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Synthesis and in vitro antitumor activity of phthalimide-based polymers containing camptothecin

Neung-Ju Lee [a,*], Su-Jin Lee [a], Seon-Hee Kim [b], Young-Soo Kang [c], Seong-Bae Moon [d], Honglae Sohn [e], Kyung-Tae Kang [f], Emmanuel A. Theodorakis [g]

[a] *Department of Premedical Sciences, College of Medicine, Kosin University, 34 Amnamdong Seoku, Pusan 602-703, South Korea*
[b] *Department of Biochemistry, College of Medicine, Pusan National University, Pusan 609-735, South Korea*
[c] *Department of Chemistry, Pukyong National University, Pusan 608-737, South Korea*
[d] *Department of Chemistry Education, Pusan National University, Pusan 609-735, South Korea*
[e] *Department of Chemistry, Chosun University, Gwangju 501-759, South Korea*
[f] *Department of Chemistry, Pusan National University, Pusan 609-735, South Korea*
[g] *Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0358, USA*

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Abstract

To improve the therapeutic efficacy of 20(s)-camptothecin (CPT) polymeric drugs containing CPT have been designed. A new CPT-conjugate, 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalimidoacetamidoglycine camptothecin ester (ETPA-gly-CPT), was synthesized by linking its hydroxyl group to the phthalimido monomer through a glycine–glycine spacer. Its homo- and copolymer with acrylic acid (AA) were prepared by photopolymerization using 2,2-dimethoxy-2-phenylacetophenone (DMP) as a photoinitiator. The monomer and its polymers were characterized by IR, ^{1}H- and ^{13}C-NMR spectra. The ETPA-gly-CPT content in poly(ETPA-gly-CPT-co-AA) obtained by elemental analysis was 40 wt.%. The number-average molecular weights of the polymers determined by gel permeation chromatography were as follows: $M_n = 15,000$ for poly(ETPA-gly-CPT), $M_n = 18,700$ for poly(ETPA-gly-CPT-co-AA). The IC_{50} values of ETPA-gly-CPT and its polymers against cancer cells were much larger than that of CPT.

Keywords: 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalimidoacetamidoglycine camptothecin ester (ETPA-gly-CPT); Camptothecin; Photopolymerization; In vitro cytotoxicity

1. Introduction

20(*S*)-Camptothecin (CPT), a pentacyclic alkaloid isolated first by Wall and co-workers in 1966 from the Chinese tree *Camptotheca acuminata*, shows potent cytotoxic activity against a range of tumor cell lines [1–3]. Due to its poor water solubility, clinical trials were begin in 1970 with the more soluble sodium salt [4]. However, administration of this drug was accompanied by severe side effects such as myelosuppression, vomiting, and diarrhea that overwrote its promising antitumor activity in animal models and halted its clinical development [5]. A renewed interest in CPT came from studies on its mechanism of action. In the late 1980s, it was discovered that CPT interacts with DNA topoisomerase I. Topoisomerase I interacts with DNA double strand to form an enzyme-linked single strand break

* Corresponding author. Tel.: +82-519-906-428; fax: +82-512-415-458.
E-mail address: njlee@ns.kosinmed.or.kr (N.-J. Lee).

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and, after unwinding the supercoiled DNA, rejoins the single strand so that DNA replication can proceed. CPT interferes with the religation by binding to the DNA–enzyme binary complex. This results in the accumulation of a reversible enzyme–CPT–DNA ternary complex, which is believed to cause cell death [6].

Polymeric drugs with anticancer drugs such as 5-fluorouracil (5-FU) [7,8], doxorubicin [9,10], paclitaxol [11], methotrexate [12,13], podophyllotoxin [14], and CPT [15,16] were obtained by covalent linking of anticancer drugs to polymers with suitable functional groups, directly or by a chemical spacer arm. To improve the therapeutic potential of CPT, thereby reducing toxicity and improving antitumor activity, polymeric delivery systems such as poly(ethylene glycol) [17,18], *N*-(2-hydroxypropyl) methacrylamide copolymer [19], poly(L-glutamic acid) [20], and phthalimide polymer [21,22] have been developed. The disadvantages of CPT can be overcome by attaching it to a polymeric support that could act as a transport form for this drug and enhance its biodistribution while keeping intact its therapeutic profile.

In the past few years we have synthesized phthalimide-based polymers of 5-FU to improve the biological profile of 5-FU [23,24]. Such polymers have been found to be efficient carriers of 5-FU, retaining its antitumor activity while decreasing its toxicity. More recently, tetrahydrophthalic acid-based polymers of CPT showed a strong antitumor activity in Balb/C mice bearing the sarcoma 180 tumor cell [25,26]. In continuation of our studies, we report herein the synthesis and antitumor activity of polymers containing CPT based on the tetrahydrophthalimide template. The monomer unit, 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalimidoacetamidoglycine camptothecin ester (ETPA-gly-CPT), was built by linking CPT to tetrahydrophthalic acid with diglycine as a spacer. Its homo- and copolymer with acrylic acid (AA) were prepared by photopolymerization. The obtained monomer and its polymers were identified by ^{1}H and ^{13}C NMR spectroscopy, and elemental analysis. The average molecular weights of the polymers were measured by gel permeation chromatography (GPC). The in vitro cytotoxicities were evaluated with human melanoma A375P cell and its metastatic A375SM cell, human colorectal carcinoma KM12C cell and its metastatic KM12SM cells as cancer cell lines, and mouse liver AC2F cell and opossum kidney OK cell as normal cell lines.

2. Experimental

2.1. Materials

Glycine (Aldrich, Wisconsin, USA), 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalic anhydride (MPA; Aldrich, Wisconsin, USA), triethylamine (TEA; Aldrich, Wisconsin, USA), camptothecin (CPT; Aldrich, Wisconsin, USA), dimethylaminopyridine (DMAP; Aldrich, Wisconsin, USA), 1-[3-(dimethylaminopropyl)]-3-ethylcarbodiimide hydrochloride (EDC; Aldrich, Wisconsin, USA), 1-hydroxybenzotriazole (HOBT; Aldrich, Wisconsin, USA), glycine *t*-butyl ester hydrochloride (H-Gly-OtBuHCl; Bachem, Torrance, USA) and 2,2-dimethoxy-2-phenylacetophenone (DMP; Aldrich, Wisconsin, USA) were used without further purification. Acrylic acid (AA; Junsei, Tokyo, Japan) was distilled under vacuum (7 Hgmm, 45 °C). Column chromatography was carried out on silica gel 60 from Merck. Thin-layer chromatography (TLC) was performed on precoated aluminum sheets of silica gel $60F_{254}$ (Merck, Art. 9385). Studies for 50% inhibitory concentration (IC_{50}) using A375P, A375SM, KM12C, KM12SM, AC2F, and OK cell lines were conducted.

2.2. Instruments

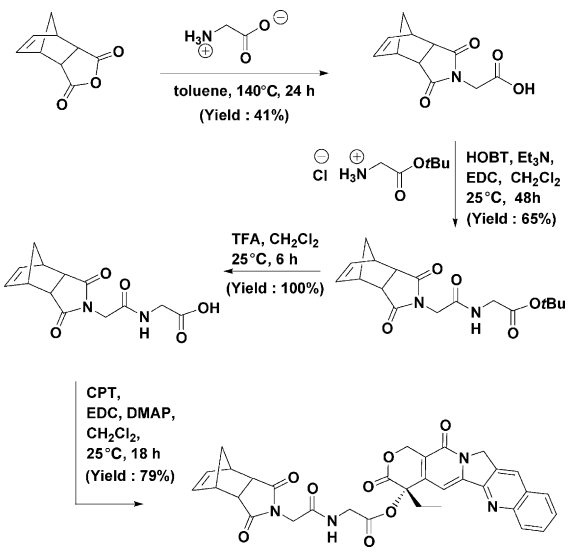
^{1}H and ^{13}C NMR spectra were recorded on a FT-300 MHz Varian Gemini 2000 spectrophotometer. Chemical shifts are given in δ-values referenced to the residual solvent peak of dimethyl sulfoxide (DMSO-d_6) at 2.50 ppm relative to tetramethylsilane (TMS). IR spectra were recorded on a Perkin–Elmer 397 spectrometer using KBr pellets. Elemental analyses were performed on a Carlo Erba model 180 elemental analyzer. Number- and weight-average molecular weights (M_n and M_w) and polydispersity (M_w/M_n) were estimated by gel permeation chromatography (GPC; Waters 410 Differential Refractometer).

2.3. Synthesis of monomer

The ETPA-gly-CPT monomer was synthesized according to Scheme 1.

2.4. 3,6-Endo-methylene-1,2,3,6-tetrahydrophthalimidoacetic acid (ETPA)

Water was distilled azeotropically with a Dean–Stark apparatus from a mixture of MPA (15.0 g, 91.4 mmol), glycine (6.9 g, 92 mmol), and TEA (1.2 ml) in toluene (150 ml). After cooling to room temperature, the reaction mixture was then concentrated under reduced pressure to near dryness. The residue was triturated with 0.1 N HCl (50 ml) and dissolved in saturated aqueous $NaHCO_3$ (60 ml). The aqueous solution was washed with ethyl acetate (45 ml), acidified to pH 2 by adding con. HCl and extracted twice with dichloromethane (50×2 ml). The dichloromethane layers were dried over anhydrous $MgSO_4$ and evaporated to obtain pure ETPEA in 41% yield: ^{1}H NMR (DMSO-d_6): δ(ppm) = 1.54 (s, 2H, $CHCH_2CH$ of MPA), 3.25 (m, 2H,



Scheme 1.

–C*H*CH=CHC*H*– of MPA), 3.42 (s, 2H, –C*H*CONCOC*H*– of MPA), 3.88 (s, 2H, –NCH_2COOH), 6.01 (s, 2H,–C*H*=C*H*– of MPA).

2.5. 3,6-Endo-methylene-1,2,3,6-tetrahydrophthalimidoacetamido glycine t-butyl ester (ETPA-gly-t-BOC)

To an ice-cooled and stirred solution of ETPA (3.0 g, 13.5 mmol), H-Gly-OtBuHCl (2.7 g, 16.3 mmol), HOBt (2.1 g, 16.3 mmol), and triethylamine (1.71 g, 17.0 mmol) in dry dichloromethane (30 ml) was added EDC (3.12 g, 16.3 mmol) and stirring was continued for 48 h at room temperature. The solution was diluted with 100 ml of dichloromethane and washed with 0.1 N HCl (30×2 ml), saturated $NaHCO_3$ (30×2 ml), and water (30×2 ml). The organic layer was dried over anhydrous $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography (CH_2Cl_2:MeOH = 60:1) to give 2.9 g (65%). ^{1}H NMR (DMSO-d_6): δ(ppm) = 1.39 (s, 9H), 1.56 (s, 2H, $CHCH_2CH$ of MPA), 3.24 (m, 2H, –C*H*CH=CHC*H*– of MPA), 3.40 (s, 2H, –C*H*CONCOC*H*– of MPA), 3.72 (d, 2H, HN–CH_2COOH), 3.84 (s, 2H, –NCH_2CONH), 6.04 (s, 2H, –C*H*=C*H*– of MPA), 8.38 (t, 1H, CON*H*CH_2–).

2.6. 3,6-Endo-methylene-1,2,3,6-tetrahydrophthalimidoacetamido glycine (ETPA-gly)

A solution of ETPA-gy-t-BOC (3.48 g, 10.4 mmo1) in a mixture of trifluoroacetic acid (8 ml) and dry dichloromethane (8 ml) was stirred at room temperature for 6 h. Toluene (30 ml) was added, and the solution was concentrated under reduced pressure. The residual trifluoroacetic acid was removed by azeotropic distillation with toluene (30×2 ml) and was triturated in *n*-hexane for 18 h to give a white solid (Yield: 100%). ^{1}H NMR (DMSO-d_6): δ(ppm) = 1.54 (s, 2H, $CHCH_2CH$ of MPA), 3.23 (m, 2H, –C*H*CH=CHC*H*– of MPA), 3.39 (t, 2H, –C*H*CONCOC*H*– of MPA), 3.74 (d, 2H, HN–CH_2COOH), 3.84 (s, 2H, –NCH_2CONH), 6.03 (s, 2H, –C*H*=C*H*– of MPA), 8.34 (t, 1H, CON*H*CH_2–).

2.7. 3,6-Endo-methylene-1,2,3,6-tetrahydrophthalimidoacetamido glycine camptothecin ester (ETPA-gly-CPT)

A mixture of ETPA–gly (252 mg, 2.0 mmol), CPT (179 mg, 0.5 mmol), DMAP(122 mg, 1.0 mmol), and EDC (192 mg, 1.0 mmol) in dry dichloromethane (10 ml) was stirred for 48 h at room temperature followed by dilution with dichloromethane (100 ml). This mixture was washed with 0.1 N HCl (30×2 ml), saturated aqueous sodium bicarbonate (30×2 ml), and water (30×2 ml). The organic layer was dried over anhydrous $MgSO_4$ and filtered, followed by removal of the solvent by evaporation under reduced pressure to give a pale yellow solid. The product was purified by flash silica gel column chromatography using CH_2Cl_2–MeOH (95:5, v/v) as eluent to give as a pale yellow solid (240 mg, Yield: 79%). ^{1}H NMR (DMSO-d_6): δ(ppm) = 0.93 (t, 3H, J = 7.5 Hz, H-18), 1.54 (s, 2H, H-c), 2.11–2.18 (m, 2H, H-19), 3.19 (s, 2H, H-b), 3.30–3.40 (m, 2H, H-d), 3.83 (d, 1H, J = 16.5 Hz, NCH_2CO) 3.87 (d, 1H, J = 16.5 Hz, NCH_2CO), 4.04 (dd, 1H, J = 18.0, 6.0 Hz, NCH_2COO), 4.22 (dd, 1H, J = 18.0, 6.0 Hz, NCH_2COO), 5.23 (s, 2H, H-5), 5.50 (s, 2H, H-17), 5.98–6.02 (m, 2H, H-a), 7.16 (s, 1H, H-14), 7.70 (t, 1H, J = 7.5 Hz, H-10), 7.88 (t, 1H, J = 7.5 Hz, H-11), 8.11 (d, 1H, J = 8.0 Hz, H-9), 8.16 (d, 1H, J = 8.0 Hz, H-12), 8.58 (t, 1H, NH), 8.64 (s, 1H, H-7). ^{13}C NMR (DMSO-d_6): δ(ppm) = 7.5, 30.4, 38.8, 40.0, 40.3, 44.2, 45.5, 50.2, 51.6, 66.3, 76.3, 95.2, 118.9, 127.7, 128.0, 128.5, 128.9, 129.8, 130.4, 131.5, 134.31, 134.36, 145.1, 146.0, 147.9, 152.4, 156.5, 166.1, 167.0, 168.7, 176.76, 176.78.

2.8. Synthesis of polymers

Poly(ETPA-gly-CPT) was synthesized by polymerization of ETPA-gly-CPT. A solution of ETPA-gly-CPT (395 mg) and DMP (10 mg) as an initiator in acetone (30 ml) was introduced into a dry Pyrex polymerization tube. The tube was flushed twice with N_2 gas, sealed and placed in a photochemical chamber where it was irradiated at 313 nm (115 V, 60 Hz power supply) at 25 °C for 72 h. After polymerization, the tube was opened and the viscous liquid obtained was slowly precipitated into a large excess of *n*-hexane (300 ml). The precipitated polymer was collected by filtration and washed several times with acetone. The obtained homopolymer was dried under reduced pressure to a constant weight. The conversion was 73%. Poly(ETPA-gly-CPT-co-AA) was

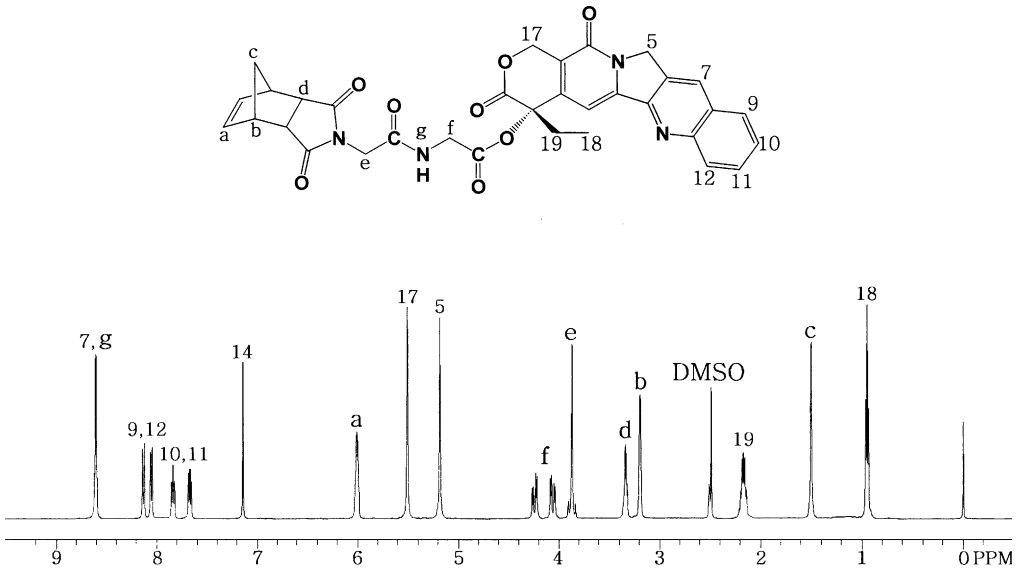


Fig. 1. The ^{1}H NMR spectrum of ETPA-gly-CPT.

obtained by copolymerization of ETPA-gly-CPT (10^{-3} mmol) with AA (10^{-2} mmol). The preparation procedure for the copolymer was the same as that described for the homopolymerization of ETPA-gly-CPT except for the monomer pairs. The copolymerization conversion was 46%.

2.9. Measurements of average molecular weight and compositions

To compare the average molecular weights of the synthesized polymers, we determined the apparent molecular weights by GPC using a microstyragel column and low polydispersity polystyrene as a standard at 40 °C. Dimethylformamide was used as an eluent. The contents of ETPA-gly-CPT moiety in the copolymer were calculated from C, H and N data obtained by elemental analysis.

2.10. In vitro antitumor activity

The cytotoxicity of ETPA-gly-CPT and its polymers against four cancer cell lines in vitro was performed with the MTT assay according to the Mosmann's method [27]. The MTT assay is based on the reduction of the soluble 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into a blue–purple formazan product, mainly by mitochondrial reductase activity inside living cells. The cells used in cytotoxicity assay were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells suspended in the medium (2×10^{4}/ml) were plated in 96 well-culture plates and incubated at 37 °C in a 5% CO_2 incubator. After 12 h, the test sample (2 μl) was added to the cells (2×10^{4}) in 96 well-plates and cultured at 37 °C for three days. The cultured cells 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 which were formed by the cellular reduction of MTT. After mixing with a mechanical plate mixer, the absorbance of each well was measured by a microplate reader using a test wavelength of 570 nm. The results were expressed as the IC_{50}, which is the concentration of the drugs inducing a 50% inhibition of cell growth of treated cells when compared to the growth of control cells. Each experiment was performed at least three times. There was a good reproducibility between replicate wells with standard errors below ±10%.

3. Results and discussion

Direct conjugation of CPT to ETPA–gly through the 20-hydroxyl group was accomplished using EDC as a coupling reagent with DMAP as a organic base. Chemical structure and purity of ETPA-gly-CPT were proven using IR, ^{1}H-NMR, and ^{13}H-NMR spectro-

Table 1
Average molecular weights and polydispersity of the polymers

Polymers	M_n	M_w	M_w/M_n
Poly(ETPA-gly-CPT)	15,000	18,300	1.22
Poly(ETPA-gly-CPT-co-AA)	18,700	24,500	1.31

Molecular weights were determined by GPC in DMF.

Table 2
In vitro cytotoxicity of ETPA-gly-CPT and its polymers against cell lines

Samples	IC_{50} (ng/ml) for cell lines[a]					
	Cancer cells			Normal cells		
	A375P[b]	A375SM[c]	KM12C[d]	KM12SM[e]	AC2F[f]	OK[g]
CPT	2.5 ± 0.2	1.8 ± 0.2	10.0 ± 0.7	6.3 ± 0.3	1.2 ± 0.1	3.5 ± 0.2
ETPA-gly-CPT	16.0 ± 1.2	9.4 ± 0.7	15.2 ± 1.6	20.9 ± 1.7	6.8 ± 0.4	38.0 ± 1.5
Poly(ETPA-gly-CPT)	80.1 ± 1.1	54.2 ± 1.6	17.3 ± 1.2	39.8 ± 1.4	20.0 ± 1.2	100.2 ± 9.2
Poly(ETPA-gly-CPT-co-AA)	50.4 ± 2.2	99.8 ± 3.5	10.2 ± 0.4	52.0 ± 2.1	14.0 ± 0.8	72.3 ± 2.4

[a]The 50% growth inhibition, [b,c]human melanoma cell, [d,e]human colorectal carcinoma cell, [f]mouse liver cell, [g]opossum kidney cell.

scopic techniques. As shown in Fig. 1, the ^{1}H-NMR spectrum of ETPA-gly-CPT showed multiplets at 5.98–6.02 ppm, which were assigned to vinyl group protons. The peaks at 0.93 and 2.11–2.18 ppm were assigned to ethyl group protons of CPT moiety in ETPA-gly-CPT. The IR spectrum of ETPA-gly-CPT showed two peaks at 1756 and 1703 cm^{-1} which were assigned to carbonyl group and a peak at 1669 cm^{-1} which are characteristic peak for vinyl group.

Poly(ETPA-gly-CPT) was synthesized via radical polymerization through the carbon–carbon double bond on the norbornene moiety. The disappearance of vinyl group peak at 1662 cm^{-1} which appeared in ETPA-gly-CPT confirmed a complete conversion of the monomer to its polymer. The peaks for the vinyl protons of monomeric ETECPT at 5.98–6.02 ppm were not observed. The FTIR spectrum of poly(ETPA-gly-CPT-co-AA) indicated absorption at 3500–2800 cm^{-1} (COOH stretching of AA moiety) and 1704 cm^{-1} (C=O stretching). The absorption peaks caused by protons of ETPA-gly-CPT moiety in poly(ETPA-gly-CPT-co-AA) were assigned to the same as those of poly(ETPA-gly-CPT). The peaks assigned to the olefinic proton of ETPA-gly-CPT and AA moiety disappeared. ETPA-gly-CPT and its polymers were soluble in acetone, DMF and DMSO and were insoluble in diethyl ether, *n*-hexane, ethanol and water. The average molecular weights and polydispersity indices of the polymers are listed in Table 1. The number average molecular weights (M_n) and the polydispersity index of poly(ETPA-gly-CPT) were 15,000 and 1.22, respectively.

The elemental analysis value of poly(ETPA-gly-CPT-co-AA) is as follows: C, 60.72; H, 3.12; N, 3.29. The ETPA-gly-CPT composition in the copolymer was calculated from N content and was 40 wt.%. The in vitro cytotoxicities of ETPA-gly-CPT and its polymers against five cancer cell lines and two normal cell lines are shown in Table 2.

As shown in Table 2, the values of 50% cytotoxicity (IC_{50}) for ETPA-gly-CPT and its polymers were in the range of 15–100 ng/ml against cancer cell lines. The cytotoxicity of monomer and its polymers against A375P cells were much lower that of free CPT. The cytotoxicity of ETPA-gly-CPT and its polymers against A375SM and KM12SM cells increased in the following order: CPT>ETPA-gly-CPT>poly(ETPA-gly-CPT)> poly(ETPA-gly-CPT-co-AA). The cytotoxicity of the copolymer against KM12C cell was comparable to that of free CPT. The in vivo study of ETPA-gly-CPT and its polymer against cancer cell lines is currently in progress in our group.

4. Conclusions

In the present study we have synthesized 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalimidoacetamidoglycine camptothecin ester (ETPA-gly-CPT) from camptothecin and 3,6-*endo*-methylene-1,2,3,6-tetrahydrophthalimidoacetamido glycine. Its homo- and copolymer with acrylic acid (AA) were prepared by photopolymerization and were identified by ^{1}H NMR and ^{13}C NMR spectroscopies. The polydispersity indices of all synthesized polymers ranged from 1.22 to 1.31. The content of ETPA-gly-CPT in poly(ETPA-gly-CPT-co-AA) was found to be 40 wt.%. The range of IC_{50} values obtained from the in vitro test for ETPA-gly-CPT, poly(ETPA-gly-CPT), and poly(ETPA-gly-CPT-co-AA) were from 9.4 to 99.8 ng/ml against cancer cell lines. In a normal cell, the cytotoxicity of monomer was stronger than those of its homopolymer and its copolymer.

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