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Antitumor and Antiangiogenic Activities of Phthalic Acid Derivative Polymers with Medium-Molecular-Weight

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α-Methoxy-3,6-*endo*-methylene-1,2,3,6-tetrahydrophthaloyl-5-fluorouracil (MMTFU), was synthesized from 5-fluorouracil (5-FU) and α-methoxy-3,6-*endo*-methylene-1,2,3,6-tetrahydrophthaloylchloride (MMTC). Poly(3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalicanhydride) [poly(MTA)] and poly(α-methoxy-3,6-*endo*-methylene-1,2,3,6-tetrahydrophthaloyl-5-fluorouracil) [poly(MMTFU)] were prepared from corresponding monomers by photo-polymerizations using 2,2-dimethoxy-2-phenylacetophenone as the photoinitiator. The synthesized MMTFU and polymers were identified by FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopies. The number average molecular weights and polydispersity indices determined by GPC were 6,200 and 1.3 for poly(MTA) and 7,700 and 2.9 for poly(MMTFU). The cytotoxicities of the prepared polymers were lower than that of 5-FU. The *in vivo* antitumor activities of the polymers against mice bearing sarcoma 180 tumor cell line were greater as compared with that of 5-FU. The antiangiogenic activity of poly(MTA) examined by the embryo chorioallantoic membrane assay was better than that of MTA.

Keywords: 3,6–*endo*-methylene-1,2,3,6–tetrahydrophthalicanhydride; α-methoxy-3,6–*endo*-methylene-1,2,3,6–tetrahydrophthaloyl-5-fluorouracil; medium molecular weight polymer; *in vitro* and *in vivo* antitumor activities; antiangiogenesis

INTRODUCTION

The high molecular weight compounds with antitumor activity are attracting much interest in the standpoint of polymer synthesis as well as drug development, because they can be expected to have some advantages such as higher specificity of actions, lower toxic side effects, and longer duration of actions as compared with low molecular weight drug compounds[1-3].

5-Fluorouracil (5-FU) has been widely used in the treatment of different solid tumors [4-6], but it also has strong side effect[7-9]. It has been known that one of the methods for the reduction of toxic side effects transforms a low molecular weight drug into a polymeric drug[10-15]. Many studies have been attempted to reduce toxic side effects of 5-FU. The studies on the synthesis of antitumor active polymers with high antitumor activities and low toxicities have been

performed in our laboratory for many years[16-34].

Antiangiognensis, which means the inhibition for the formation of new blood vessels from pre-existing blood, is important factor for growth inhibition of solid tumor cells. Because tumor cells is supplied nutrients and release waste products through newly formed blood vessels [35-37]. Furthermore, antiangiogenesis is essential for prevention of metastasis of tumor cells. Consequently, antiangiogenesis may lead to the inhibition of the tumor cell growth and metastasis. Thus, synthesis of polymer with antitumor and antiangiogenesis activities is the very important stategy to overcome a cancer.

The aim of this study is to synthesize new polymers with antitumor and antiangiogenesis activities. In this work, MMTFU was synthesized from 5-fluorouracil and α-methoxy-3,6-*endo*-methylene-1,2,3,6-tetrahydrophthaloyl chloride (MMTC) which was prepared from 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalic anhydride (MTA). Poly(MTA) and poly(MMTFU) were prepared by photopolymerizations. The structure of MMTFU, poly(MTA), and poly(MMTFU) were identified by IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopies. The average molecular weights of the synthesized polymers were determined by GPC. The *in vitro* cytotoxicities of MTA, MMTFU, poly(MTA) and poly(MMTFU) were evaluated with mouse mammary carcinoma (FM3A),

mouse leukemia (P388), and human histiocytic lymphoma (U937) as a cancer cell lines and mouse liver cells (AC2F) as a normal cell line. The *in vivo* antitumor activities of the synthesized samples against mice bearing sarcoma 180 tumor cell line were evaluated. The antiangiogenesis of MTA, MMTFU, poly(MTA), and poly(MMTFU) were examined by the embryo chorioallantoic membrane (CAM) assay.

EXPERIMENTAL

Materials

5-FU (Aldrich Co.Milwaukee,WI), MTA (Fluka Co.), and DMP (Aldrich Co.) were used without further purification. All other chemicals were reagent grade and used without further purification. For *in vitro* test, P388, FM3A, and U937 as cancer cell lines and AC2F as a normal cell line were used. For *in vivo* test, Balb/C mouse and sarcoma 180 cell line was purchased from the Center of Genetic Engineering (Korea Institute of Science and Technology).

For CAM assay, fertilized chick eggs were obtained from Han-shin Farm

(Kimhae, Korea). Fat emulsion (10%) was purchased from Green Cross Pharm. Co. (Seoul, Korea). Thermanox coverslips were purchased from Nunc Inc. (Naperville, IL, U.S.A).

Measurements

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a FT-300 MHz Varian Gemini 2000 spectrophotometer. IR spectra were obtained with a Jasco FT/IR-5300 spectrophotometer by using KBr pellet and Neat for analysis. Elemental analysis was performed by elemental analyzer (Carlo Erba : Model EA1180). Photopolymerizations were carried out under the irradiation UV light (λ_{max}=313 nm) in a photochemical chamber. The number and weight average molecular weights were determined by gel permeation chromatography (GPC: Waters 410).

Preparation of Monomer and Homopolymers

α-Methoxy-3,6-Endo-Methylene-1,2,3,6-Tetrahydrophthaloyl-5-Fluoro-uracil (MMTFU)

The monomer, MMTFU was synthesized according to previous paper[29]. The

yield and m.p. of synthesized MMTFU were 51 % and 127 ℃, respectively. Elemental analysis: Cald. (%) for $C_{14}H_{13}N_2O_5F$: C, 54.5; H, 4.3; N, 9.1, Found (%); C, 53.5; H, 4.3; N, 10.1.

Poly(3,6-Endo-Methylene-1,2,3,6-Tetrahydrophthalicanhydride) [Poly (MTA)] and Poly(α-Methoxy-3,6-Endo-Methylene-1,2,3,6-Tetrahydrophthaloyl-5-Fluorouracil) [poly(MMTFU)]

Poly(MTA) and poly(MMTFU) were synthesized by the same procedure as previous paper[29]. The conversions of poly(MTA) and polt(MMTFU) were 34 % and 75 %, respectively.

Measurement of Average Molecular Weight

The number (M_n) and weight (M_w) average molecular weights and polydispersity indices of the homopolymers were measured by GPC DMF as an eluent at 40 ℃.

Biological Activity Test

In Vitro Cytotoxicity Test and In Vivo Antitumor Activity Test

The *in vitro* cytotoxicities of synthesized monomers and homopolymers were determined by the same procedure as that described in the previous paper[33,37]. The 50% cytotoxic dose (IC_{50}) was defined as the concentration of samples that reduced the absorbance of the treated cells by 50%.

The *in vivo* antitumor activity of synthesized samples were evaluated by mice bearing sarcoma 180 tumor cells according to previous paper[30-33].

CAM Assay for Monomers and Polymers

The fertilized chicken eggs used in this study were kept in humidified incubator at 37 ℃. After 3 days incubation, about 1 ml of albumin was aspirated from the eggs with an 18-gauge hypodermic needle through the small hole drilled at the narrow end of the eggs, allowing the small CAM and yolk sac to drop away from the shell membrane. On day 4, the shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away.[38] Embryos with chorioallantois of 3～5 mm in diameter were employed for the assay of antiangiogenetic activity. Five microliters of an aqueous, salt-free solution of each sample, were applied to sterile Thermanox 15-mm disks and allowed dry under laminar flow conditions.[39] The loaded-disks were inverted and applied to the CAM surface

of 4.5 day old embryos through the windows. The air sac ends of the embryo with shells were covered with scotch tape. Two days later, an appropriate volume of a 10% fat emulsion was injected using a 33-gauge needle into the 6.5 day embryo chorioallantois so that the vascular network of CAM stood out against the white background of lipid. At least 20 eggs were used for each dose of agent. Finally, the chorioallantois was microphotographed.

RESULTS AND DISCUSSION

Identification of Monomer and Polymers

The structures of synthesized monomer and polymers were confirmed by FT-IR and ^{1}H-NMR spectroscopies[29]. In ^{1}H-NMR spectrum (DMSO-d_6) of poly(MTA), the methine protons of anhydride ring appear at 3.4 ppm and the methylene, and methine protons of cyclopentane ring at 1.6 and 1.8 ppm. The peak at 2.9 ppm was assigned to the methine protons of the polymer skeleton and the peak at 6.3 ppm as assigned to the olefinic protons of the monomer disappeared. In ^{13}C-NMR spectrum (DMSO-d_6) of poly(MTA), the carbonyl

carbons appear at 175.4 ppm, the methine carbons of anhydride ring at 47.8 and 49.2 ppm, and the methine carbons in polymer backbone at 41.4 and 41.0 ppm.

Solubility of Monomers and Polymers

The solubilities of the synthesized monomers and homopolymers were listed in Table I. The synthesized samples were good in DMSO, DMF, and acetone. MMTFU and poly(MMTFU) were poorly soluble in water. The synthesized samples were insoluble in diethyl ether except MTA.

TABLE I. The Solubility of Monomers and Polymers

Solvents	Samples			
	MTA	Poly(MTA)	MMTFU	Poly(MMTUF)
Water	S[a]	S	PS	PS
DMSO	S	S	S	S
DMF	S	S	S	S
acetone	S	S	S	S
diethyl ether	PS[b]	IS	IS	IS

[a]S= Soluble, [b]PS= Poorly soluble, [c]IS= Insoluble

Average Molecular Weights of Homopolymers

The average molecular weights of the synthesized polymers were listed in TABLE II. The number and weight average molecular weights and

polydispersity indices determined with GPC were 4,700, 6,200, and 1.3 for poly(MTA) and 7,700, 22,100, and 2.9 for poly(MMTFU).

TABLE II. The Average Molecular Weights of The Synthesized Polymers

Polymers	M_n[a] ($\times 10^4$)	M_w[a] ($\times 10^4$)	M_w/M_n
Poly(MTA)	0.47	0.62	1.3
Poly(MMTFU)	0.77	2.21	2.9

[a]The number (M_n) and weight (M_w) average molecular

In Vitro Antitumor Activity and Cytotoxicity

As shown in TABLE III, 5-FU against cancer cell lines were in the range of 0.04 to 6.61. In normal cell line, the toxicities of the prepared polymers were lower than that of 5-FU.

Table 3. The Cytotoxicity of Samples against Cancer and Normal Cell line.

Samples	IC_{50} (μg/mL) for Cell Lines[a]			
	Cancer Cells			Normal Cells
	FM3A[a]	P388[b]	U937[c]	AC2F[e]
5-FU	0.03	0.04	0.05	0.16
MTA	1.35	1.35	1.35	5.52
MMTFU	0.04	0.07	0.04	0.01
Poly(MTA)	1.95	1.95	1.95	9.95
Poly(MMTFU)	4.11	6.61	2.62	17.21

[a]The 50% growth inhibition concentration (IC_{50})

[b]Mouse mammary carcinoma cell. [c]Mouse leukemia cell.

[d]Human histiocytic lymphoma cell. [e]Mouse liver cell.

***In Vivo* Antitumor Activity against Mice Bearing Sarcoma 180**

The *in vivo* antitumor activities of the monomers and polymers against mice bearing sarcoma 180 tumor cell line were listed in TABLE IV, and 5-FU was used for comparison. The ratio, T/C was used as the index of the antitumor activity :

$$T/C(\%) = \frac{\text{Survival time of mice treated with polymer (T)}}{\text{Survival time of mice in a control group (C)}} \times 100$$

The life spans (T/C) of mice treated with MMTFU and poly(MMTFU) were longer than those of control group and mice treated with 5-FU at 800 and 80 mg/kg. T/C values of MTA and poly(MTA) were similar to those of 5-FU at 80 amd 0.8 mg/kg. But T/C values of MTA and poly(MTA) at 800 mg/kg were much lower than that of 5-FU due to the toxic side effect at higher concentration. The highest antitumor activity (T/C value) was 317% for MMTFU at 80 mg/kg. The dosage of 80 mg/kg, which exhibited the highest T/C value for MMTFU, is about 2.3 times lower than the optimum dosage of free 5-FU. This means that MMTFU has excellent antitumor activity and very low toxicity.

TABLE IV. The *In Vivo* Antitumor Activity of The Synthesized Samples

Samples	Dosage (mg/kg)	Mean Survival Time (day)		T/C (%)
Control	-	14.7 ±	2.3	100
	saline	15.7 ±	0.5	100
5-FU	800.0	5.9 ±	0.3	39
	80.0	21.3 ±	2.8	140
	0.8	20.3 ±	1.8	134
MTA	800.0	0.2 ±	0.7	1
	80.0	10.2 ±	0.8	67
	0.8	17.3 ±	1.2	114
MMTFU	800.0	23.2 ±	5.7	148
	80.0	49.8 ±	10.1	317
	0.8	20.8 ±	3.7	133
Poly(MTA)	800.0	9.7 ±	1.3	64
	80.0	19.6 ±	2.1	129
	0.8	26.3 ±	2.3	173
Poly(MMTFU)	800.0	20.6 ±	4.3	131
	80.0	25.0 ±	1.8	159
	0.8	20.2 ±	4.2	129

Antiangiogeneic Activities of Monomer and Polymer

The microphotographs of MTA and poly(MTA) by the CAM assay were shown in FIGURE 1. Shown in FIGURE 1, Poly(MTA) and MTA have angiogenic activities. Antiangiogenic activity of poly(MTA) was better than that of MTA due to the T/C value of poly(MTA) is higher than that of MTA. This result can be explained in terms of higher cytotoxicity of MTA.

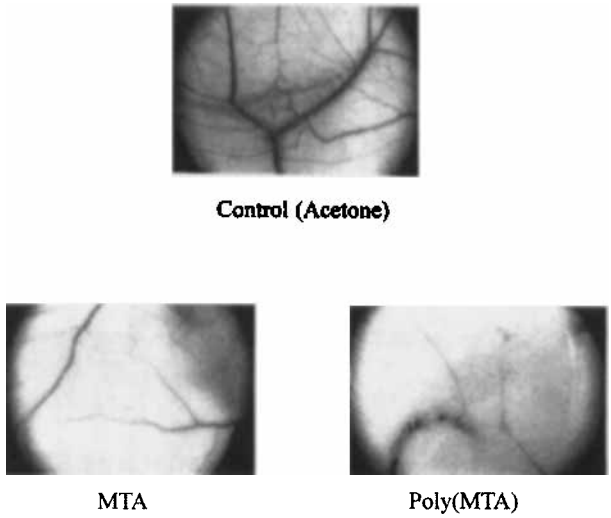

FIGURE.1 The Microphotographs of control (a), MTA (b), and poly(MTA) (c) on embryonic angiogenesis in CAM (× 100).

CONCLUSIONS

MMTFU was prepared by the reaction of 5-FU and MMTC, and poly(MTA) and poly(MMTFU) were synthesized by the photopolymerizations using DMP as the photoinitiator at 25 ℃ for 48 hr. The structures of the synthesized monomer and polymers were identified by FT-IR and ^{1}H-, ^{13}C-NMR spectroscopes. The average molecular weights and polydispersity indices of the

synthesized polymers determined with GPC were as follows : M_n=4700, M_w=6200, M_w/M_n=1.3 for poly(MTA), M_n=7700, M_w=22100, M_w/M_n=2.9 for poly(MMTFU). The IC_{50} values of the prepared samples containing 5-FU against cancer cell lines were in the range of 0.04 to 6.61. In normal cell line, the toxicities of the prepared polymers were lower than that of 5-FU. The *in vivo* antitumor activities of the monomers and polymers were evaluated by the survival time with sarcoma 180 tumor-bearing mice. The life spans (%) of mice treated with the polymers were 173 for poly(MTA) at 0.8 mg/kg and 159 for poly(MMTFU) at 80 mg/kg. In CAM assay, antiangiogenic activity of poly(MTA) is higher than that of MTA.

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