Water-Soluble Poly(ethylene glycol) Prodrug of Pemetrexed: Synthesis, Characterization, and Preliminary Cytotoxicity

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ABSTRACT: Pemetrexed is a novel antifolate of antimetabolite with multiple enzyme targets involved in both pyrimidine and purine synthesis. It has entered the clinical usage due to favorable profiles especially in the cancer treatment of mesothelioma and non–small-cell lung carcinoma. But it presents numerous challenges associated with poor water solubility and unstability in its original form of glutamic acid. The aim of this study is to solubilize pemetrexed by designing and synthesizing its aqueous-soluble prodrug using high aqueous-soluble polymeric carrier poly(ethylene glycol) (PEG). A new type of soluble pemetrexed prodrug was synthesized with dihydroxyl PEG and a single amino acid linkage, and was extensively characterized using ^{1}H-NMR, ^{13}C-NMR, Fourier-transform infrared, and matrix-assisted laser desorption time of flight mass spectrometry. In addition, the prodrugs were evaluated for the drug loading capability, the aqueous solubility, and the preliminary *in vitro* cytotoxicity. The results indicate that the new PEGylated pemetrexed conjugates possess enhanced water solubility and stability, and provide another feasible choice of the pharmaceutical form of pemetrexed in the clinical application. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 444–451, 2009

Key words: conjugated polymers; drug delivery systems; hydrophilic polymers; water-soluble polymers; synthesis

INTRODUCTION

Pemetrexed (LY231514: *N*-{4-[2-(2-amino-4,7-dihydro-4-oxy-3H-pyrrolo[2,3-D] pyrimidine-5-ly)-ethyl]-benzoly}-L-glutamic acid) is a promising folated-based antimetabolite (Fig. 1) with activity against numerous tumor types such as mesothelioma and non–small-cell lung carcinoma in particular.[1–3] Pemetrexed has been shown to inhibit multiple folate-requiring enzymes including dihydrofolate reductase, thymidylate synthase, and glycinamide ribonucleotide formyltransferase, which are essential for both pyrimidine and purine synthesis.[1] However, this compound, in the form of free acid, presents numerous challenges associated with poor water solubility, unstability upon light, heat and moisture, and strong tendency of degradation.[2] In addition, it was discovered that a simple, isotonic saline solution of pemetrexed is not pharmaceutically acceptable for commercial purposes due to degradation of the solution to form unacceptable related substances.[3] Although pemetrexed in the form of disodium salt could only solve the above drawbacks to some extent, it still seems a promising candidate for dealing with a variety of solid tumors in the clinical stage. The research, searching for the more suitable solubilization type of pemetrexed with characteristics of high aqueous solubility and more stable storage, has never ceased. To date, numerous attempts have been made. Sun discussed some pharmaceutically acceptable nontoxic metal and organic ammonium salts of pemetrexed, such as calcium, magnesium, dimethylammonium, triethylammonium, and meglumine, some of

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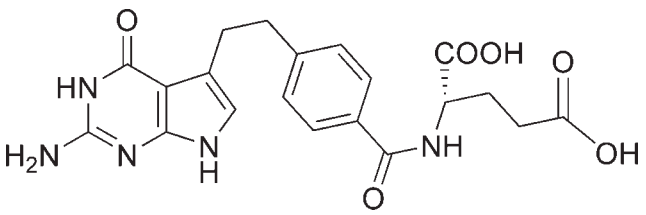


Figure 1 Chemical structure of pemetrexed.

which are even more water-soluble than pemetrexed disodium.[4] On the basis of Sun's work, Mao reported the findings of pemetrexed ethylenediamine salt, which has good aqueous solubility and stability and is easy to produce.[5] However, more or less drawbacks still exist in the above-mentioned types of pemetrexed, leading to their failure as extensively used pharmaceutical agents.

Until now, very few investigations, according to our knowledge, have been done on solubilizing pemetrexed with water-soluble polymers by using a strategy of polymeric prodrug delivery system (PDDS). In recent years, the potential of synthetic macromolecules as carriers of anticancer drugs has attracted extensive interest. Polymeric prodrugs of various cytotoxic agents with soluble polymers such as dextrans, polypeptides, and especially with N-(2-hydroxypropyl)methacrylamide copolymer and poly (ethylene glycol) (PEG) have been extensively investigated.[6] A prodrug is a biologically inactive derivative of a parent drug molecule that usually requires hydrolysis or enzymatic transformation within the body to release the active drug. Usually, a prodrug has improved delivery properties over the parent molecule. A conjugation of a drug with a polymer provides a "polymeric prodrug." Polymeric prodrugs have a great many advantages over their low molecular weight precursors.[7]

Among synthetic polymers, high molecular weight PEG plays an outstanding part as a drug vehicle. As a water-soluble, biocompatible, nontoxic, and nonimmunogenic material, it has been accepted by FDA for human intravenous, oral, and dermal applications. The antitumor drugs conjugated with PEG usually exhibit favorable pharmacokinetics and tissue distribution. Furthermore, the PEGylated prodrug strategy is showing emerging potential in passive tumor targeting because high molecular weight conjugates generally accumulate at the tumor site due to the "enhanced permeability and retention" effect.[8] A wide variety of biologically relevant molecules including proteins, enzymes, lipids, and small organic drugs have been conjugated to PEG to take advantage of these properties. The PEG conjugate approach has been applied in the anticancer agents paclitaxel, camptothecin, and doxorubicin to enhance the water-solubility and plasma half-life of the drugs.[9] The camptothecin PEG conjugate prothecan has recently been evaluated in a phase II clinical trial. As a versatile candidate for prodrug conjugation, PEG also has sound chemical features. Either monomethoxy PEG (mPEG) or dihydroxyl PEG has available functionable groups for coupling directly or through a linker to drugs or other biological components.

In this article, we report on the synthesis, characterization and preliminary *in vitro* cytotoxicity of a new kind of water-soluble PEG prodrugs for pemetrexed. PEG was used as solublizing moiety to enhance the solubility of pemetrexed and amino acids were employed as linkers to modulate the release of pemetrexed from prodrugs. Both PEG and amino acids are biocompatible and display no undesired side effects. This design would optimize its delivery characters of the parent drug, render the pemetrexed conjugates a better bioavailability, and further the great potential of pemetrexed for better clinical application.

EXPERIMENTAL

Materials

Pemetrexed (purity > 95%) was prepared in our group. PEG (MW ~ 4000, 20,000) was purchased from Nanjing Chemical Reagent Company (China). Dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), and trifluoroacetic acid (TFA) were obtained from Sinopharm Chemical Reagent Co., Ltd, China. All commercially available solvents and reagents were used without further purification. Melting points were determined in open capillaries and were uncorrected using a RY-1 melting point detector which was purchased from Tianjin Tianfen Analysis Instrument Factory. UV absorbance was observed using a UV-Vis spectrophotometer purchased from Shanghai Huxi Analysis Instrument Factory Co., Ltd.

Preparation of PEGylated pemetrexed prodrugs

Synthesis of N-*tert*-butoxycarbonyl amino acids (Boc-AA)

Boc-AA were prepared according to the literature by Keller et al.[10] Briefly, glycine (7.5 g, 0.1 mol) was dissolved in a solution of sodium hydroxide (4.4 g, 0.11 mol) in 110 mL of water and 75 mL of *tert*-butyl alcohol. To the well-stirred clear solution was added di-*tert*-butyl dicarbonate (21.8 g, 0.1 mol) dropwise during a period of 1 h. The reaction was brought to completion by further stirring overnight at room temperature. The resulting mixture was extracted with pentane and the organic phase was extracted with saturated aqueous sodium bicarbonate solution.

The combined aqueous layers were acidified with 2*N* HCl to pH 2 and then extracted with ethyl ether. The combined organic layers were washed with water, dried over anhydrous sodium sulfate and filtered. The solvent was removed under reduced pressure to give the remaining yellowish oil, which was treated with hexane and allowed to stand overnight to obtain the partially crystallizing product as white powder of N-*tert*-Butoxycarbonyl glycine (Boc-Gly) (14.3 g, yield 81.7%, m.p. 86–87°C).

The N-*tert*-Butoxycarbonyl leucine (Boc-Leu) was synthesized using the same method affording a similar white powder (yield 74.6%, m.p. 85–87°C).

Synthesis of PEG-amino acid derivatives

The synthesis of PEG-amino acid derivatives was mainly based on the procedures of Zalipsky et al.[11] To a solution of PEG_{20000} (40 g, 2 mmol), Boc-Gly (0.77 g, 4.4 mmol), and DMAP (0.12 g, 1 mmol) dissolved in 100 mL of dichloromethane (DCM (100 mL) was added dropwise a solution of DCC (1.2 g, 4.8 mmol) in DCM. After reacting at room temperature for 24 h, the mixture was filtrated and concentrated to oil at reduced pressure. Addition of cool acetone followed by the subsequent filtration was to remove some insoluble byproducts. The concentrated filtrate, which was dissolved in DCM (40 mL), was added dropwise into well-stirred cool diethyl ether (150 mL) and allowed to stand overnight, affording a white powder (36 g). The N-*tert*-butoxycarbonyl protective group was removed by dissolving the above precipitate in 50 mL of 1:1 (v/v) TFA/DCM stirring for 1 h at room temperature. The mixture was adjusted to pH 8 with saturated NaOH, extracted with chloroform, dried over anhydrous sodium sulfate and concentrated. The residue was poured into a large amount of cool diethyl ether. The precipitate was recrystallized from isopropanol twice, affording a white powder of PEG_{20000}-Gly (33 g, yield 81.5%).

The PEG_{4000}-Leu was prepared using the same method obtaining the similar white powder (yield 82.3%).

Synthesis of PEGylated pemetrexed prodrugs

Pemetrexed (1.4 g, 3 mmol), DCC (0.62 g, 3 mmol), DMAP (0.06 g, 0.5 mmol) were dissolved in DMF (15 mL). After stirring for 20 min, PEG_{4000}-Leu (5 g, 1.025 mmol) in DMF (10 mL) was added. The reaction mixture was allowed stirring violently overnight. The filtrate was concentrated to dryness and the residue was dissolved in water followed by extraction with chloroform. The combined organic layer was evaporated to dryness, and the obtained solid was recrystallized from isopropanol twice, affording PEG_{4000}-Leu-PM (2.1 g, yield 35%, m.p. 50–55°C.)

The PEG_{20000}-Gly-PM was synthesized using the similar method obtaining a white powder (yield 43.1%, m.p. 61–65°C).

Characterization

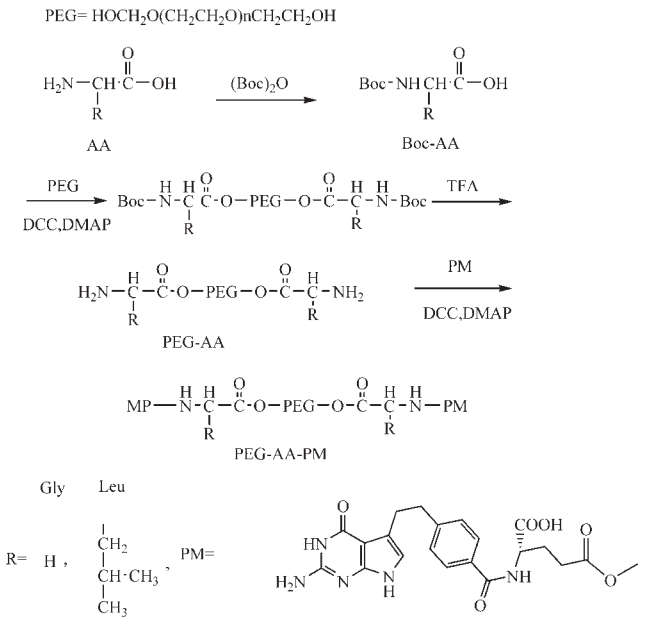
The Fourier-transform infrared (FT-IR) spectrum of PEGylated pemetrexed prodrugs was recorded on a Shimadzu FT-IR-8400S FT-IR spectrometer using KBr pallets. The ^{1}H-NMR and ^{13}C-NMR spectra were determined on a Bruker (AVACE) AV-500 spectrometer using $CDCl_3$ as solvent. Chemical shifts (δ) were given in parts per million using tetramethylsilane as an internal reference. Mass spectra were performed on Agilent 1100-MS spectrometer. Matrix-assisted laser desorption time of flight mass spectrometry (MALDI TOF MS) was carried out on Bruker Daltonics Ultraflex MALDI TOF Mass Spectrometer in the State Key Lab of Coordination Chemistry, Nanjing University. The targeted compounds were all purified by preparative HPLC (Shimadzu LC-8A) before structure characterization.

Analysis of drug loading capability

The UV absorbance of native pemetrexed in DMF was determined at 254 nm for seven different concentrations ranging from 4 to 20 μg/mL to provide the standard plot of absorbance versus concentration. PEGylated pemetrexed prodrugs were dissolved in DMF at an approximate concentration. The UV absorbance of target compounds at 254 nm was determined. Then, the content of pemetrexed in the prodrug was obtained using this value and employing the above standard plot. Dividing this value by the sample concentration provided the percentage of pemetrexed in the prodrug. The value of drug loading capability (the content of native drug in the prodrug) could also be obtained.

Measurement of the solubility of prodrugs

PEGylated pemetrexed prodrugs were prepared by the methods described above. The solubility was estimated by adding water in small portions until the dissolution occurred. At room temperature, an exact amount of targeted compounds (100 mg) was weighed into a volumetric flask (2 mL). A portion of distilled water (25 μL each time) was added using continuously adjustable pipette, which was followed by sonication for 30 s. When dissolution occurred and a flowable liquid could be observed, the total volume of water was obtained. On the basis of the amount of water added, the solubility of PEGylated pemetrexed prodrugs could be calculated.



Scheme 1 Synthetic route of PEGylated pemetrexed prodrugs.

Preliminary *in vitro* cytotoxicity

The *in vitro* cytotoxicity of PEGylated pemetrexed prodrugs were evaluated in four tumor cell lines of human lung carcinoma (A549), human poorly differentiated gastric adenocarcinoma (BGC-823), human hepatoma carcinoma (SMMC-7721), and human promyelocyte leukemia (HL-60). The assay was adapted for 96-well plates. Briefly, a 0.25% trypsin solution was added to the cells in exponential growth phase to give the cell suspension at a proper concentration of 2 $\sim$ 4 $\times$ 10^4 cells/mL. The cell suspension (180 μL) was transferred to each well and the plates were incubated at 37°C in a humidified atmosphere and gassed continuously with 5% CO_2 in air. After 1 day, when the cells were adherent to the dishes, the cultures were replenished with fresh medium containing the test substances at a concentration of 20 μL/well. A 20 μL tetrazolium salt (MTT) solution was then added to each well, allowing their reaction for 4 h. After removing the supernatant, 150 μL of DMSO was added to each well, followed by shaking for 5 min. The absorption values were detected on the ELISA Reader at a wavelength of 570 nm and the corresponding cell inhibition rate (CI %) was calculated by the equation: CI % = (OD value of negative group-OD value of positive group)/OD value of positive group $\times$100%.

RESULTS AND DISCUSSION

Design, synthesis, and characterization of PEGylated prodrugs

PEGylated pemetrexed produrgs were successfully prepared by using the synthetic route as shown in Scheme 1. The method of choice for coupling bioactive components to the PEG backbone is mainly by esterification. This type of esterification can be divided into two main approaches: (a) activation of the hydroxy end group through transformation into a good leaving group and subsequent attack by the carboxylate component, such as formation of PEG-isourea and PEG-tosylate; and (b) activation of the carboxy component and subsequent attack by PEG hydroxy end groups, such as the direct coupling by DCC. Particularly, it was demonstrated that DMAP-catalyzed attachment of DCC-activated N-*tert*-butoxycarbonyl protected amino acids proceeds quantitatively with PEG under very mild conditions.[11] The mechanistic pathway for the reaction was postulated that the initial formed carbodiimide adducts could change into the activated form of a cyclic anhydride, which then reacts with a nucleophile (e.g., alcohol or amine) to give stable products.[9] Therefore, we employed this DCC/DMAP method in the synthesis of both PEG-amino acid derivatives and the final PEGylated pemetrexed prodrugs.

Esterification of PEG-OH with amino acids provides a simple way for introduction of terminal NH_2 groups, which could subsequently be coupled to the anticancer agent. Furthermore, the used amino acid could play an important role in the steric hindrance between the PEG backbone and the anticancer drug, thus modulating the release of pemetrexed from prodrugs. In term of steric hindrance, two classic amino acids were chosen, glycine as the simplest linker and leucine as a larger one having much more steric hindrance.

PEGylated pemetrexed prodrug with Leu as the linker (PEG_{4000}-Leu-PM) was taken as an example to illustrate the structure characterization. Figure 2 shows the FT-IR spectra of PEG_{4000} and PEG_{4000}-Leu-PM. From the prodrug's spectrum, it was found

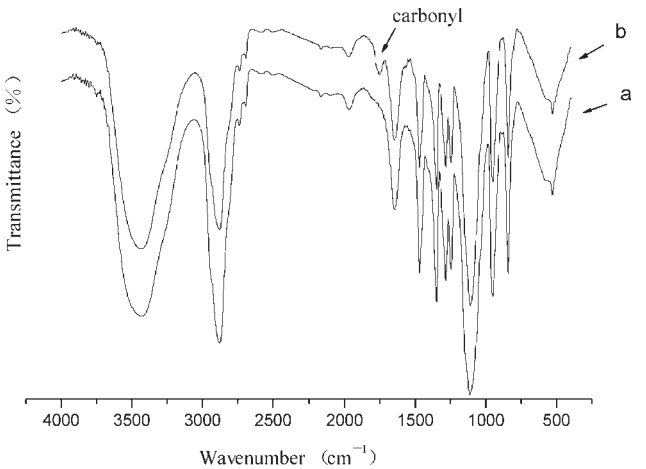


Figure 2 FT-IR spectra of (a) PEG_{4000} and (b) PEG_{4000}-Leu-PM.

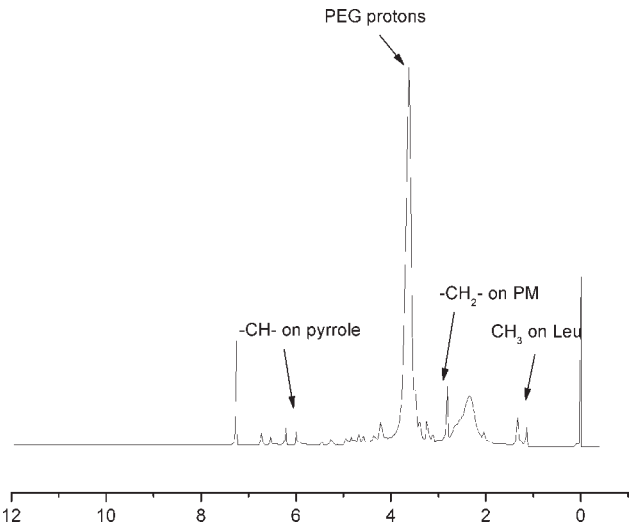


Figure 3 ^{1}H-NMR spectra of PEG_{4000}-Leu-PM.

that a distinctive absorption bands appear at 1751.24 cm^{-1} compared with that of PEG_{4000}. This peak could be assigned to the characteristic absorption of carbonyl group between the pemetrexed molecule and the PEG-Leu conjugate. In addition, the ^{1}H-NMR spectrum of this prodrug was given in Figure 3. The peak assignments of PEG_{4000}-Leu-PM were as follows: ^{1}H-NMR ($CDCl_3$) δ: 3.5–3.8 (CH_3 of Leu), 2.0–2.6 (—CH— and —CH_2— of Leu, —CH_2— of glutamate in PM), 2.8–3.1 (—CH_2— of PM), 4.0–5.0 (—OCH_2CH_2— of PEG closest to COOH, —*N*—CH— of Leu, —NH— of PM), 5.99 (—CH— of pyrrole in PM), 6.2 (—NH—), 6.5–6.8 (Ar—H). The ^{1}H-NMR assignments belonging to the pemetrexed molecule were very difficult to identify clearly because of its relatively rather low content in the prodrug. Bur it is still possible for us to conform that the pemetrexed molecule has been conjugated to PEG backbone with a Leu linker, since all the targeted compounds were all purified by preparative HPLC before structure characterization.

From the ^{13}C-NMR spectra of PEG_{4000}-Leu-PM (Fig. 4), the strong peaks at 70 and 77 ppm belong to the polyethylene glycol backbone. The other relatively weak signal was assigned to the pemetrexed molecule. For example, the peaks at 40 and 28 ppm belong to the alkyl carbons close to the carboxyl group of glutamic acid part. The 24 and 22 ppm signal were assigned to the alkyl carbons between pyrimidine and benzene moiety.

Though not quantitative, mass spectrometry is an ideal tool in determining the true MW of various PEG conjugate species. MALDI TOF MS is now an established method for not only determining the true MW of PEG conjugate species, but also in identifying the individual species contained in a particular preparation. The TOF Mass spectra result from Figure 5 showed that the MW4000 PEG starting material (MW 3500 ~ 4500 actually), after being conjugated to the pemetrexed molecule, was found to be increased to about 4200 Da in its molecular weight. Considering the molecular weight distribution of macromolecules, the enhanced molecular weight

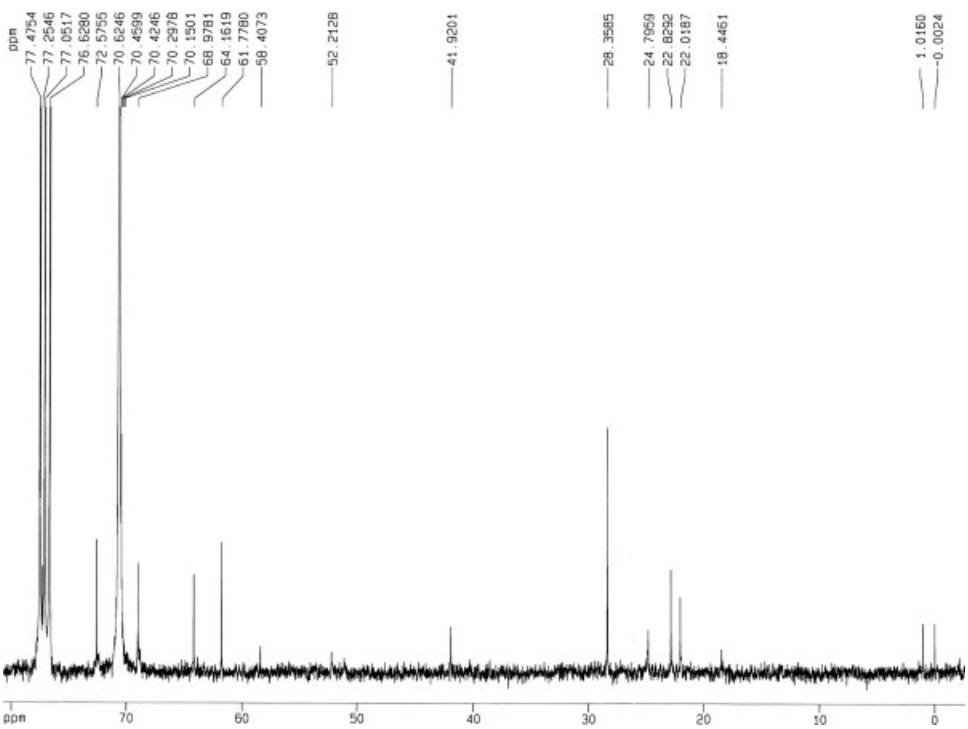


Figure 4 ^{13}C-NMR spectra of PEG_{4000}-Leu-PM.

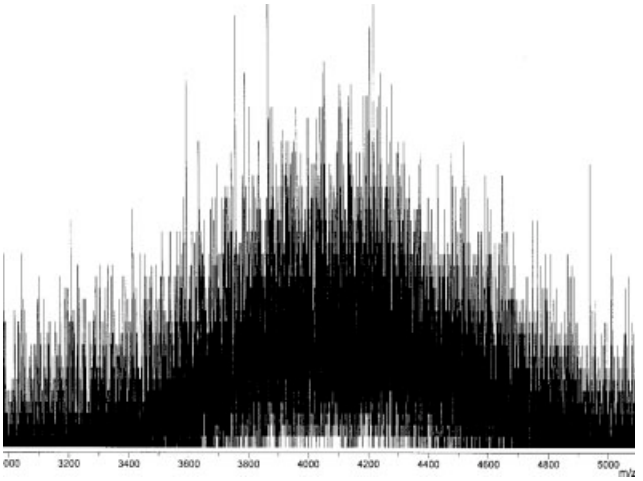


Figure 5 MALDI TOF MS of PEG_{4000}-Leu-PM.

could reasonably be due to the amino acid and pemetrexed molecules which have been conjugated to PEG backbone.

Drug loading capability

A prodrug is a form of a drug that remains inactive during its delivery to the site of action and is activated by the specific conditions in the targeted site. Therefore, it is the parent drug that has the therapeutic effect. Drug loading capability is an important parameter in the design of polymeric prodrugs for better therapeutic efficiency.

In the PEGylated pemetrexed conjugates, PEG backbone and the amino acid linker have no ultraviolet absorption. So all the absorption is contributed to the conjugated pemetrexed molecule and the prodrug absorption intensity has a positive correlation with the concentration of the parent drug. According to the method which had been set up, the standard plot was obtained (Fig. 6) and the drug loading capability data of these prodrugs were shown in Table I. If all the available hydroxy groups on PEG backbone were covalently coupled to pemetrexed, the drug loading capability would be 16.93% for PEG_{4000}-Leu-PM and 4.08% for PEG_{20000}-Gly-PM, respectively. However, the observed values suggested that only about 50% of functional groups of PEG were successfully conjugated with pemetrexed, which was due to the high steric hindrance of the long chain polymer, influencing the efficiency of chemical conjugation.

Native PEG has only two available hydroxy groups for the conjugation with the anticancer drug or other biological components. This limits the loading capacity of this kind of polymeric carriers, which is one of the major disadvantages of linear PEG polymers. In our future work, introducing branched spacer to the polymeric carrier system will be an effective method to increase the amount of binding sites, thus enhancing the drug loading capability of prodrugs.

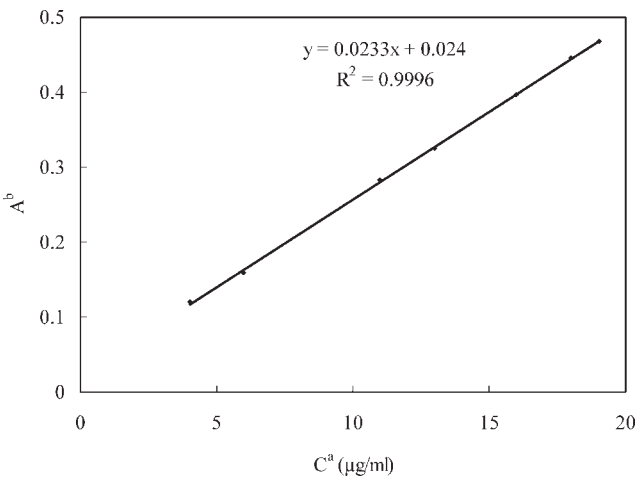


Figure 6 Standard plot of UV ABS versus pemetrexed contents.

Water solubility of PEGylated pemetrexed conjugates

The poor solubility of biologically active compounds is often a limiting factor in their applicability, which might influence the transport of drugs *in vivo* and lead to a low bioavailability. The excellent solubilization ability makes PEG a versatile candidate for the prodrug conjugation. Therefore, the solubility enhancement is an important task in pharmaceutical technology. Solubilizing pemetrexed with salt forms such as disodium salts can solve this problem to some extent, but they may introduce some other drawbacks simultaneously. For example, excessive

TABLE I
Drug Loading Capability of PEGylated Pemetrexed Prodrugs

Sample	C^a (μg/mL)	A^b	Calculated[c] (%)	Observed[d] (%)
PEG_{4000}-Leu-PM	84.0	0.218	16.93	9.57
PEG_{20000}-Gly-PM	356	0.237	4.08	2.47

[a] Concentration of pemetrexed prodrugs in DMF.
[b] UV absorbance of native pemetrexed in DMF determined at 254 nm.
[c] Drug loading capability of the conjugates if all of the binding sites on polymeric carriers were conjugated with pemetrexed.
[d] Drug loading capability actually obtained of pemetrexed prodrugs in this study.

TABLE II
Preliminary *in vitro* Cytotoxicity of Pemetrexed, PEG_{4000}-Leu-PM and PEG_{20000}-Gly-PM

Compound	C [10^{-6} mol L^{-1}]	Inhibitivity (%)			
		BGC	HL60	SMMC	A549
PM	0.1	39.19	63.06	18.70	17.67
	1	47.97	84.03	42.28	30.93
	10	52.75	87.05	29.01	31.98
PEG_{4000}-Leu-PM	0.1	13.43	9.12	0.00	7.36
	1	36.35	66.44	7.55	17.47
	10	40.55	72.20	37.69	3.24
PEG_{20000}-Gly-PM	0.1	14.72	0.00	0.96	8.30
	1	16.23	0.32	6.57	0.00
	10	33.32	57.31	86.77	15.44

intake of sodium ion is very likely to raise the blood pressure, which is not suitable for patients with high blood pressure.

Since observing the dissolving process of high molecular weight PEG is not difficult, it is efficient to measure the solubility of prodrug by directly observation as the literature has reported[12] Results show that the aqueous solubility of PEG_{4000}-Leu-PM and PEG_{20000}-Gly-PM was about 650 mg/mL and 125 mg/mL, respectively. Pemetrexed was highly solubilized by conjugation with PEG in comparison to native pemetrexed with very little aqueous solubility. It was indicated that PEG's molecular weight dominates the solubility of the final prodrug to such a large extent that the solubility of prodrug was very close to that of PEG polymers.

Preliminary *in vitro* cytotoxicity

The inhibitory effect of pemetrexed, pemetrexed-PEG conjugates was evaluated in a preliminary *in vitro* cytotoxicity assay on four cell lines including A549, BGC-823, SMMC-7721 and HL-60. Table II shows that both the native pemetrexed and the target prodrugs had very weak inhibition against A549, for all their inhibitivity values were below 50%. The anticancer agent pemetrexed was perhaps not a suitable choice for inhibiting A549 cell growth in term of original mechanism of biological function. All the tested compounds possessed rather weak inhibition towards SMMC except PEG_{20000}-Gly-PM having 86.77% inhibitivity at the highest concentration of 10^{-5} mol/L. This surprising fact may infer that this high molecular weight PEG conjugate with pemetrexed has more tendency to be phagocytized by lung cancer cells. In the case of BGC, both prodrugs PEG_{4000}-Leu-PM and PEG_{20000}-Gly-PM were weaker than the free pemetrexed in inhibitory ability. But this effect was still not strong even at the highest concentration of 10^{-5} mol/L. The data on the HL60 cell line gave a good example in illustrating the effect of PEG's molecular weight on *in vitro* cytotoxicity. Pemetrexed has the strongest inhibition at all three concentrations. PEG_{4000}-Leu-PM was a little weaker than the free drug, showing strong inhibition at the two higher concentrations. PEG_{20000}-Gly-PM, compared with the above two, had the lowest inhibitory effect and only showed a moderate inhibition at the highest concentration. This result is consistent with the findings of Riebeseel et al. on MTX-PEG conjugates.[9] MTX, short for methotrexate, is another classical antifolate. In their work, evaluation of the *in vitro* cytotoxicity of the MTX-PEG conjugates revealed that the most effective compound tested in the assays was the free drug MTX itself, and as the PEG molecular weight of prodrugs increased (varying in their molecular weight from MW 750 to MW 40,000), the inhibitory effect decreased. In contrast to the *in vitro* results, the high molecular weight MTX-PEG conjugates exhibited the highest *in vivo* antitumor activity. In addition, in Greenwald's review,[12] it was also mentioned that water-soluble mPEG 5000 paclitaxel-7-carbamates 10^3 less active than the native drug *in vitro* and nontoxic in mice. It was suggested that this may be attributed to two reasons: the large PEG blocks activity at the target cells or the PEGylated paclitaxel does not reach the cells in sufficient concentration to produce meaningful results.

CONCLUSIONS

Water-soluble PEGylated pemetrexed prodrugs using glycine or leucine as linkers were successfully synthesized and extensively characterized by FT-IR, ^{1}H-NMR, ^{13}C-NMR and MALDI TOF MS. The results of drug loading capability, water solubility and preliminary *in vitro* cytotoxicity were obtained. The solubility of PEG_{4000}-Leu-PM and PEG_{20000}-Gly-PM was enhanced remarkably to at least 650 and 125 mg/mL, respectively. PEG_{20000}-Gly-PM showed a considerable inhibitory effect on SMMC cell lines whereas pemetrexed exhibited a weak activity. In the case of HL-60 cell line, it was found that as the PEG molecular weight of prodrugs increased the inhibitory effect decreased. It would facilitate the development of dosage form design of pemetrexed and its better clinical application. We predict that the high molecular weight PEG carriers will improve both the circulation half-life of the conjugate and the percentage dose accumulated in the tumor tissue. Although these effects can bring about improvement in antitumor efficacy *in vivo* but cannot be measured in cell culture assays, further investigation in animal models is warranted.

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