# Synthesis and Biological Activities of Copolymers of *N*-Glycinyl Maleimide with Methacrylic Acid and Vinyl Acetate

GI-TAK GAM,[1] JIN-GON JEONG,[1] NEUNG-JU LEE,[2] YOUNG-WOO LEE,[3] CHANG-SIK HA,[1] and WON-JEI CHO[1,*]

[1]Department of Polymer Science & Engineering, College of Engineering, Pusan National University, Pusan 609-735, [2]Department of Premedical Sciences, Kosin University, Pusan 602-702, and [3]Department of Neurosurgery, College of Medicine, Pusan National University, Pusan 609-735 Korea

**SYNOPSIS**

Copolymerizations of *N*-glycinylmaleimide (GMI) with methacrylic acid (MA) and vinyl acetate (VAc) were carried out in 2-butanone using lauroylperoxide as an initiator at 70°C. Synthesized GMI, poly(GMI-*co*-MA), and poly(GMI-*co*-VAc) were characterized by IR and $^1$H-NMR spectroscopies, elemental analysis, and gel permeation chromatography. The *in vitro* cytotoxicities of poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) were evaluated using K-562 human leukemia cells and HeLa cells. From the cytotoxicity data against HeLa cells, the copolymers are less cytotoxic than monomeric GMI at dosage of 0.02, 1.0, and 5.0 mg/mL. Copolymers were very effective at any dosage tested. The *in vivo* antitumor activities of poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) were also evaluated against mice bearing sarcoma 180. Monomeric GMI and its copolymers showed higher antitumor activity than 5-fluorouracil (5-FU) at any dosage tested. © 1995 John Wiley & Sons, Inc.

## INTRODUCTION

The polymer drugs (or polymeric drugs) have received much attention in recent years. The copolymer of divinyl ether with maleic anhydride (DIVEMA) first reported by Butler[1] has been extensively studied for its broad biological activities such as antitumor, antiviral, antibacterial, interferon-inducing, and antifungal activities.[3] Afterward, many attempts[4-9] were made to obtain a polymeric drug like DIVEMA. Several studies have been made also in this laboratory to develop a polymeric antitumor agent.[10-15] Breslow[2] prepared DIVEMA with narrow molecular weight distribution by the photopolymerization technique in solvent with or without a photoinitiator.[16-18]

The aim of this study is to obtain a new biologically active polymer from *N*-glycinylmaleimide (GMI). GMI was expected to show considerably high biological activity because it has amino acid moiety in the repeating unit and its anionic character after hydrolysis is similar to that of DIVEMA.

In this work, GMI was synthesized by the reaction of maleic anhydride and glycine. Poly(GMI-*co*-MA) (MA, methacrylic acid) and poly(GMI-*co*-VAc) (VAc, vinyl acetate) were prepared by the copolymerizations of the corresponding monomer pairs. The structure of monomeric GMI, poly(GMI-*co*-MA), and poly(GMI-*co*-VAc) were identified by IR and $^1$H-NMR spectroscopies.

*In vitro* cytotoxicities of the prepared copolymers were evaluated with K-562 human leukemia cells and HeLa cells as a target cell. *In vivo* antitumor activities against sarcoma 180 were also investigated using tumor bearing Balb/C mice.

## EXPERIMENTAL

### Materials

Maleic anhydride (MAH, Junsei Chem.) was recrystallized from chloroform. Glycine (Junsei Chem.) was recrystallized from distilled water. Lauroylperoxide (LPO), MA, and VAc were purified by conventional methods.

* To whom correspondence should be addressed.

Journal of Applied Polymer Science, Vol. 57, 219–225 (1995)
 CCC 0021-8995/95/020219-07

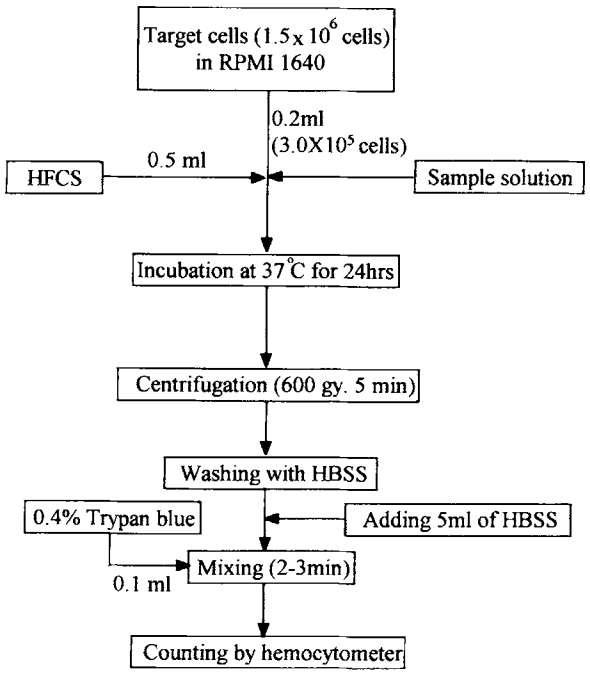


**Figure 1** Procedure to determine the cytotoxicity by the dye exclusion method.

For the *in vitro* test, K-562 human leukemia and HeLa cell lines were used as target tumor cells. 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 obtained on a Jasco FT/IR-5300 spectrophotometer using a KBr disc. $^1$H-NMR spectra were recorded on a FT-300 MHz Bruker A-3000 spectrophotometer. Average molecular weights were determined by gel permeation chromatography (GPC; Water, Water-410).

Elemental analysis was performed on a Carlo Erba Instruments model EA1108 elemental analyzer.

## Synthesis of GMI

A mixture of 41.7 g (0.425 mol) of MAH and 31.9 g (0.425 mol) of glycine in 680 mL of acetic acid was placed in a round-bottom flask. The mixture was stirred at room temperature for 3 h. The white precipitate was filtered, washed with cold water (50 mL), and dried. Crystallization from water afforded analytically pure glycinyl maleamic acid (GMA) (yield, 95%). The melting point of GMA was 193°C.[19,20] GMA (2.91 g) was suspended in 500 mL of dry toluene and treated with 3.55 g of triethylamine. The mixture was refluxed with concomitant removal of the produced water through a Dean–Stark apparatus for 1.5 h. The toluene solution containing the reaction product was decanted from the brown-colored oil. Toluene was removed by evaporation to yield the triethylammonium salt of GMI as a hygroscopic solid. The solid was acidified to pH 2 with HCl, extracted with ethyl acetate, and dried

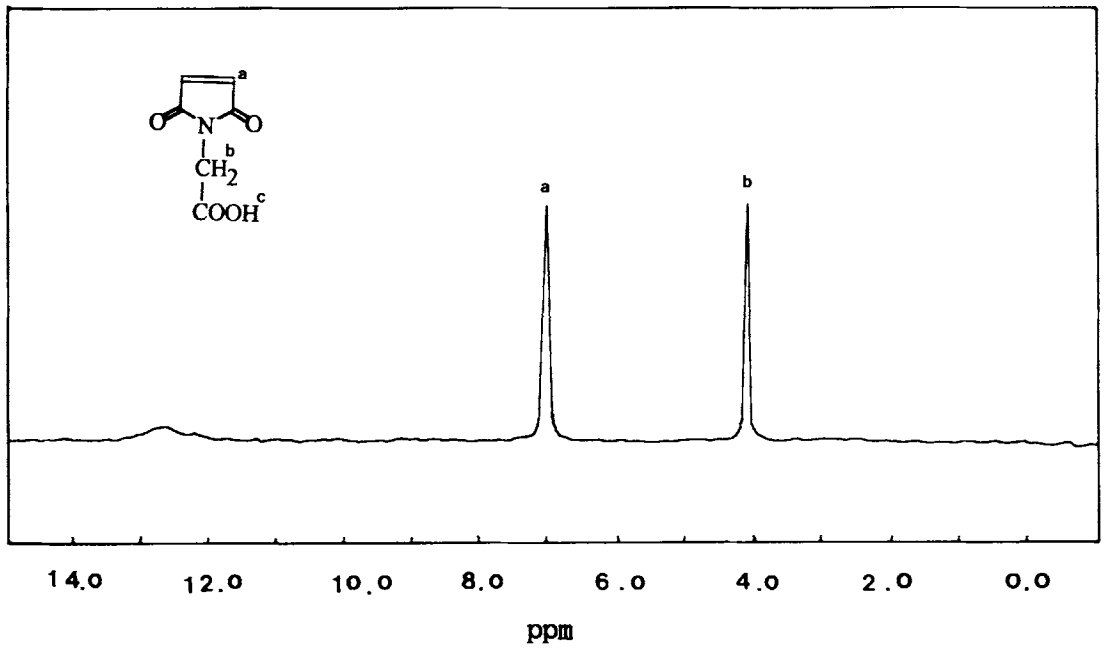


**Figure 2** $^1$H-NMR spectrum of GMI (300 MHz, DMSO-$d_6$).

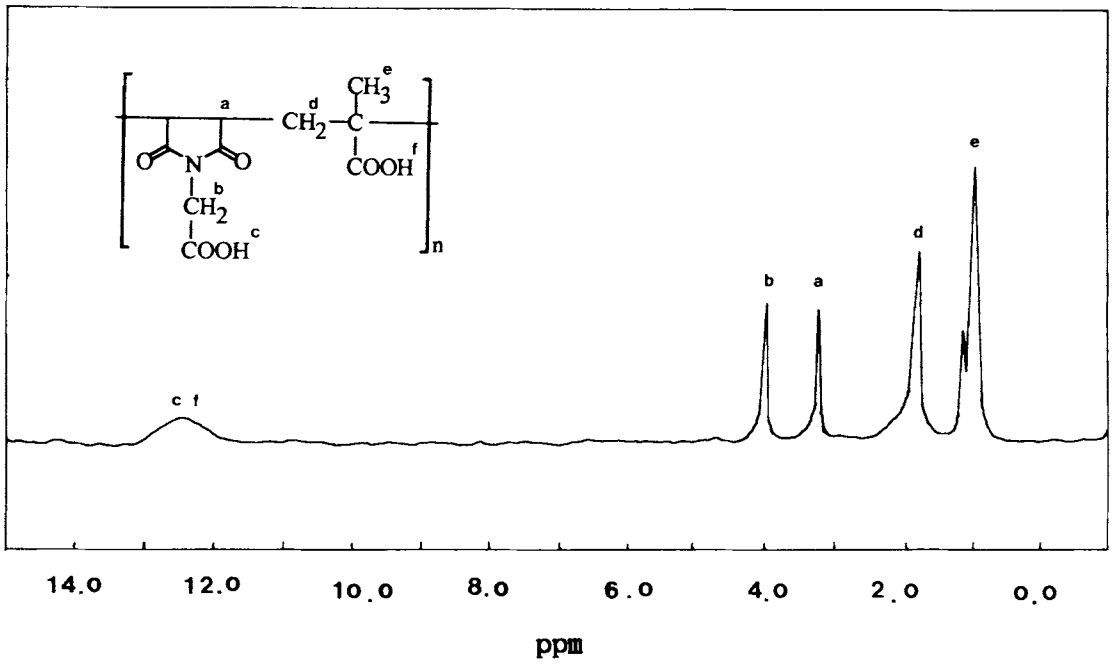


**Figure 3** $^1$H-NMR spectrum of poly(GMI-*co*-MA) (300 MHz, DMSO-$d_6$).

with magnesium sulfate. Ethyl acetate was removed *in vacuuo* to give GMI (yield, 31%). The melting point of GMI was 119°C.[19,20]

ANAL. Calcd for $C_6H_5NO_4$: C = 46.46%; H = 3.25%; N = 9.03%. Found: C = 46.36%; H = 3.12%; N = 9.08%.

## Synthesis of Copolymers

### *Poly(GMI-co-MA)*

GMI (0.78 g, 0.005 mol), 0.42 mL of MA (0.005 mol) and 0.04 g of LPO were dissolved in 9.0 mL of 2-butanone, placed in a polymerization tube, and

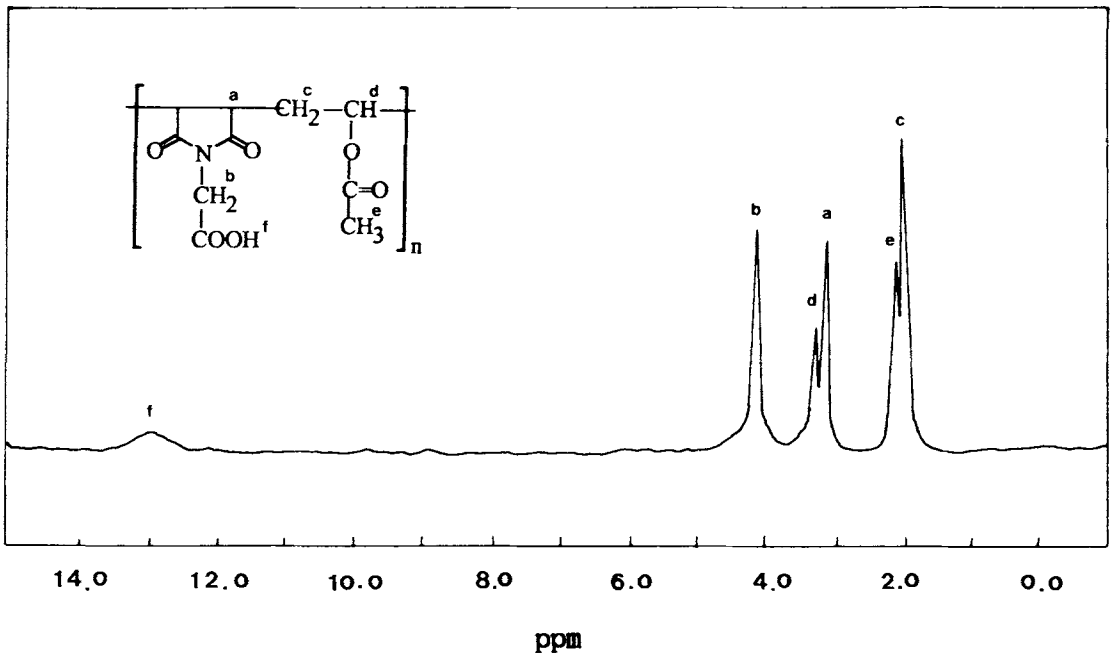


**Figure 4** $^1$H-NMR spectrum of poly(GMI-*co*-VAc) (300 MHz, DMSO-$d_6$).

**Table I Solubility of GMI, Poly(GMI-*co*-MA), and Poly(GMI-*co*-VAc)**

| Solvent | GMI | Poly(GMI-*co*-MA) | Poly(GMI-*co*-VAc) |
|---|---|---|---|
| Water | ○ | ○ | △ |
| Methanol | △ | ○ | ○ |
| 1,4-Dioxan | △ | × | × |
| Diethylether | × | × | × |
| Tetrahydrofuran | ○ | △ | △ |
| Chloroform | △ | × | × |
| Acetone | ○ | × | ○ |
| 2-Butanone | ○ | × | × |
| *n*-Hexane | × | × | × |
| Toluene | × | × | × |
| *N*,*N*-Dimethylformamide | ○ | ○ | ○ |

○, good solubility; △, partially soluble; ×, insoluble.

dried. The tube was sealed after flushing twice with bubbling purified gaseous nitrogen and placed in the regulated thermostat bath at 70 ± 0.5°C for 48 h. The precipitated poly(GMI-*co*-MA) was filtered and washed twice with 2-butanone. Then the polymer was collected by filtration and dried until a constant weight in a vacuum oven at 30°C (yield, 46.1%). The elemental analysis found: C = 42.31%; H = 5.39%; N = 2.73%.

### *Poly(GMI-co-VAc)*

GMI (0.78 g, 0.005 mol), 0.46 mL of VAc (0.005 mol) and 0.04 g of LPO in 9.0 mL of 2-butanone was placed in a dried polymerization tube. Copolymerization of GMI and VAc was carried out by a similar method to that used for the copolymerization of GMI and MA. Results of elemental analysis were found: C = 42.80%, H = 4.38%, N = 5.39%.

## Measurement of Average Molecular Weight

Number average molecular weight ($\overline{M_n}$) and weight average molecular weight ($\overline{M_w}$) of poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) were determined by GPC using a microstyragel column and monodisperse polystyrene as a standard at 40°C. The used eluent was tetrahydrofuran. The concentration of polymers was 0.1% or less.

## Analysis of Copolymers

The content of GMI moiety in copolymers were calculated from C, N, and H data using a Carlo Erba Instruments Model EA1108 elemental analyzer.

## Assay of Biological Activity

### *Cytotoxicity of GMI and Its Copolymers*

The procedure to investigate the *in vitro* cytotoxicity is as follows (Fig. 1): The assay was carried out in 96-well flat-bottom tissue culture plates. Target cells were K-562 human leukemia cells and HeLa cells. The cells were suspended at $3 \times 10^5$ cells/mL in a culture medium containing HFCS (heat inactivated fetal calf serum). The target cells (0.7 mL) and a polymer solution (4.3 mL) were added to each well of the plate. The assay was run six times. The plates were incubated at 37°C in a 5% $CO_2$ incubator for 24 h and then the cell suspension was centrifuged at 600 × *g* at room temperature for 5 min. After centrifugation, the precipitated cells were washed twice with HBSS (Hanks' balanced salt solution), and then 5 mL of HBSS were added. Viability of the cells was determined with trypan blue by the dye exclusion method. The percent cytotoxicity was calculated by the following equation.

$$\text{cytotoxicity (\%)} = \frac{\text{no. control cells} - \text{no. sample cells}}{\text{no. control cells}} \times 100.$$

**Table II Average Molecular Weights of Poly(GMI-*co*-MA) and Poly(GMI-*co*-VAc)**

| Copolymer | $M_n$ | $M_w$ | $M_w/M_n$ |
|---|---|---|---|
| Poly(GMI-*co*-MA) | 8400 | 13500 | 1.61 |
| Poly(GMI-*co*-VAc) | 4900 | 7000 | 1.43 |

Eluent: THF; PS standard; Waters, Water-410.

**Table III Content of GMI Moiety in Poly(GMI-*co*-MA) and Poly(GMI-*co*-VAc)**

| | Poly(GMI-*co*-MA) | Poly(GMI-*co*-VAc) |
|---|---|---|
| Element composition (%)[a] | | |
| C | 42.31 | 42.80 |
| H | 5.39 | 4.38 |
| N | 2.73 | 5.39 |
| GMI fraction in copolymer (%) | 24.8 | 55.1 |

[a] Determined by elemental analysis.

### *Antitumor Activity of GMI and Its Copolymers*

To evaluate the antitumor activity of GMI and its copolymers, mice bearing sarcoma 180 tumor cells were used. Balb/C mice ($n$ = 10) were first intraperitonearly (i.p.) implanted with sarcoma 180 cells ($2 \times 10^5$). The animals were then treated with a saline of sample at 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) also were 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 only sarcoma 180 cells. The ratio (T/C) of survival times 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.

## RESULTS AND DISCUSSION

### Identification of Monomer and Polymers

The IR spectrum of GMI shows characteristic absorption peaks at: 3100 $cm^{-1}$ (— OH in acid group); 1775 and 1690 $cm^{-1}$ (C=O); 1620 $cm^{-1}$ (— CH=CH —); and 1440 $cm^{-1}$ (— $CH_2$ —). $^1$H-NMR spectrum of GMI showed methene protons in double bond at 7.06 and 7.1 ppm, methylene protons at 4.1 ppm, and a proton of carboxylic acid at 12.7 ppm (Fig. 2).

The IR spectrum of poly(GMI-*co*-MA) shows characteristic absorption bands at 3400 $cm^{-1}$ (— OH in acid group of GMI and MA), 1745 $cm^{-1}$ (C=O of GMI) and 1450 $cm^{-1}$ (— $CH_3$ of MA). The absorption peak assignable to the C=C bond of monomeric GMI was not observed at 1620 $cm^{-1}$. The $^1$H-NMR spectrum of poly(GMI-*co*-MA) is shown in Figure 3. The methine protons, methylene protons, and a proton of carboxylic acid of GMI moiety in poly(GMI-*co*-MA) were characterized by peaks at 3.2, 4.03, and 12.60 ppm. The peaks at 1.77, 1.04, 0.94, and 12.60 ppm were assigned to methylene protons, methyl protons, and a proton of carboxylic acid of MA moiety in poly(GMI-*co*-MA). The peaks as assigned to the olefinic proton of comonomers at 7.06 and 7.10 ppm disappeared.

The IR spectrum of poly(GMI-*co*-VAc) shows the same characteristic absorption bands of GMI as those of poly(GMI-*co*-MA). The absorption at 1465 $cm^{-1}$, and 1234 and 1176 $cm^{-1}$ were assigned to the methylene and carboxylic group of VAc moiety, respectively. Figure 4 is the $^1$H-NMR spectrum of poly(GMI-*co*-VAc). The peaks due to GMI units are the same as those of poly(GMI-*co*-MA). The peaks at 2.03 and 3.4 ppm were assigned to methylene and methine protons of VAc units, respectively.

**Table IV *In Vitro* Cytotoxicity of GMI, Poly(GMI-*co*-MA), and Poly(GMI-*co*-VAc) Against K-562 Human Leukemia Cells**

| Sample Concentration (mg/mL) | Cytotoxicity (%) | | |
|---|---|---|---|
| | GMI | Poly(GMI-*co*-MA) | Poly(GMI-*co*-VAc) |
| 5.0 | 100.0 | 99.5 | 90.5 |
| 1.0 | 83.1 | 31.4 | 38.0 |
| 0.1 | 15.3 | 20.8 | 21.9 |
| 0.02 | 20.0 | 0.0 | 19.5 |

**Table V *In Vitro* Cytotoxicity of GMI, Poly(GMI-*co*-MA), and Poly(GMI-*co*-VAc) Against HeLa Cells**

| Sample Concentration (mg/mL) | Cytotoxicity (%) | | |
|---|---|---|---|
| | GMI | Poly(GMI-*co*-MA) | Poly(GMI-*co*-VAc) |
| 5.0 | 100.0 | 44.4 | 81.5 |
| 1.0 | 86.8 | 35.1 | 54.9 |
| 0.1 | 19.2 | 29.8 | 19.2 |
| 0.02 | 21.8 | 0.0 | 1.9 |

## Solubility, Average Molecular Weights, and Composition of Polymers

Solubilities of monomeric GMI, poly(GMI-*co*-MA), and poly(GMI-*co*-VAc) are listed in Table I. Poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) are soluble in polar solvents such as water, dimethylformamide (DMF), and methanol, but insoluble in chloroform.

The average molecular weights of poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) measured by GPC are listed in Table II. Number average molecular weight of synthesized poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) were 8400 and 4900, respectively.

The content of GMI moiety in copolymers calculated from C, N, and H data by elemental analysis was listed in Table III. GMI content in poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) was 24.8 and 55.1%, respectively.

## *In Vitro* Cytotoxicity of GMI and Its Copolymers

K-562 human leukemia cells and HeLa cells were treated with GMI, poly(GMI-*co*-MA), and poly(GMI-*co*-VAc) at various concentrations of the samples (0.02–5 mg/mL). Tables IV and V show the effect of sample concentration on the *in vitro* cytotoxicity against the target cells. Poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) showed less cytotoxicity against K-562 cells than GMI at higher dosages (1.0 and 5.0 mg/mL; Table V). Copolymers also showed less cytotoxicity against HeLa cells than monomeric GMI at higher dosages (1.0 and 5.0 mg/mL; Table V).

## *In Vivo* Antitumor Activity of GMI and Copolymers Containing GMI

Results of *in vivo* antitumor activity of GMI, poly(GMI-*co*-MA), and poly(GMI-*co*-VAc) against sarcoma 180 are listed in Table VI. 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 dosages, but is remarkably short at a high dosage (800 mg/kg).

**Table VI *In Vivo* Antitumor Activity Against Sarcoma 180**

| Drug | No. of Mice | Dose (mg/kg) | Survival Time (Days) | T/C (%) |
|---|---|---|---|---|
| Control | 10 | — | 14.7 ± 2.3 | 100 |
| | 10 | Saline | 15.7 ± 0.5 | 100 |
| 5-FU | 10 | 800.0 | 5.9 ± 0.3 | 39 |
| | 10 | 80.0 | 21.3 ± 1.3 | 140 |
| | 10 | 0.8 | 20.3 ± 1.8 | 134 |
| GMI | 10 | 800.0 | 1.7 ± 0.1 | 11 |
| | 10 | 80.0 | 34.7 ± 4.1 | 228 |
| | 10 | 0.8 | 22.6 ± 1.3 | 149 |
| Poly(GMI-*co*-MA) | 10 | 800.0 | 30.0 ± 6.6 | 197 |
| | 10 | 80.0 | 29.9 ± 10.6 | 197 |
| | 10 | 0.8 | 20.8 ± 1.6 | 137 |
| Poly(GMI-*co*-VAc) | 10 | 800.0 | 27.0 ± 2.1 | 178 |
| | 10 | 80.0 | 24.4 ± 1.6 | 161 |
| | 10 | 0.8 | 29.1 ± 2.8 | 191 |

Groups: T, polymer treated; C, control.

5-FU has enhanced survival at low dosage (0.8 and 80 mg/kg) but has not proven to be effective at high dosage (800 mg/kg) because of its side toxicity.

The survival times of mice treated with GMI, poly(GMI-*co*-MA), and poly(GMI-*co*-VAc) was not shorter than those of treated with 5-FU. Poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) showed more effective antitumor activity than 5-FU at low dosages. The antitumor activity of monomeric GMI was higher than that of 5-FU at low dosages (0.8 and 80 mg/kg). The antitumor activity of poly(GMI-*co*-MA) was similar to that of poly(GMI-*co*-VAc) at all dosages.

## CONCLUSIONS

Poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) were prepared by the copolymerization of GMI with MA and VAC in 2-butanone using LPO at 70°C. The average molecular weights of copolymers synthesized determined by GPC were:

1. poly(GMI-*co*-MA): $\overline{M_n}$ = 8400, $\overline{M_w}$ = 13500, $\overline{M_w}/\overline{M_n}$ = 1.61;
2. poly(GMI-*co*-VAc): $\overline{M_n}$ = 4900, $\overline{M_w}$ = 7000, $\overline{M_w}/\overline{M_n}$ = 1.43.

The contents of the GMI units in poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) were 24.8 and 55.1%, respectively.

Cytotoxicities against K-562 human leukemia cells and HeLa cells of poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) were lower than that of monomeric GMI at higher doses (1.0 and 5.0 mg/mL).

Antitumor activity of poly(GMI-*co*-MA) and poly(GMI-*co*-VAc) against sarcoma 180 was higher than that of 5-FU at all dosages tested.

The antitumor activity of monomeric GMI was higher than that of 5-FU at low dosages (0.8 and 80 mg/kg).

This work was financially supported by the Korea Science and Engineering Foundation (Grant No. 931-0300-014-2).

## REFERENCES

1. G. Butler, *J. Polym. Sci.*, **48,** 279 (1960).
2. D. S. Breslow, E. I. Edwards, and N. R. Newberg, *Nature,* **346,** 160 (1973).
3. G. Butler and A. Zampini, *J. Macromol. Sci. Chem.,* **A11,** 491 (1977).
4. R. M. Ottenbrite, E. Goodell, and A. Munson, *Polymer,* **18,** 461, (1977).
5. R. M. Ottenbrite, K. Kuus, and K. Kaplan, *Polym. Prepr., Am. Chem. Soc., Div. Polym. Chem.,* **24,** 25 (1983).
6. R. M. Ottenbrite, *J. Macromol. Sci. Chem.,* **A22,** 819 (1985).
7. F. W. Bassett, Jr. and O. Vogel, *J. Polym. Sci., Polym. Chem. Ed.,* **21,** 891 (1983).
8. T. Sato, Junzo Sunamoto, and R. M. Ottenbrite, *J. Bioactive Comp. Polym.,* **1,** 448 (1986).
9. N. J. Lee, S. H. Oh, C. S. Ha, J. K. Lee, and W. J. Cho, *J. In Korean Ind. Eng. Chem.,* **1,** 190 (1990).
10. N. J. Lee, C. S. Ha, and W. J. Cho, *Polymer (Korea),* **15,** 211 (1991).
11. N. J. Lee, I. S. Kim, C. S. Ha, and W. J. Cho, *J. Korean Ind. Eng. Chem.,* **2,** 156 (1991).
12. M. S. Shim, N. J. Lee, C. S. Ha, and W. J. Cho, *Polymer (Korea),* **15,** 489 (1991).
13. W. M. Choi, I. S. Kim, C. S. Ha, W. J. Cho, and N. J. Lee, *J. Bioactive Compatable Polym.,* **7,** 39 (1992).
14. N. J. Lee, C. S. Ha, and W. J. Cho, *J. Macromol. Sci.-Pure Appl. Chem.,* **A29,** 161 (1992).
15. M. S. Melzer, *Eur. J. Cancer,* **16**(1), 15 (1980).
16. A. S. Sartoelli and D. G. Johns, *Handbook of Experimental Pharmacology,* Vol. 38, Parts 1 and 2, Springer, Berlin, 1974/1975.
17. P. Ehrlich, in *Readings in Pharmacology,* L. Shuster (Ed.), Little, Brown, Boston, 1962, p. 231.
18. H. Ringsdorf, *J. Polym. Sci. Symp.,* **51,** 135 (1975).
19. T. Oishi, Y. Otsubo, and M. Fujimoto, *Polym. J.,* **24,** 527–537 (1992).
20. T. Oishi, A. Karmori, and M. Fujimoto, *J. Macromol. Sci.—Pure Appl. Chem.,* **A29,** 231 (1992).

*Received September 28, 1994*
*Accepted December 22, 1994*