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Synthesis and biological activity of medium range molecular weight polymers containing *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalimidocaproic acid

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Abstract: A new monomer, *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalimidocaproic acid (ETCA), was prepared by reaction of maleimidocaproic acid and furan. The homopolymer of ETCA and its copolymers with acrylic acid (AA) or with vinyl acetate (VAc) were obtained by photopolymerizations using 2,2-dimethoxy-2-phenylacetophenone as an initiator at 25 °C. The synthesized ETCA and its polymers were identified by FTIR, ^{1}H NMR and ^{13}C NMR spectroscopies. The apparent average molecular weights and polydispersity indices determined by gel permeation chromatography (GPC) were as follows: $M_n = 9600\,g\,mol^{-1}$, $M_w = 9800\,g\,mol^{-1}$, $M_w/M_n = 1.1$ for poly(ETCA); $M_n = 14300\,g\,mol^{-1}$, $M_w = 16200\,g\,mol^{-1}$, $M_w/M_n = 1.2$ for poly(ETCA-*co*-AA); $M_n = 17900\,g\,mol^{-1}$, $M_w = 18300\,g\,mol^{-1}$, $M_w/M_n = 1.1$ for poly(ETCA-*co*-VAc). The *in vitro* cytotoxicity of the synthesized compounds against mouse mammary carcinoma and human histiocytic lymphoma cancer cell lines decreased in the following order: 5-fluorouracil (5-FU) $\geq$ ETCA $>$ polymers. The *in vivo* antitumour activity of the polymers against Balb/C mice bearing sarcoma 180 tumour cells was greater than that of 5-FU at all doses tested.

Keywords: *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalimidocaproic acid (ETCA); medium molecular weight; photopolymerization; *in vitro* cytotoxicity; *in vivo* antitumour activity

INTRODUCTION

Polymeric materials are commonly examined for possible use in chemotherapy for the treatment of cancer and other diseases. On the basis of their structural motif, polymeric anticancer agents can be broadly classified into two classes. In the first category are included polymer–drug conjugates, in which a biologically active substance is bonded to a polymeric matrix and is solely responsible for the physiological effect. Such polymeric carriers aim to overcome the problems associated with the inherent lack of specificity of drugs by providing site-specific drug targeting and increased drug concentration at the desired sites. In the second class are included polymers whose therapeutic potential is solely attributable to their macromolecular matrix. These polymers are not conjugated with drugs and, moreover, their monomeric units do not exhibit any significant biological activity.

One of the best examples of the latter category is the copolymer of divinyl ether with maleic anhydride (DIVEMA), which has been extensively studied for its macromolecular structure and biological activities. The structural feature of the hydrolysed form of DIVEMA contains the carboxylic group as a hydrophilic part and sugar moieties such as a pyran or furan ring as a hydrophobic part. This polymer has antitumour, antiviral, antibacterial, interferon-inducing and antifungal activities.[1–3] Nonetheless, despite its rich pharmacological profile, DIVEMA was also shown to have significant side effects such as pyrogenicity, thrombocytopenia, inhibition of microsomal enzymes, sensitization to endotoxin, liver damage, organomegaly and depression of the reticuloendothelial system. Following initial studies, several attempts have been made to narrow the molecular weight distribution of DIVEMA, thereby minimizing its side effects and optimizing its pharmacological properties.[4–10]

Over the past few years, research from our own

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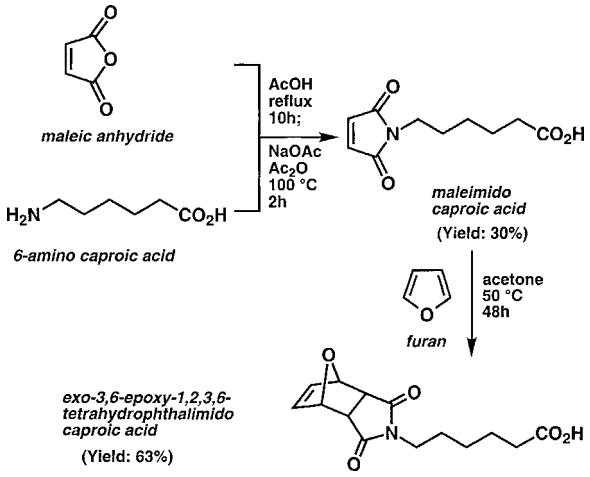
laboratories has been focused on the development of bioactive polymers with high antitumour activity and low toxicity. With this goal in mind, we have reported the synthesis and biological activity of polymeric drugs containing glycinylmaleimide,[11] tetrahydrophthalic glycinylimide,[12] alanylmaleimide,[13] tetrahydrophthalimide,[14] adducts of furan and maleic anhydride.[15]

Herein we report the synthesis and antitumour activity of a new monomer, *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalimidocaproic acid, which was prepared from maleimidocaproic acid and furan. Its homopolymer and copolymers with acrylic acid (AA) or vinyl acetate (VAc) were prepared by photopolymerization. ETCA, poly(ETCA), poly(ETCA-*co*-AA) and poly(ETCA-*co*-VAc) were identified by FTIR, ^{1}HNMR and ^{13}CNMR spectroscopies. The contents of ETCA in poly(ETCA-*co*-AA) and poly(ETCA-*co*-VAc) were determined by elemental analysis. The apparent average molecular weights of the polymers were measured by gel permeation chromatography (GPC). The *in vitro* cytotoxicities were evaluated with mouse mammary carcinoma (FM3A), mouse leukaemia (P388), and human histiocytic lymphoma (U937) as cancer cell lines and mouse liver cells (AC2F) as a normal cell line. The *in vivo* antitumour activities of the synthesized samples were tested against mice bearing the sarcoma 180 tumour cell line.



Scheme 1. Synthesis of ETCA.

EXPERIMENTAL

Materials

Maleic anhydride (Fluka, Wisconsin, USA), furan (Aldrich, Wisconsin, USA), 6-aminocaproic acid (Aldrich) and 2,2-dimethoxy-2-phenylacetophenone (DMP; Aldrich) were used without further purification. Acrylic acid (AA; Junsei, Tokyo, Japan) and vinyl acetate (VAc; Junsei) were purified by conventional methods. All other chemicals were reagent grade and used without further purification. P388, FM3A and U937 as cancer cell lines and AC2F as a normal cell line were used for *in vitro* tests. Balb/C mice and sarcoma 180 cell line for *in vivo* testing were purchased from the Center of Genetic Engineering, Korea Institute of Science and Technology.

Instruments

IR spectra were obtained with a JASCO (Maryland, USA) FTIR-5300 spectrophotometer using KBr pellets. ^{1}HNMR and ^{13}CNMR spectra were recorded on a FT-300 MHz Varian Gemini (California, USA) 2000 spectrophotometer. GPC analysis was conducted with a Waters (Massachusetts, USA) 510. Elemental analysis was performed on a Carlo Erba Model EA1180 elemental analyser.

Synthesis of monomer

The monomer, ETCA, was synthesized according to Scheme 1. A suspension of maleic anhydride (6.13 g, 62.5 mmol) and 6-aminocaproic acid (8.20 g, 62.5 mmol) in 300 ml of glacial acetic acid was heated under reflux for 10 h. The resulting solution was cooled to room temperature. Then, acetic anhydride (100 ml) and sodium acetate (0.14 g) were added and heated at 100 °C for 2 h. After evaporation of the solvent, the residue was dissolved in 25 ml of water. The aqueous solution was washed three times with 35 ml of ethyl ether and the combined organic layers were dried over anhydrous $MgSO_4$. The solvent was removed under vacuum, and the residue recrystallized from ethanol to give pure maleimidocaproic acid (yield 30%). A solution of 1.70 g (24.9 mmol) of furan in 5 ml of acetone and a solution of 3.14 g (14.8 mmol) of maleimidocaproic acid in 30 ml of acetone were mixed in a three-necked flask equipped with magnetic stirrer and nitrogen inlet, and the mixed solution was stirred at 50 °C for 48 h. The solution was concentrated under vacuum conditions and precipitated in excess diethyl ether; the precipitate was collected and dried to give pure ETCA in 63% yield, m p 117–118 °C.

Polymerization

Poly(ETCA) was prepared by photopolymerization of ETCA with DMP as an initiator. In a typical procedure, ETCA (3.0 g, 8.3 mmol) and DMP (0.12 g, 0.5 mmol) were dissolved in 70 ml of acetone. The solution was poured into a dry Pyrex polymerization tube and degassed twice by purging with purified nitrogen gas. The tube was sealed and placed in a photochemical chamber using 313 nm UV lamps at 25 °C for 72 h. The obtained polymer solution was slowly dropped into 500 ml of petroleum ether to precipitate the polymer. The precipitated polymer was collected by filtration and then dried at room temperature under vacuum. The solid product was washed several times with an excess of hot 2-butanone and dried under reduced pressure to a constant weight. The conversion was 50%. Copolymers of ETCA with AA and with MMA were prepared by photopolymerization with DMP as an initiator. ETCA (2.45 g, 8.8 mmol), AA (0.6 g, 8.3 mmol) and DMP (0.15 g, 0.6 mmol) were dissolved in acetone, the solution was introduced into a dry Pyrex polymeriza-

tion tube and degassed by purging with purified N_2 gas. The preparation procedures for poly(ETCA-*co*-AA) and poly(ETCA-*co*-VAc) were the same as that described for the homopolymerization of ETCA except for the monomer pairs used. The conversions to poly(ETCA-*co*-AA) and poly(ETCA-*co*-VAc) were 60% and 55%, respectively.

Measurement of average molecular weight and composition

The apparent molecular weights of the polymers were determined by GPC using a microstyragel column and low polydispersity polystyrene as a standard at 40 °C. Dimethylformamide was used as an eluent. The comonomer composition for each copolymer was calculated from elemental analysis (*N* %).

In vitro cytotoxicity

The cytotoxic effects of ETCA and its polymers against cancer cells *in vitro* were tested using a standard MTT assay.[16] The MTT assay is based on the reduction of the soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into a blue-purple formazan product, mainly by mitochondrial reductase activity inside living cells. All the cell lines used for the cytotoxicity assay were maintained in RPMI-1640 medium containing 10% heat inactivated foetal calf serum. The synthesized compounds were dissolved in the minimum quantity of dimethyl sulfoxide (DMSO) and diluted with phosphate-buffered saline just before use. The solution was added to the cells (2×10^4) in 96-well microtiter plates and cultured for 3 days at 37 °C. The plates were mixed with 20 µl of MTT solution and incubated for 4 h at 37 °C. The supernatant was carefully removed from each well and 100 µl of DMSO was added to each well to dissolve the formazan crystals that were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, the plates were read on an ELISA-reader using a 570 nm filter. All measurements were done in triplicate. The cytotoxicity is represented as the IC_{50}, which denotes

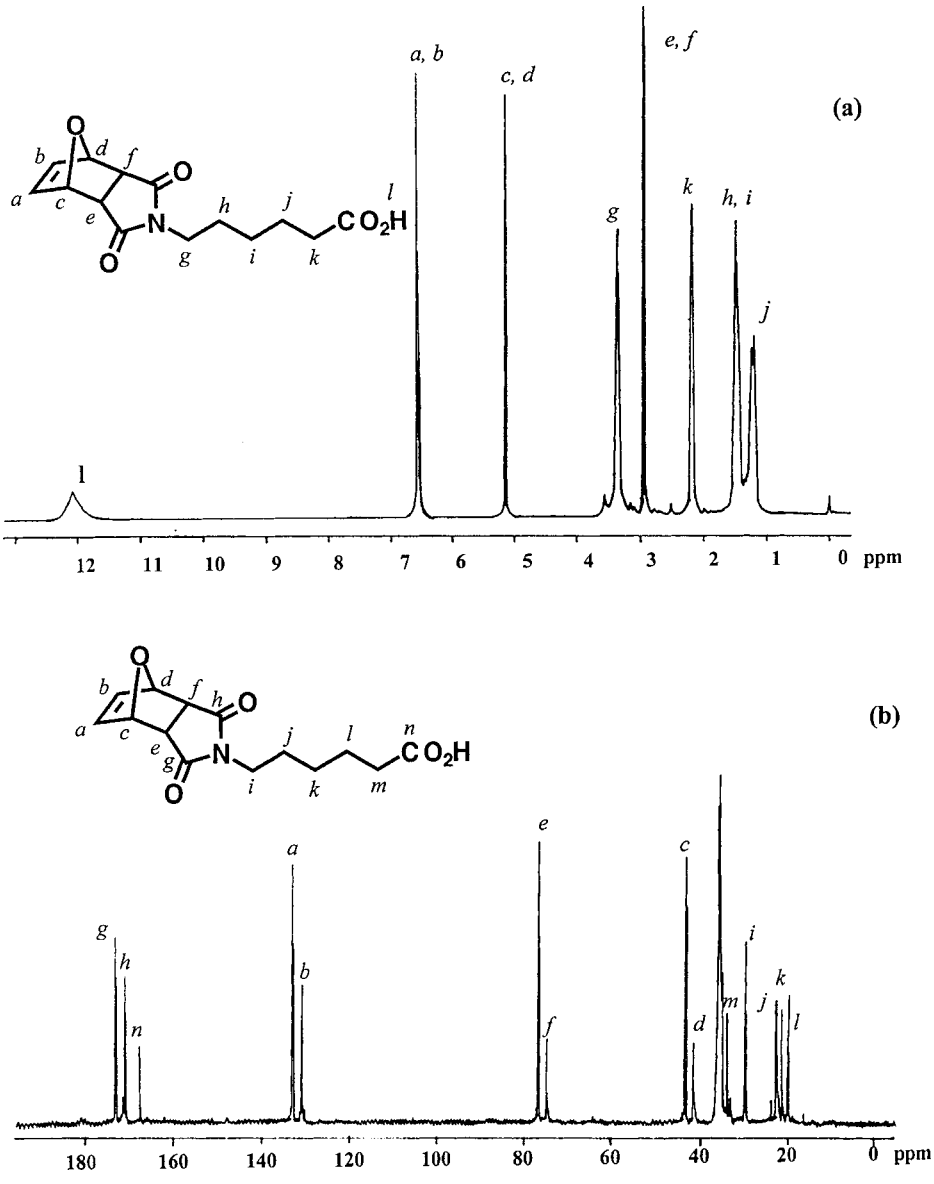


Figure 1. (a) ^{1}HNMR and (b) ^{13}CNMR spectra of ETCA.

Table 1. Solubility of ETCA and its polymers[a]

	Solvent						
Sample	*Water*	*DMSO*	*DMF*	*Acetone*	*2-Butanone*	*THF*	*Diethyl ether*
ETCA	PS	S	S	S	S	S	I
Poly(ETCA)	PS	S	S	PS	I	I	I
Poly(ETCA-*co*-AA)	PS	S	S	PS	I	I	I
Poly(ETCA-*co*-VAc)	PS	S	S	PS	I	I	I

[a] S, soluble; PS, poorly soluble; I, insoluble.

the concentration of 50% growth inhibition. There was good reproducibility between replicate wells with standard errors of less than 10%.

In vivo antitumour activity

For the antitumour activity test, sarcoma 180 cells (2×10^5) isolated from ascitic fluid of tumour-bearing mice were injected intraperitoneally into Balb/C mice (6 weeks, 25 g). Every day for four consecutive days starting from 24 h after the cell injection, the synthesized compounds in physiological saline were administered intraperitoneally to each group. The different doses tested were: 0.8, 80 and 800 mg/kg. Each sample-treated group for each dose consisted of ten mice. Mice were observed until the time of death. For comparison, the antitumour activity of 5-FU was also tested by the same method. The control group was divided into two groups: one subgroup was treated with sarcoma 180 cells together with neat saline by replacing the sample solution; the other subgroup was treated with sarcoma 180 cells alone. The comparative antitumour effect of various dosages on the median survival time in days for treated *versus* control groups was expressed as *T/C*%.

RESULTS AND DISCUSSION

Identification of monomer and polymers

The structures of synthesized monomer and polymers were confirmed by IR, ^{1}H NMR and ^{13}C NMR spectroscopies. The FTIR characteristic absorption peaks for ETCA appeared at 1725 and 1710 cm^{-1} (C=O stretching), and 1640 cm^{-1} (C=C stretching). ^{1}H NMR and ^{13}C NMR spectra of ETCA are shown in Fig 1. ^{1}H NMR (DMSO-d_6): δ (ppm) = 12.1 (s, 1H, COO$\underline{H}$), 6.6 (s, 2H, C$\underline{H}$=C$\underline{H}$), 5.2 (s, 2H, —C$\underline{H}$, —C$\underline{H}$), 2.9 (s, 2H, —C$\underline{H}$—CO, —C$\underline{H}$—CO), 3.4 (s, 2H, —CON—C$\underline{H}_2$—CH$_2$), 2.2 (t, 2H, —C$\underline{H}_2$COOH), 1.5 (t, 4H, —C$\underline{H}_2$—CH$_2$, —CH$_2$—C$\underline{H}_2$), 1.2 (t, 2H, —CH$_2$—C$\underline{H}_2$,— CH$_2$); ^{13}C- NMR (DMSO-d_6): δ (ppm) = 175.2 (—CH$_2$—$\underline{C}$O—CH$_2$), 171.5 (—CH$_2$—$\underline{C}$O—CH$_2$), 167.8 (—CH$_2$—$\underline{C}$O—OH—), 134.3 (—$\underline{C}$H=CH—), 131.4 (—CH=$\underline{C}$H—), 76.4 (—CH—C$\underline{H}$—CO—), 75.6 (—CH—C$\underline{H}$—CO—), 44.1 ($\underline{C}$H—O—CH), 41.7 (CH—O—$\underline{C}$H), 33.3 (—$\underline{C}$H$_2$COOH), 29.1 (—N—$\underline{C}$H$_2$—), 23.7 (—CH$_2$—$\underline{C}$H$_2$ —CH$_2$—), 21.8 (—CH$_2$—$\underline{C}$H$_2$—CH$_2$—), 19.9 (—CH$_2$—$\underline{C}$H$_2$—CH$_2$—).

The FTIR spectrum of poly(ETCA) showed the characteristic absorption peaks at 1730 and 1710 cm^{-1} (C=O stretching) with disappearance of vinyl absorptions at 1660 cm^{-1} due to ETCA monomer. The ^{1}H NMR spectrum of poly(ETCA) showed methine protons of the polymer backbone at 0.9 ppm, methylene protons of aminocaproic acid at 3.4, 2.2, 1.4 and 1.2 ppm with disappearance of the vinyl absorption peak at 6.1 ppm, which was ascribed to the ETCA monomer. The FTIR spectrum of poly(ETCA-*co*-AA) indicated absorption peaks at 3300–2950 cm^{-1} (COOH stretching of AA moiety) and 1730–1700 cm^{-1} (broad, C=O stretching). The ^{1}H NMR spectrum of the copolymer indicated several characteristic peaks of the acid proton of the AA moiety at 12.6 ppm and methine protons in the polymer backbone at 1.0 ppm. The peaks assigned to the olefinic proton of ETCA and AA moiety at 6.0 ppm and 7.1 ppm respectively, disappeared. In the ^{1}H NMR spectrum of poly(ETCA-*co*-VAc), the absorption peaks due to protons of the ETCA moiety were assigned to the same as those of poly(ETCA). The 2.9, 2.3 and 2.0 ppm peaks were assigned to methine, methylene and methyl protons of VAc moiety in copolymer, respectively. Peaks due to vinyl protons in ETCA and VAc were not observed.

Solubility of monomer and polymers

The solubility of the synthesized compounds is shown in Table 1. ETCA was very soluble in DMSO, DMF, acetone, 2-butanone and THF. Polymers were insoluble or poorly soluble in 2-butanone and diethyl ether.

Average molecular weights and compositions of polymers

The apparent average molecular weights of the synthesized polymers are listed in Table 2. The number- (M_n) and weight- (M_w) average molecular weights of the polymers were in the range 9600–17 900 g mol^{-1} and 9800–18 300 g mol^{-1}, respectively, and molecular weight distributions (M_w/M_n) were typically below

Table 2. Apparent average molecular weights[a] and polydispersity of polymers

Polymer	M_n (g mol^{-1})	M_w (g mol^{-1})	M_w/M_n
Poly(ETCA)	9600	9800	1.1
Poly(ETCA-*co*-AA)	14300	16200	1.2
Poly(ETCA-*co*-VAc)	17900	18300	1.1

[a] Molecular weights were determined by GPC in DMF.

1.2. Ottenbrite *et al*[9] reported that medium size molecular weight polymers (in the 10000–30000 g mol^{-1} range) play a significant role in biological activity. Therefore, we controlled the molecular weights of the polymers by using the photopolymerization method on the basis of our previous study,[12–14] and the molecular weights of the synthesized polymers were in a reasonable range to exhibit antitumour activity.

The elemental analysis results for the copolymers are as follows. Poly(ETCA-*co*-AA): C, 53.8; H, 5.2; N, 10.2. Poly(ETCA-*co*-VAc): C, 49.9; H, 3.8; N, 12.2. The ETCA composition in poly(ETCA-*co*-AA) and poly(ETCA-*co*-VAc) was 65 mol% and 68 mol%, respectively.

In vitro cytotoxicity of ETCA and its polymers

The cytotoxicities of ETCA and its polymers *in vitro* were tested with P388, FM3A and U937 as cancer cell lines, and AC2F as a normal cell line. As shown in Table 3, the IC_{50} values of the synthesized samples against cancer cell lines were in the range 6.3 to more than 100 μg ml^{-1}. For U937, the IC_{50} value of ETCA was 7.4 μg ml^{-1}, and those of poly(ETCA), poly(ETCA-*co*-AA) and poly(ETCA-*co*-VAc) were all greater than 100 μg ml^{-1}. The cytotoxicities of 5-FU and synthesized samples against FM3A and U937 cancer cell lines decreased in the following order: 5-FU ≥ ETCA > polymers. The cytotoxicities of the synthesized polymers against the AC2F normal cell line were much lower than those of 5-FU.

In vivo antitumour activity against mice bearing sarcoma 180

The *in vivo* antitumour activities of ETCA and its polymers against the sarcoma 180 tumour cell line were tested together with 5-FU for comparison. The results are listed in Table 4. The ratio *T*/*C* was used as an index of antitumour activity.

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

As shown in Table 4, the *T*/*C* values of mice treated with the polymers were longer than those of the control group and 5-FU at all doses tested. To estimate the effect of the concentration of the synthesized polymers, it is desirable to refer to the fact that the optimum dosage of 5-FU for a human is 15 mg kg^{-1} daily for 5 days every month. The dosage of 0.8 mg kg^{-1} is about 19 times lower than the optimum dosage of 5-FU. At a dose of 0.8 mg kg^{-1}, the *T*/*C* values of the polymers were 174% for poly(ETCA), 167% for poly(ETCA-*co*-AA), and 244% for poly(ETCA-*co*-VAc). The *T*/*C* value of 5-FU at the highest dose (800 mg kg^{-1}) is 39%. Because toxicity of 5-FU at this dose exceeds its therapeutic activity, the survival time is shortened.

Table 3. *In vitro* cytotoxicity of 5-FU, ETCA and polymers against cancer and normal cell lines

	IC_{50}[a] (μg ml^{-1})			
	Cancer cell line			Normal cell line
Sample	FM3A[b]	P388[c]	U937[d]	AC2F[e]
5-FU	0.03	0.04	0.05	0.16
ETCA	9.7	6.3	7.4	28
Poly(ETCA)	≥100	≥100	≥100	≥100
Poly(ETCA-*co*-AA)	≥100	≥100	≥100	≥100
Poly(ETCA-*co*-VAc)	≥100	≥100	≥100	≥100

[a] IC_{50} denotes the 50% growth inhibition concentration.
[b] Mouse mammary carcinoma cell.
[c] Mouse leukemia cell.
[d] Human histiocytic lymphoma cell.
[e] Mouse liver cell line.

However, the *T*/*C* values of the synthesized polymers are 3–12 times greater than that of 5-FU, indicating that the polymers have higher antitumour activity and much lower toxicity than 5-FU.

CONCLUSIONS

The apparent number average molecular weights and molecular weight distributions of the obtained polymers were in the range 9600–17900 g mol^{-1} and 1.1–1.2, respectively. The contents ETCA units in poly(ETCA-*co*-AA) and poly(ETCA-*co*-VAc) were 65 mol% and 68 mol%, respectively. The *in vitro* toxicities of the polymers against cancer and normal cell lines were much lower than that of 5-FU. The antitumour activities of all polymers were higher than those of 5-FU at the dosages used.

Table 4. *In vivo* antitumour activity of 5-FU, ETCA and polymers

Sample	Dose (mg kg^{-1})	Mean survival time (days)	T/C[a] (%)
Control	–	14.7 ± 2.3	100
	Saline	15.7 ± 0.5	100
5-FU	800	5.9 ± 0.3	39
	80	21.3 ± 2.8	140
	0.8	20.3 ± 1.8	134
ETCA	800	70.0 ± 0.6	476
	80	25.9 ± 0.1	176
	0.8	31.6 ± 5.1	214
Poly(ETCA)	800	38.4 ± 12.0	261
	80	26.9 ± 0.2	179
	0.8	25.6 ± 0.78	174
Poly(ETCA-*co*-AA)	800	21.7 ± 0.92	147
	80	24.8 ± 0.73	168
	0.8	24.6 ± 0.74	167
Poly(ETCA-*co*-VAc)	800	73.8 ± 13.2	502
	80	26.2 ± 0.02	178
	0.8	35.9 ± 5.68	244

[a] *T/C* (%) represents the percentage ratio of the survival time of mice treated with polymer (*T*) to control (*C*) mice.

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