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Synthesis of Poly(Ethylene Glycol) with Sulfadiazine and Chlorambucil End Groups and Investigation of Its Antitumor Activity

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Abstract—α-Amino-ω-hydroxyl-poly(ethylene glycol) (PEG) with different molecular weight (M_r = 2100, 4400, 7200) were synthesized and used as carrier for the combination of sulfadiazine and chlorambucil. In vivo, all these polymer drugs with sulfadiazine and chlorambucil at each end are water soluble and showed the higher antitumor activity against Lewis lung cancer than the same polymers but without the sulfadiazine. The best one is the sample with molecular weight of 2100. In vitro, however, for the samples with same molecular weights, the polymer drugs with and without sulfadiazine showed the similar results against C6 human breast cancer cells. No obvious difference was found.

Poly(ethylene glycol) (PEG) has been used widely as antitumor drug carrier because of its excellent water-solubility, biocompativility and low immunogenecity. Compared with low molecular weight antitumor drugs, PEG modified antitumor drugs can be expected to achieve high water solubility and overcome the problem of side effects by improving the body distribution and prolongation of their activities. Ouchi et al.[1–4] reported several prodrugs of PEG end capped with antitumor agents such as 5-fluorouracil and doxorubicin, which showed high antitumor activities.

The selectivity of traditional chemotherapeutic agents for cancer cells is often low, resulting in unacceptable toxicity to normal tissues. To solve this problem, great efforts have been made to develop tumor-targeting drug-delivery systems. Sulfapyrazine has been shown to concentrate selectively in the walker carcinoma growing in rats.[5] Similarly sulfadiazine was found concentrated in the Yoshida sarcoma.[6] However, when the small drug as mustard connected with the sulfadiazine, all these modified drugs lost the ability to selectively take up by tumor cells..[7] Recently, the work in Huang's group showed if the sulfadiazine was attached on poly(-ethylene glycol)'s (M_r = 2000) end at the site of sulfonamido group rather than the aromatic group, its selectivity could be preserved.[8–13] Therefore, the sulfonamide moiety appears to be a useful directing group toward the tumor tissues.

Chlorambucil is an effective antitumor drug and widely used in the treatment of tumors, but it is sparingly soluble in water and exhibits severe side effects. Till now, there has no report about the PEGylation of chlorambucil.

Based on these facts, we designed and synthesized a novel kind of antitumor drug using α-amino-ω-hydroxyl-PEG with different molecular weight as carrier in which one end is sulfadiazine, the other is chlorambucil. As a comparison, the same polymer drugs but without sulfadiazine moiety were also prepared. The preliminary investigation was focused on their antitumor activity in vitro and in vivo.

Chemistry

As shown in Scheme 1, α-amino-ω-hydroxyl-poly(ethylene glycol) (PEG) with different molecular weight (M_r = 2100,

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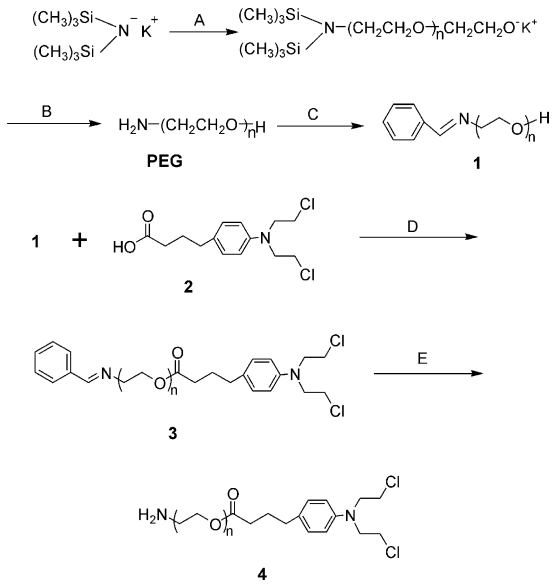
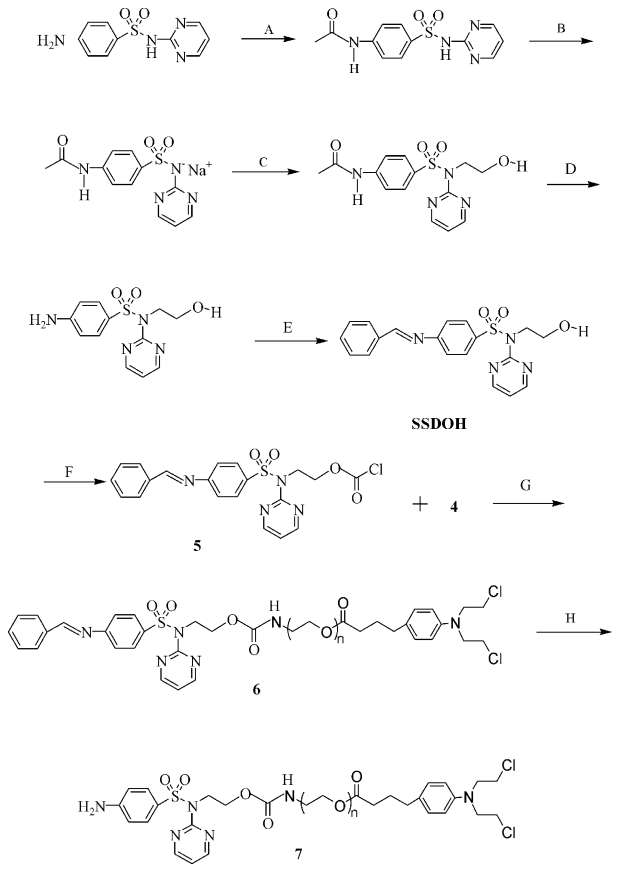


Scheme 1. Reagents and conditions: (A) ethylene oxide, THF, 50 °C, 15 h; (B) HCl (0.1 M); (C) benzaldehyde, toluene, N_2 atmosphere, 100 °C, 6 h; (D) dicyclohexyl carbodiimide (DCC), anhydrous pyridine, N_2 atmosphere, rt, 48 h; (E) acetic acid glacial, rt, 3 h.

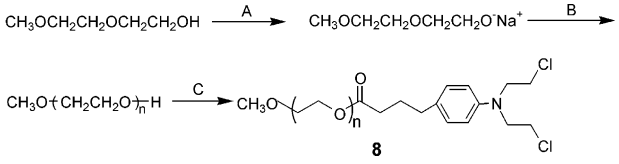
4400, 7200) were synthesized by the method of Yokoyama et al.[14] The polydispersity value was about 1.20–1.25 (M_w/M_n). Benzaldehyde was selected to protect the amino group of PEG as depicted in Scheme 1 and compound **1** was synthesized. Compound **3** was obtained by coupling of chlorambucil (**2**) with compound **1** in the presence of DCC at room temperature under nitrogen atmosphere. Subsequent hydrolysis of **3** was carried out by stirring for 3 h at room temperature in methylene chloride-acetic acid solution. The excess of acetic acid was got rid of by adding triethylamine and filtering, and compound **4** was obtained after concentration.

2-[N^1-2-Pyrimidinyl-(*p*-benzylidene)aminobenzenesulfonamido]ethanol (SSDOH) was synthesized by the method of Zhaohua Huang et al.[15] as depicted in Scheme 2 in which, the solution of SSDOH in pyridine was added dropwise into the solution of bis(trichloromethyl) carbonate in methylene chloride. After being stirred at 0 °C for 30 min, the solution of compound **5** was produced. This solution was applied to the next step of reaction directly by adding the solution of **4** in methylene chloride. The reaction mixture was stirred at 0 °C for 30 min, then at room temperature for 2 h. The solution was filtrated, concentrated carefully below 60 °C and precipitated in diethylether for three times and compound **6** was obtained. The target compound **7**[16] was obtained after hydrolysis of **6** according to the same route of that of **3**. By the similar method, three kinds of polymer drugs: SPEG(21)-CBL, SPEG(44)-CBL and SPEG(72)-CBL with the molecular weight of 2100, 4400 and 7200, respectively, were synthesized.

As comparison, monomethoxy-poly (ethylene glycol's) (MPEG) with different molecular weight (M_r = 2100, 4500, 7200) shown in Scheme 3 were synthesized and their polydispersities were about in the range of 1.05–



Scheme 2. Reagents and conditions: (A) acetyl chloride, pyridine, 0 °C, 2 h; (B) NaOH (aq., 1.0 equiv); (C) *N*, *N*-dimethylformamide (DMF), $BrCH_2CH_2OH$ (1.0 equiv), 80 °C, 8 h; (D) HCl (1.2 M), reflux, 1 h; (E) benzaldehyde, N_2 atmosphere, 100 °C, 6 h; (F) bis(trichloromethyl) carbonate (1.0 equiv), methylene chloride, pyridine, 0 °C, 30 min; (G) methylene chloride, pyridine, 0 °C, 30 min, rt, 2 h; (H) acetic acid glacial, rt, 3 h.



Scheme 3. Reagents and conditions: (A) Na, THF, N_2 atmosphere, 60 °C, 72 h; (B) ethylene oxide, THF, 60 °C, 48 h; (C) chlorambucil, dicyclohexyl carbodiimide (DCC), anhydrous pyridine, N_2 atmosphere, rt, 48 h.

1.09 (M_w/M_n). The compounds **8** [MPEG(21)-CBL, MPEG(44)-CBL and MPEG(72)-CBL with the molecular weight of 2100, 4400 and 7200][17] were obtained by reaction of MPEG with chlorambucil according to the same route of that of **3**.

The contents of sulfadiazine and chlorambucil for **7**, and chlorambucil for **8** determined by ^{1}H NMR were listed in Tables 1 and 2, respectively. As shown in Table 1, the contents of chlorambucil in SPEG(44)-CBL and SPEG(72)-CBL exceeded 100%.This may be caused by the diol by-product, which is always present in the synthesis of PEG with amino and hydroxyl end groups and its separation is very difficult and time-consuming, and its content and the polydispersivity value depends

Table 1. The contents of sulfadiazine (SD) and chlorambucil (CBL) in compound **7**

Drug	Content of SD (%)	Content of CBL (%)
SDPEG(21)-CBL	43	61
SDPEG(44)-CBL	59	110
SDPEG(72)-CBL	85	129

Table 2. The contents of chlorambucil(CBL) in compound **8**

Drug	Content of CBL (%)
MPEG(21)-CBL	92
MPEG(44)-CBL	60
MPEG(72)-CBL	59

upon the molecular weight (higher for high-mass PEG).[18] The excess chlorambucil could react with diol and lead to this result. However, as we indicated in following discussion this phenomenon did not affect the reliability of our final conclusion.

In vitro, the cytotoxic assays of SPEG(21)-CBL, SPEG(44)-CBL and SPEG(72)-CBL were performed on the C6 human breast cancer cells.[19] As shown in Figure 1, all these polymer drugs exhibited good antitumor activity against C6 human breast cancer cells. According to the contents of chlorambucil in the conjugates, IC_{50} values for the polymer drugs were determined from Figure 1 and listed in Table 3. Compared with that of chlorambucil, its IC_{50} value against C6 human breast cancer cells is in the range of $10^{-8} \sim 10^{-9}$ mol/L,[20] it was found that the immobilization of chlorambucil to PEG did not have fatal effect on the cytotoxic activity of chlorambucil, and the activity of chlorambucil is reserved.

The IC_{50} values also suggested that in vitro the two kinds of polymer drugs with similar molecular weight showed similar antitumor activity against C6 human breast cancer cells.

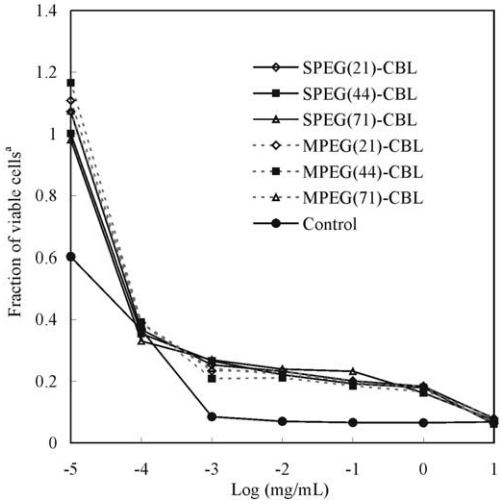


Figure 1. Semi-log plots of viability of cells versus drug concentration.
[a]The fraction of viable cells = OD $values_{treated}$/OD $values_{control}$.

Table 3. IC_{50} values of the polymer drugs

Drug	IC_{50} (10^{-8} mol/L)
SPEG(21)-CBL	4.92
SPEG(44)-CBL	1.18
SPEG(71)-CBL	0.543
MPEG(21)-CBL	3.59
MPEG(44)-CBL	2.67
MPEG(72)-CBL	1.53

In order to evaluate the toxicity of polymer drugs, LD_{50} values are measured and listed in Table 4.[21] It showed that the toxicity of polymer drugs no matter with sulfadiazine or without sulfadiazine is dropped greatly, the LD_{50} values of them are 10–16 times greater than chlorambucil.

In vivo antitumor activity of these polymer drugs on Lewis lung carcinoma planted in mice was tested.[22] All the polymer drugs were evaluated in each assay using a consecutive (qd×5) schedule, and the parameters: inhibition against the growth of Lewis lung carcinoma was used to assess their efficacy. The doses in the condition of equal mole for six drugs were as following: SPEG(21)-CBL: 25 mg/kg, SPEG(44)-CBL: 62.5 mg/kg, SPEG(71)-CBL: 100 mg/kg, MPEG(21)-CBL: 22.6 mg/kg, MPEG(44)-CBL: 59.3 mg/kg, MPEG(71)-CBL: 96.7 mg/kg. As shown in Figure 2, in vivo all three kinds of SPEG-CBL exhibited higher antitumor activity comparing with the corresponding MPEG-CBL but a little lower than the chlorambucil. However in vitro cytotoxic activity of these SPEG-CBL drugs showed approximately equal activity comparing with MPEG-CBL with the similar molecular weight, no sharp difference could be found. So in vivo the high antitumor activity of SPEG-CBL could be attributed to the targeting action of sulfadiazine moiety. On other hand, as we mentioned before, although the CBL contents of SPEG-CBL are not reliable because of the existence of diol for the samples of SPEG(44)-CBL and SPEG(71)-CBL, but it was observed that although CBL contents of SPEG(21)-CBL in all samples are lowest, but it showed the highest antitumour activity, so the activity of polymer drugs is also dependent on the molecular weight of PEG. The further research is undergoing.

In conclusion, a novel kind of antitumor drug (SPEG-CBL) is synthesized by fastening the sulfadiazine and chlorambucil onto the different ends of PEG ($M_n = 2100$, 4400, 7100). Although in vitro these polymer drugs

Table 4. LD_{50} of polymer drugs and chlorambucil[a]

Drugs	LD_{50} (mg kg^{-1})**
Chlorambucil	49.54
SPEG(21)-CBL	476.82
SPEG(44)-CBL	680.13
SPEG(72)-CBL	723.54
MPEG(21)-CBL	510.43
MPEG(44)-CBL	765.94
MPEG(72)-CBL	801.47

**$p < 0.05$.
[a]See References and Notes for details: 10 mice/group.

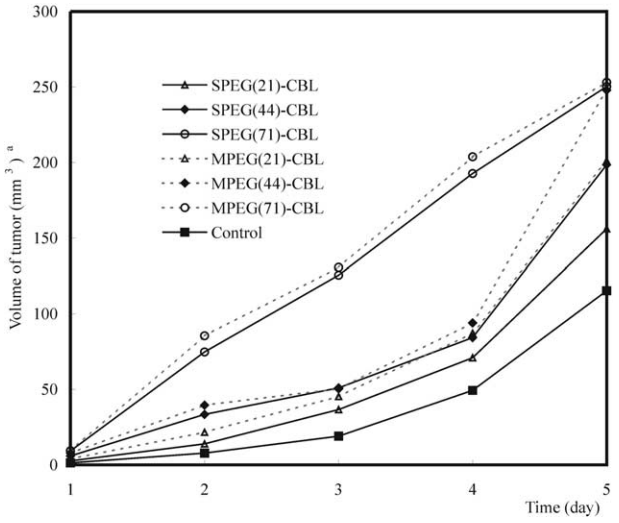


Figure 2. Curves of drug inhibition against tumor growth. [a]The volume of tumor was determined by the following equation: volume = 1/2×length×width^2.

showed higher antitumor activity against C6 human breast cancer cells, however, no sharp difference was found comparing with the same polymers but without the sulfadiazine. In vivo, however, SPEG-CBL exhibited higher antitumor activity for Lewis lung carcinoma than the corresponding MPEG-CBL, that means SPEG-CBL with sulfadiazine end group showed the obviously directing action.

Acknowledgements

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16. Selected data for compound **7**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.87 (m, 2H, $CH_2CH_2CH_2$), 2.30–2.40 (4H, $CH_2CH_2CH_2$), 3.40–3.80 (CH_2CH_2O of PEG backbone), 5.64 (br s, 2H, NH_2), 6.71 (d, 2H, phenyl of chlorambucil), 4.1 (t, 4H, CH_2OCO) 7.07 (d, 2H, phenyl of chlorambucil), 8.47 (q, 1H, pyrimidyl), 8.81 (d, 2H, pyrimidyl).
17. Selected data for compound **8**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.90 (m, 2H, $CH_2CH_2CH_2$), 2.34 (t, 2H, $OCCH_2CH_2CH_2$), 2.55 (t, 2H, $CH_2CH_2CH_2Ph$), 3.38 (s, 3H, CH_3O), 3.50–3.80 (CH_2CH_2O of PEG backbone), 4.23 (t, 2H, CH_2OCO), 6.62 (d, 2H, phenyl), 7.06 (d, 2H, phenyl).
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19. In vitro cytotoxity of these polymer drugs was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The polymer drugs were sterilized and then seven sample solutions with the concentration of 10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} mg/mL respectively were prepared. The human breast cancer cells were cultured in Dulbecco Eagle's minimum essential medium (DMEM) supplemented with 100 u/mL penicillin, 100 u/mL streptomycin and 10% inactivated calf serum at 37 °C in a humidified atmosphere of 5% CO_2. The cells harvested from log phase were digested by 0.25% trypsin and diluted to 1×10^5 cells/mL by DMEM culture solution containing serum. Then the cells were seeded into a 96-well plate for 200 μL per well. The drug solution was added to each well for 20 μL. The plate was kept in CO_2 incubator at 37 °C in a humidified atmosphere of 5% CO_2 for prescribed time. Then the cultured cells in each well were mixed with 20 μL of MTT solution of 5 mg/mL in DMEM culture solution without serum and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. The top clear solution was got away and 150 μL dimethylsulfoxide (DMSO) was added to dissolve the formazan for each well. After shaken for 10 min by plate shaker, the OD value of each well was measured on an ELISA spectrophotometer at 490 nm.
20. The cytotoxic activity of chlorambucil was evaluated by the same method as that described for the polymer drugs.
21. For the assessment of the acute toxicity, the TA1 mice were randomly divided into five groups (20/group and female/male = 1:1), polymer drugs and chlorambucil were injected ip×1 into TA1 mice at five different dose levels on day 0. Then the behavior and death distribution of the test mice were recorded. The highest death rate appeared on day 1 and the condition of the survivals was good after 2 weeks. LD_{50} was calculated by using Bliss method.
22. Lewis lung carcinoma cells were maintained by intraperitioneal passage at weekly intervals in C57 mice. In experiments with tumor cells, 1×10^6 cells in 0.1 mL were transplanted intracutaneously into the right forelimb of C57 mice (10 mice per group) on day 0. The polymer drugs were sterilized and dissolved in normal saline. The mice bearing the Lewis lung carcinoma cells were treated by tail vein injection with a consecutive (qd×5) schedule at various doses from day 1. From day 6, the volume of the Lewis lung carcinoma was measured to evaluate the in vivo antitumor activity of the polymer drugs.