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Syntheses and Biological Activities of *N*-Alaninylmaleimide and Its Polymers

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SYNTHESES AND BIOLOGICAL ACTIVITIES OF *N*-ALANINYLMALEIMIDE AND ITS POLYMERS

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Key Words: *N*-Alaninylmaleimide; Poly(*N*-alaninylmaleimide); Poly(*N*-alaninylmaleimide-*co*-methacrylic acid); Poly(*N*-alaninylmaleimide-*co*-vinyl acetate); Average molecular weight; In-vitro cytotoxicity; In-vivo antitumor activity

ABSTRACT

A new monomer, *N*-alaninylmaleimide (AMI), was synthesized by the reaction of maleic anhydride and β-alanine. Poly(*N*-alaninylmaleimide) [poly(AMI)], poly(*N*-alaninylmaleimide-*co*-methacrylic acid) [poly(AMI-*co*-MA)], and poly(*N*-alaninylmaleimide-*co*-vinyl acetate) [poly-

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(AMI-*co*-VAc)] were synthesized by photopolymerizations. The synthesized AMI and its polymers were characterized by IR and ^{1}H-NMR spectroscopies, elemental analysis, and gel permeation chromatography (GPC). The average molecular weights as determined by GPC were as follows: $\overline{M}_n$ = 4200, $\overline{M}_w$ = 4300, $\overline{M}_w/\overline{M}_n$ = 1.02 for poly(AMI); $\overline{M}_n$ = 4000, $\overline{M}_w$ = 4100, $\overline{M}_w/\overline{M}_n$ = 1.03 for poly(AMI-*co*-MA); and $\overline{M}_n$ = 21,500, $\overline{M}_w$ = 23,300, $\overline{M}_w/\overline{M}_n$ = 1.08 for poly(AMI-*co*-VAc). In-vitro cytotoxicities of polymers against mouse mammary carcinoma (FM-3A/s), mouse leukemia (P-388/s), and human histiocytic lymphoma (U-937/s) cell lines were lower than those of AMI. The in-vivo antitumor activities of polymers against sarcoma 180 cells were greater than those of 5-FU, and the toxicities of polymers were much less than that of 5-FU.

INTRODUCTION

Polymer drugs can be expected to have some advantages such as higher specificity of actions, longer durations of actions, and lower toxic side effects compared with low molecular weight drugs. The copolymer of divinyl ether with maleic anhydride (DIVEMA), first reported by Butler, has been extensively studied for its broad biological activities such as antitumor, antiviral, antibacterial, interferon-inducing, and antifungal activities [1–4]. Many workers [5–7] have tried to obtain a polymeric drug like DIVEMA. We have reported on studies of the syntheses and biological activities of polymeric antitumor agents with the above advantages for polymer drugs [8–16].

The aim of this study is to obtain a new monomer, *N*-alaninylmaleimide (AMI), and its polymers for their potential biological activity. AMI was expected to show considerably high biological activity because it has an amino acid moiety in the repeating unit and its anionic character after hydrolysis is similar to that of DIVEMA.

In this work, AMI was obtained by the reaction of maleic anhydride and β-alanine. Poly(*N*-alaninylmaleimide) [Poly(AMI)], poly(*N*-alaninylmaleimide-*co*-methacrylic acid) [poly(AMI-*co*-MA)], and poly(*N*-alaninylmaleimide-*co*-vinyl acetate) [poly(AMI-*co*-VAc)], were prepared by photopolymerization. The structures of monomeric AMI, poly(AMI), poly(AMI-*co*-MA), and poly(AMI-*co*-VAc) were identified by IR and ^{1}H-NMR spectrophotometry.

In-vitro cytotoxicities of synthesized polymers were evaluated against mouse mammary carcinoma cell (FM-3A/s), mouse leukemia cell (P-388/s), and human histiocytic lymphoma cell (U-937/s). In-vivo antitumor activities against sarcoma 180 were also investigated by using Balb/C mice-bearing tumor.

EXPERIMENTAL

Materials

β-Alanine and dimethoxy benzoin (DMB) were used as received from Junsei without further purification. Maleic anhydride (MAH), 2-butanone, toluene, mathacrylic acid (MA), and vinyl acetate (VAc) were purified by conventional purifi-

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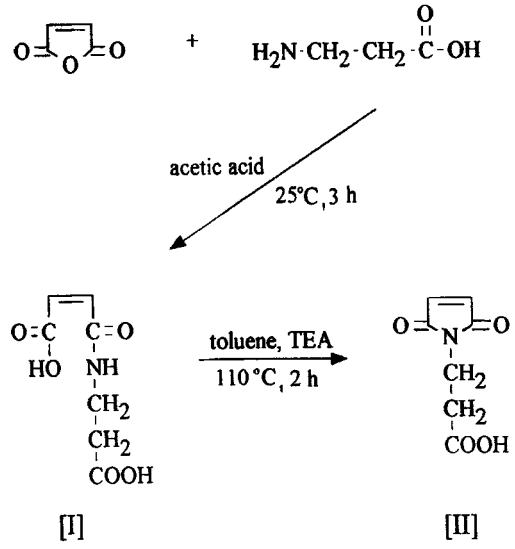
cation methods. P-388, FM-3A, and U-937 cells as target cell lines for in-vitro testing were used. For the in-vivo test, Balb/C mice and sarcoma 180 cell lines were purchased from the Center of Genetic Engineering (Korea Institute of Science and Technology).

Instruments

IR spectra were taken on a Jasco FT/IR-5300 spectrophotometer using a KBr pellet. ^{1}H-NMR spectra were recorded on a FT-300 MHz Bruker A-3000 spectrophotometer. The average molecular weights were measured by a Waters-410 gel permeation chromatograph (GPC). Elemental analyses were carried out with a Carlo Erba model EA1108 analyzer.

Synthesis of AMI

The synthesis procedure of AMI is shown in Scheme 1. A solution of MAH (19.6 g, 0.2 mol) in acetic acid (90 mL) was added to a solution of β-alanine (17.8 g, 0.2 mol) in acetic acid (225 mL), and the solution was stirred at room temperature for 3 hours. The white precipitate was filtered, washed with methanol (100 mL), and dried. The white powder was recrystallized from water to give pure alaninyl maleamic acid (AMA) (yield: 88%) (Scheme 1 [I]). The melting point of AMA was 161°C. A mixture of 2.6 g (0.015 mol) of AMA and 2.9 g (0.028 mol) of triethylamine in 400 mL of dry toluene was refluxed with concomitant removal of the water produced through a Dean-Stark apparatus for 2 hours. The toluene solution containing the reaction product was decanted from the brown-colored oil. Toluene was removed by evaporation to give the triethylammonium salt of AMI as a hygroscopic solid. The solid was acidified to pH 2 with HCl, extracted with ethyl acetate,



[I] [II]

SCHEME 1.

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and dried with anhydrous $MgSO_4$. Ethyl acetate was removed in vacuo to give AMI (yield: 30%) (Scheme 1 [II]). The melting point of AMI was 105°C. Elemental analysis: Calculated (%) for $C_7H_7NO_4$: C, 49.7; H, 4.17; N, 8.28. Found (%): C, 48.6; H, 4.97; N, 7.64.

Syntheses of Polymers

Synthesis of Poly(AMI)

0.85 g (5 mmol) of AMI and 0.027 g of DMB as an initiator were dissolved in 18 mL of 2-butanone and acetone (1V/2V) and the solution was introduced into a dry Pyrex polymerization tube. After the solution was degassed twice by purging with purified N_2 gas, the tube was sealed and placed in a photochemical chamber reactor using 313 nm lamps at 25 ± 1.0°C for 72 hours. The obtained polymer solution was precipitated in diethyl ether. The precipitated polymer was filtered and washed twice with 2-butanone and acetone (1V/2V). Then the polymer was collected by filtration and dried until a constant weight under vacuum (conversion: 35%).

Synthesis of Poly(AMI-*co*-MA)

Poly(AMI-*co*-MA) was prepared by the photocopolymerization of AMI and MA with DMB as an initiator: 0.85 g (5 mmol) of AMI, 0.215 g (2.5 mmol) of MA, and 0.041 g of DMB were dissolved in 18 mL of 2-butanone and acetone (1V/2V), and the solution was introduced into a dry Pyrex polymerization tube. The procedure of photopolymerization of AMI and MA was the same as that described for the homopolymerization of AMI except for monomer pairs (conversion: 27%). The elemental analysis found (%): C, 49.9; H, 5.16; N, 6.85.

Synthesis of Poly(AMI-*co*-VAc)

0.85 g (5 mmol) of AMI, 0.43 g (5 mmol) of VAc, and 0.055 g of DMB were dissolved in 18 mL of 2-butanone and acetone (1V/2V), and the solution was introduced into a dry Pyrex polymerization tube. The copolymerization procedure of AMI and VAc was the same as that described for that of AMI and MA. The conversion was 21%. The elemental analysis found (%): C, 23.07, H, 2.37; N, 3.96.

Determination of Molecular Weight

Number ($\overline{M}_n$) and weight ($\overline{M}_w$) average molecular weights of polymers were measured by GPC using a microstyragel column with monodisperse polystyrene as a standard at 40°C. DMF was used as an eluent.

Elemental Analysis of Copolymers

The contents of AMI moiety in copolymers were calculated from C, N, and H data.

Biological Activity Test

In-Vitro Cytotoxicity of AMI and Its Polymers

The in-vitro cytotoxicities of AMI and its polymers were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium (MTT) assay [17]. The synthesized samples were added to J-82, P-388, FM-3A, and U-937 cell lines (2×10^4

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cells/mL) in 96-well microtiter plates and cultured for 3 days at 37°C. The cultured cells were mixed with 20 μL of MTT solution and incubated at 37°C for 4 hours. The supernatant was removed from each well and 100 μL of 100% DMSO was added to solubilize the formazan crystals which were formed by the cellular reduction of MTT.

After mixing by a mechanical plate mixer, absorbance spectra were measured on a ELISA Processor II Microplate Reader at a wavelength of 570 nm.

Antitumor Activity of AMI and Its Polymers

To evaluate the antitumor activity of AMI and its polymers, mice-bearing sarcoma 180 tumor cells were used. Balb/C mice were first intraperitonearly (i.p.) implanted with sarcoma 180 cells (2×10^5 cells/mL). The mice were then treated with a sample saline on Days 1-4. Three different dosages were tested: 0.8, 80, and 800 mg/kg. For comparison, antitumor activities of free 5-fluorouracil (5-FU) were also tested by the same method. A control group was divided into two groups. One group was treated with sarcoma 180 cells along with the same volume of saline, and the other group was treated with sarcoma 180 cells. The ratio (T/C) of survival time of the polymer-treated (T) to that of control groups (C) was used as the index of the antitumor activity. Each group consisted of 10 animals.

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RESULTS AND DISCUSSION

Characterization of Monomer and Polymers

The IR spectrum of AMA shows characteristic absorption peaks at 3500-2700 (−OH in acid group), 3290 (NH), 1715 (C=O), and 1620 (−CH=CH−). ^{1}H-NMR spectrum of AMA shows methene protons due to a double bond at 6.42 ppm, methylene protons at 3.37 and 2.52 ppm, and a carboxylic acid proton at 9.10 ppm.

The IR spectrum of AMI shows characteristic absorption peaks at 3400-3100 (−OH in acid group), 1775 and 1690 (C=O), and 1620 (−CH=CH−). ^{1}H-NMR spectrum of AMI shows methene protons due to a double bond at 7.01 ppm, methylene protons at 3.61 and 2.50 ppm, and a carboxylic acid proton at 11.7 ppm (Fig. 1).

The IR spectrum of poly(AMI) shows characteristic absorption peaks at 3400-3100 (−OH in acid group) and 1775 and 1690 cm^{-1}(C=O). ^{1}H-NMR spectrum of poly(AMI) shows methine protons in the polymer backbone at 3.31 ppm, methylene protons at 3.65 and 2.52 ppm, and a carboxylic acid proton at 11.8 ppm (Fig. 2).

The IR spectrum of poly(AMI-*co*-MA) shows characteristic absorption bands at 3400 (−OH in acid group of AMI and MA), 1745 (C=O of AMI), and 1450 cm^{-1} (−CH_3 of MA). The ^{1}H-NMR spectrum of poly(AMI-*co*-MA) is shown in Fig. 3. The methine protons, methylene protons, and carboxylic acid proton of the AMI moiety in poly(AMI-*co*-MA) were identified with peaks of 3.31, 3.65 and 2.52, and 12.4 ppm. The methylene protons, methyl protons, and carboxylic acid protons due to the MA moiety in poly(AMI-*co*-MA) were identified with peaks of 1.04, 2.02, and 12.4 ppm, respectively. The characteristic peaks of the synthesized copolymer were identified with the disappearance of olefinic peaks at 7.0 ppm.

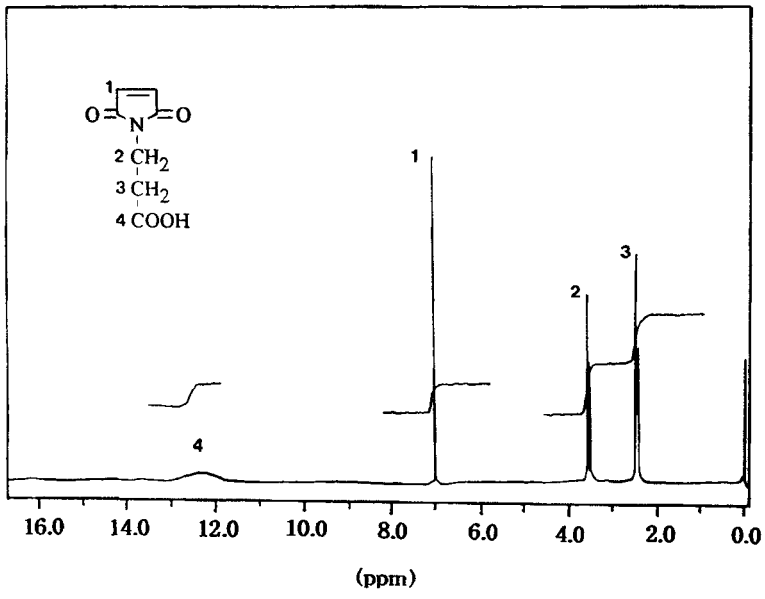

FIG. 1. ^{1}H-NMR spectra (DMSO-d_6) of AMI.

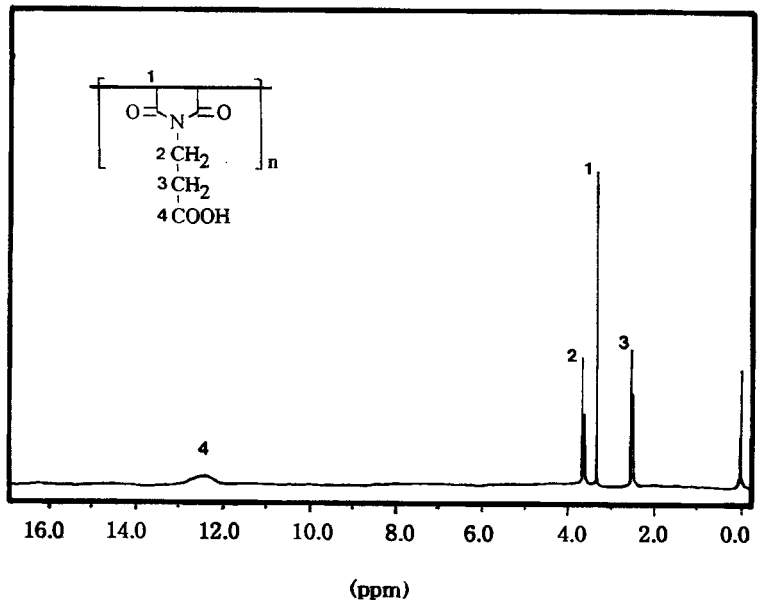

FIG. 2. ^{1}H-NMR spectra (DMSO-d_6) of poly(AMI).

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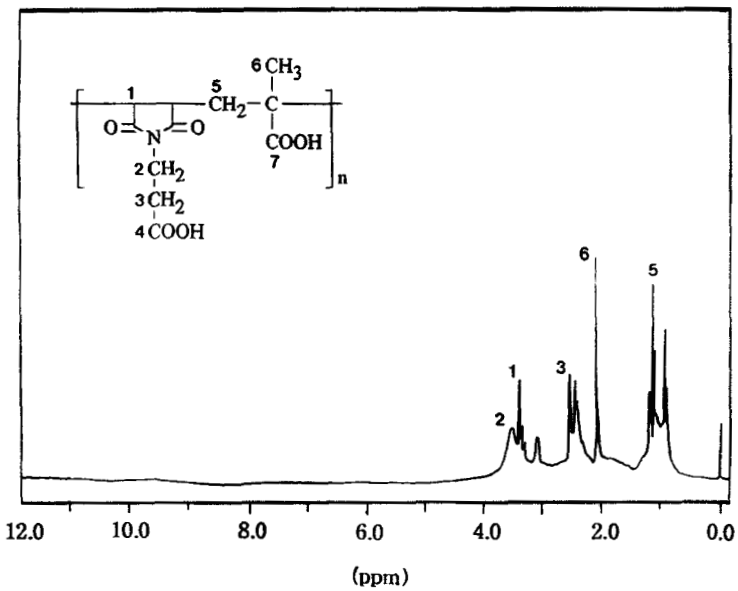

FIG. 3. ^{1}H-NMR spectra (DMSO-d_6) of poly(AMI-*co*-MA).

The IR spectrum of poly(AMI-*co*-VAc) shows the same characteristic absorption bands of AMI as those of poly(AMI-*co*-MA). The absorption at 1464 and at 1234 and 1176 cm^{-1} were assigned to the methylene and ester group of VAc moiety, respectively. Figure 4 is the ^{1}H-NMR spectrum of poly(AMI-*co*-VAc). The peaks due to AMI units are the same as those of poly(AMI-*co*-MA). The peaks at 2.06,

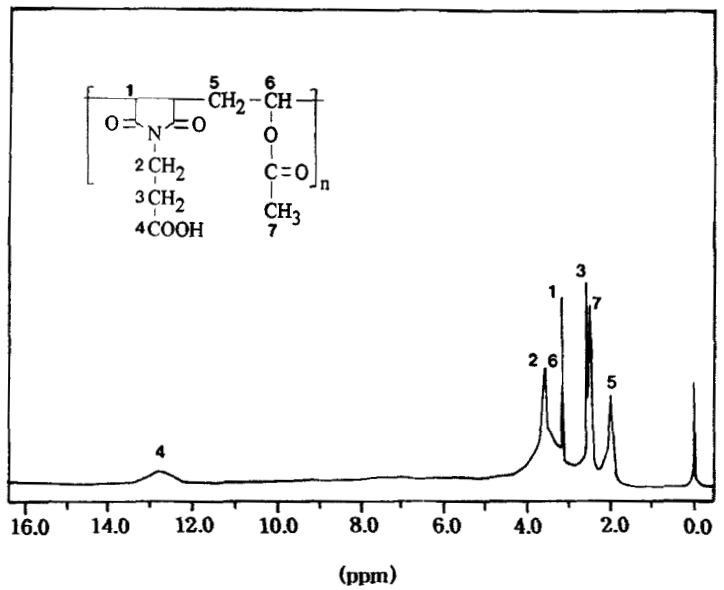

FIG. 4. ^{1}H-NMR spectra (DMSO-d_6) of poly(AMI-*co*-VAc).

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TABLE 1. Solubility of AMI and Its Polymers[a]

Solvent/samples	AMI	Poly(AMI)	Poly(AMI-*co*-MA)	Poly(AMI-*co*-VAc)
Water	○	○	○	○
Dimethylsulfoxide	○	○	○	○
N,N-Dimethylformamide	○	○	○	○
Methanol	○	△	△	△
Acetone	○	△	△	○
Tetrahydrofuran	○	x	△	○
Chloroform	x	x	x	x
1,4-Dioxane	○	x	x	△
2-Butanone	△	x	x	x
Diethyl ether	x	x	x	x
Toluene	x	x	x	x

[a]○: good solubility; △: poor solubility; x: insoluble.

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3.39, and 2.44 ppm were assigned to methylene, methine, and methyl protons of VAc units, respectively.

Solubility of Polymers

Solubilities of AMI, poly(AMI), poly(AMI-*co*-MA), and poly(AMI-*co*-VAc) are listed in Table 1. Poly(AMI) and poly(AMI-*co*-MA) were soluble in water, DMF, and DMSO, but insoluble in THF, 2-butanone, chloroform, and toluene. Poly(AMI-*co*-VAc) was soluble in water, acetone, THF, DMF, and DMSO, but insoluble in chloroform, 2-butanone, and toluene.

Average Molecular Weights and Compositions of Polymers

GPC measurements of polymers with polystyrene as the calibration standard showed narrow molecular weight distributions. $\overline{M}_n$ values of poly(AMI), poly(AMI-*co*-MA), and poly(AMI-*co*-VAc) were 4200, 4000, and 21,500, respectively. The detailed molecular weights and polydispersity (PD) indices are listed in Table 2.

The contents of the AMI moiety in copolymers calculated from the C, N, and H compositions are listed in Table 3. AMI contents in poly(AMI-*co*-MA) and poly(AMI-*co*-VAc) were 83 and 48%, respectively.

TABLE 2. The Average Molecular Weights of Polymers

Polymer	$\overline{M}_n$	$\overline{M}_w$	$\overline{M}_w/\overline{M}_n$[a]
Poly(AMI)	4,200	4,300	1.02
Poly(AMI-*co*-MA)	4,000	4,100	1.03
Poly(AMI-*co*-VAc)	21,500	23,300	1.08

[a]Determined by GPC.

TABLE 3. Elemental Compositions and Contents of AMI Moiety in Poly(AMI-*co*-MA) and Poly(AMI-*co*-VAc)

Sample	E.A., %[a]			AMI unit in polymer, mol%[b]
	C	H	N	
AMI	48.64	4.97	7.64	
Poly(AMI-*co*-MA)	49.94	5.16	6.85	83
Poly(AMI-*co*-VAc)	23.07	2.37	3.96	48

[a]E.A.: Elemental analysis.
[b]Calculated from elemental analysis.

In-Vitro Cytotoxicity of AMI and Its Polymers

Cytotoxicities of AMI and its polymers were evaluated against FM-3A/s, P-388/s, and U-937/s. Table 4 shows the results of in-vitro cytotoxicity. All of the synthesized polymers showed less cytotoxicity than AMI.

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In-Vivo Antitumor Activity of AMI and Its Polymers

Results of in-vivo antitumor activity of AMI, poly(AMI), poly(AMI-*co*-MA), and poly(AMI-*co*-VAc) against sarcoma 180 are listed in Table 5. In this table the antitumor activity of 5-FU is also shown for comparison.

The life span of mice treated with 5-FU is longer than that of the control group at low doses (34 and 40% increase at 0.8 and 80 mg/kg), but 5-FU reduced life span by 61% at a high dose (800 mg/kg). 5-FU showed an efficient antitumor activity at low doses but an undesirable toxicity at a high dose.

The life span of mice treated with polymers was longer than that of 5-FU and the control group at all doses. At 800 mg/kg doses, poly(AMI), poly(AMI-*co*-MA), and poly(AMI-*co*-VAc) increased life span by 64, 55, and 158%, respectively. At

TABLE 4. In-Vitro Cytotoxicity of AMI and Its Polymers Against Tumor Cell Lines

Compound	IC_{50}, μg/mL[a]		
	FM-3A/s[b]	P-388/s[c]	U-937/s[d]
AMI	25	40	48
Poly(AMI)	72	>100	>100
Poly(AMI-*co*-MA)	96	>100	>100
Poly(AMI-*co*-VAc)	>100	>100	>100

[a]IC_{50}: 50% inhibitory concentration.
[b]Mouse mammary carcinoma cell.
[c]Mouse leukemia cell.
[d]Human lymphoma cell.

TABLE 5. In-Vivo Antitumor Activity of AMI and Its Polymers

Samples	Dose, mg/kg	Survival times, day	T/C, %[a]
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 ± 1.3	140
	0.8	20.3 ± 1.8	134
AMI	800.0	1.6 ± 1.8	11
	80.0	37.2 ± 3.5	245
	0.8	24.3 ± 2.4	160
Poly(AMI)	800.0	25.0 ± 1.6	164
	80.0	24.0 ± 1.8	158
	0.8	26.5 ± 2.5	174
Poly(AMI-*co*-MA)	800.0	23.5 ± 1.7	155
	80.0	25.6 ± 1.3	168
	0.8	31.5 ± 1.9	207
Poly(AMI-*co*-VAc)	800.0	39.2 ± 3.7	258
	80.0	31.1 ± 1.7	205
	0.8	27.1 ± 2.2	178

[a]T/C (%) = [(survival time of treated mice)/(survival time of control)] × 100.

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0.8 mg/kg doses, poly(AMI), poly(AMI-*co*-MA), and poly(AMI-*co*-VAc) increased life span by 74, 107, and 78%, respectively. It means that the antitumor activities of polymers were greater than those of 5-FU, and the toxicities of polymers were much less than those of 5-FU.

CONCLUSIONS

1. Poly(AMI) was prepared by the photopolymerization of AMI in 2-butanone by using DMB a an initiator at 25°C. Poly(AMI-*co*-MA) and poly(AMI-*co*-VAc) were prepared by the photocopolymerizations of AMI with MA or VAc in 2-butanone using DMB at 25°C.
2. The average molecular weights of polymers synthesized were as follows: Poly(AMI): $\overline{M}_n$ = 4200, $\overline{M}_w$ = 4300, $\overline{M}_w/\overline{M}_n$ = 1.02. Poly(AMI-*co*-MA): $\overline{M}_n$ = 4000, $\overline{M}_w$ = 4100, $\overline{M}_w/\overline{M}_n$ = 1.03. Poly(AMI-*co*-VAc): $\overline{M}_n$ = 21,500, $\overline{M}_w$ = 23,300, $\overline{M}_w/\overline{M}_n$ = 1.08.
3. The contents of the AMI units in poly(AMI-*co*-MA) and poly(AMI-*co*-VAc) were 83 and 48%, respectively.
4. Cytotoxicities of polymers against FM-3A/s, P-388/s, and U-937/s were lower than those of AMI.

5. Antitumor activities of samples were increased in the following order: 5-FU < AMI < poly(AMI) < poly(AMI-*co*-VAc) < poly(AMI-*co*-MA) (at 0.8 mg/kg doses); 5-FU < poly(AMI) < poly(AMI-*co*-MA) < poly(AMI-*co*-VAc) < AMI (at 80 mg/kg doses); AMI < 5-FU < poly(AMI-*co*-MA) < poly(-AMI) < poly(AMI-*co*-VAc) (at 800 mg/kg doses).

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