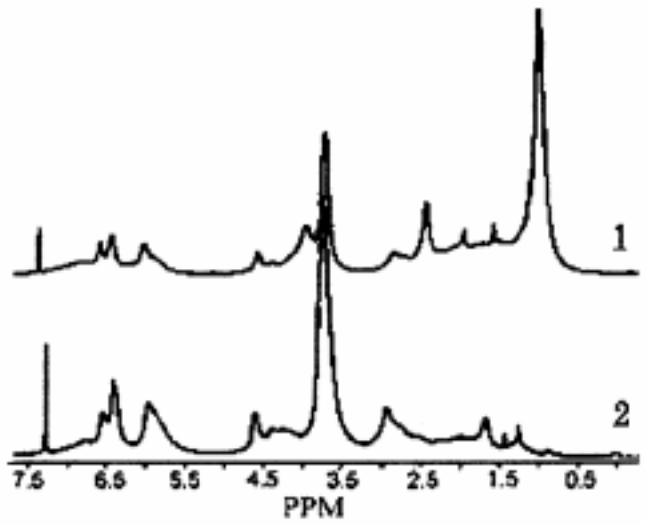

Figure 2 ^{1}H-NMR spectra of homo- and copolymer of APPT with NIPAAm (1) Copolymer with NIPAAm (The PAPPT content (mol-%)=26.4%); (2) Homopolymer.

copolymerization of APPT with monomer of higher hydrophilicity such as N-isopropylacrylamide (NIPAAm) and acrylamide (AAm), which are often used in the preparation of polymeric hydrogels. In addition, the comonomers are expected to improve the flexibility of polymer chains since they are smaller in size.

The copolymers of APPT with NIPAAm/AAm were made by free radical polymerization in chloroform. The products were characterized by ^{1}H-NMR and IR spectra. Figure 2 and 3 show the ^{1}H-NMR and IR spectra of the copolymer of APPT with NIPAAm, respectively. In addition to the absorption band of PNIPAAm at 1656cm^{-1}, typical for amides, absorption bands at 1783 and 1732cm^{-1} could be seen, which are typical for two -CO-O- groups of the chain element of poly(4 α -acrylpodophyllotoxin). The peak at 3.7and 5.96 ppm in the ^{1}H-NMR spectra were assigned to the protons of the -OCH_3 and -O-CH_2-O- groups of podophyllotoxin skeleton, the peak at 3.99 and 1.12 ppm to the CH-proton and CH_3-proton of N-isopropyl group. All signals of ^{1}H-NMR spectra show broadening.

Table1 Copolymerization of APPT with NIPAAm/AAm

Sample	PAPPT content (mol-%)		Weight conversion	*M*w	*M*w/*M*n
	Feed	Polymer			
PAPPT/ PNIPAAm	10.0	9.3	78.6	102900	2.46
	30.0	26.4	70.2	89600	1.96
	50.0	43.7	59.7	53800	1.61
	70.0	73.1	46.2	38700	2.23
PAPPT/ PAAm	10.0	8.1	82.3		
	30.0	23.8	71.5		
	50.0	45.2	55.7		
	70.0	68.6	41.2		

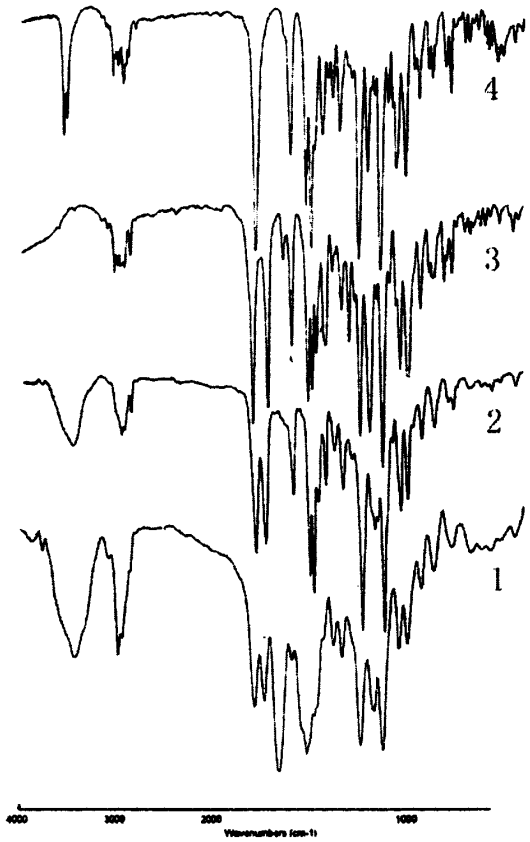

Figure 3 . IR spectra of podophyylotoxin, APPT, homo- and copolymer of APPT with NIPAAm. (1) Homopolymer; (2) Copolymer with NIPAAm (The PAPPT content (mol-%) =26.4%); (3) APPT; (4) podophyllotoxin.

The chemical composition of copolymers was determined by analysis of nitrogen element (Table 1). The data in the Table 2 showed that, the chemical composition of the resulting copolymer were close to the original monomer composition added to the solution prior to polymerization. This indicated that the content of drug in the copolymer was controlled by the original monomer composition added.

Since the copolymers of APPT with AAm were not dissolved in THF, chloroform and benzene, the molecular weight of the copolymers of new monomer with NIPAAm was only determined by GPC (Table 1). The results unequivocally indicated the formation of polymer, not oligomer. While the results showed that the molecular weight of copolymer increased with decreasing the new monomer content, this was due to steric hindrance of the podophyllotoxin skeleton.

The HL60 cells were treated with various concentration of podophyllotoxin, APPT, homopolymer and copolymer with NIPAAm (The PAPPT content (mol-%) =43.7%) for 24h. The inhibition ratio of podophyllotoxin on proliferation in HL60 cells is slightly bigger than that of homopolymer and slightly smaller than that of APPT (Table 2). This indicated that the change of antitumor activity was smaller when APPT and its homopolymer were derived from podophyllotoxin. The copolymer with NIPAAm (The PAPPT content (mol-%) =43.7%) did not exhibit any antitumor activity at present condition of experiment, it was probably due to chain of copolymer with NIPAAm formed a special microstructure in the environment of cells culture, the chain element of poly(4 α -acrylpodophyllotoxin) was inside and the chain element of

PNIPAAm was outside in this special microstructure, so the podophyllotoxin element could not directly contact with HL60 cells, and it possibly would exhibit antitumor activity only after the fracture of acrylate bond. For the antitumor activity of copolymer, further studies are necessary.

Table 2. Effect of drug on proliferation in HL60 cells

Drug concentration /μg.mL^{-1}	Podophyllotoxin A_{570nm}	IR/%	APPT A_{570nm}	IR/%	Homopolymer A_{570nm}	IR/%	Copolymer A_{570nm}	IR/%
Control	0.51±0.02		0.51±0.02		0.51±0.02		0.51±0.02	
0.74	0.41±0.01[a]	19.6	0.41±0.03[a]	19.6	0.47±0.03[b]	7.3	0.50±0.02[b]	2.0
2.22	0.39±0.03[a]	23.5	0.40±0.03[a]	21.6	0.45±0.02[b]	12.0	0.52±0.01[b]	0.0
6.67	0.32±0.01[a]	37.3	0.36±0.03[a]	29.4	0.37±0.02[a]	27.6	0.50±0.01[b]	2.0
20.00	0.30±0.02[a]	41.2	0.21±0.01[a]	58.8	0.32±0.02[a]	37.5	0.53±0.02[b]	0.0

(1) a: $P<0.01$; b: $P>0.05$. (2) Copolymer: copolymeration with NIPAAm (The PAPPT content (mol-%) =43.7%). (3) IR: the inhibition ratio(%) =(1−A_{570nm} of given drug group / A_{570nm} of control group) × 100%.

Conclusion

The APPT was synthesized by attaching a polymerizable acrylic group at position C-4 of the podophyllotoxin,and It was found that the configuration of H protons on podophyllotoxin skeleton of new monomer did not isomerize by NMR spectra. Homo- and copolymers of APPT with NIPAAm/AAm were prepared by solution free radical polymerization using AIBN as initiator and characterized by IR, ^{1}H-NMR and GPC. The composition of copolymer was determined by analysis of nitrogen element. It was found that the composition in the final products was close to the original monomer composition prior to copolymerization. The homopolymer had low molecular weight because of the steric hindrance of the podophyllotoxin skeleton, but it was not oligomer. The molecular weight of copolymer increased with decreasing APPT content. The change of antitumor activity was smaller when APPT and its homopolymer were derived from podophyllotoxin. The copolymer with NIPAAm (The PAPPT content (mol-%) =43.7%) did not exhibit any antitumor activity at present condition of experimentation.

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