Simply mixing with poly(ethylene glycol) enhances the fraction of the active chemical form of antitumor drugs of camptothecin family

Tianyuan Ci a, Ting Li a, Guangtao Chang a, Lin Yu a,b, Jiandong Ding a,b,*

a State Key Laboratory of Molecular Engineering of Polymers, Department of Macromolecular Science, Advanced Materials Laboratory, Fudan University, Shanghai 200433, China
b Key Laboratory of Smart Drug Delivery of Ministry of Education and PLA, School of Pharmacy, Fudan University, Shanghai 201203, China

A R T I C L E   I N F O

Article history:
Received 14 October 2012
Accepted 3 December 2012
Available online 13 December 2012

Keywords:
Poly(ethylene glycol) (PEG)
Drug-material interaction
Anticancer
Camptothecin (CPT)
Topotecan (TPT)
10-Hydrocamptothecin (HCPT)

A B S T R A C T

This paper reveals a new function of poly(ethylene glycol) (PEG) – a common polymer in pharmaceutics – enhancement of the active chemical form of the antitumor drugs of camptothecin (CPT) analogs. All of members in the CPT family confront the severe problem of hydrolysis of the active lactone ring to the inactive carboxylate, leading to not only less efficiency but also more toxicity. Herein, we report that the equilibrium fraction of the active lactone form could be enhanced significantly by simply mixing the drug solution with PEG. For instance, while the equilibrium lactone fraction of topotecan (TPT) was only a bit more than 10% under neutral pH at 37 °C, it was increased to nearly 50% in the presence of 40 wt.% PEG. Two CPT family members, TPT and 10-hydrocamptothecin, and six PEG agents with molecular weight from 200 to 5000, were tested, and the phenomenon was confirmed to be universal. The underlying reason was further discussed. The in vivo drug efficacy was also observed in a solid tumor model in mice.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Poly(ethylene glycol) (PEG) is one of the most important polymers in pharmaceutics. This polymer is well known in the strategy of PEGylation by covalently binding drugs to increase the solubility of some insoluble drugs and/or prolong the circulation of some drugs or nanocarriers in the blood [1,2]. Free PEG molecules are often applied as porogen or solvent etc. in formulations [3,4]. PEG chains also serve as the hydrophilic part in many copolymers potentially applied in drug delivery systems [5–12] or other biomedical fields [13–15]. To date, a lot of commercialized drug formulations contain PEG. Herein, we report a new function of PEG based on our recent finding that addition of PEG significantly enhances the fraction of the active form of drugs in the family of camptothecin (CPT).

CPT and its derivatives such as topotecan (TPT) and 10-hydrocamptothecin (HCPT) are one famous category of topoisomerase I inhibitors and have been applied in tumor treatments [16–25]. Among them, two water-soluble analogs, TPT and irrinotecan, have been approved by Food and Drug Administration (FDA). Nevertheless, all of the members in the CPT family are faced with a severe “stability” problem. The hydrolysis of the active lactone ring to the inactive carboxylate reduces the drug efficacy, and meanwhile the inactive form can lead to side effects [26–28]. The transformation between the two forms is pH dependent. Unfortunately, the equilibrium favors the inactive form at physiological pH. For instance, the equilibrium lactone fraction of TPT at neutral pH is only a bit more than 10% [29]. While many efforts have been made to overcome this problem [30–37], the approach in this report might be the simplest, based upon the phenomenon schematically presented in Fig. 1. The present paper is aimed to report this novel phenomenon and reveal the underlying reason.

2. Experimental section

2.1. Materials

TPT and HCPT were products of ChengDu LanBei Chemical Technology Ltd. and Shanghai Knowshine Pharmachemicals Inc., respectively. PEG series of molecular weight (MW) 200 (PEG200), 400 (PEG400), 1000 (PEG1000), 2000 (PEG2000), and methoxy PEG series of molecular weight 2000 (MPEG2000) and 5000 (MPEG5000) were bought from Sigma-Aldrich. For MPEG, one of the two hydroxyl end groups of PEG is capped by the methoxy group since the end capping does not alter the main chemical structure of the polymer, MPEG is often included into PEGylation by covalently binding drugs to increase the solubility of some insoluble drugs and/or prolong the circulation of some drugs or nanocarriers in the blood [1,2]. Free PEG molecules are often applied as porogen or solvent etc. in formulations [3,4]. PEG chains also serve as the hydrophilic part in many copolymers potentially applied in drug delivery systems [5–12] or other biomedical fields [13–15]. To date, a lot of commercialized drug formulations contain PEG. Herein, we report a new function of PEG based on our recent finding that addition of PEG significantly enhances the fraction of the active form of drugs in the family of camptothecin (CPT).

CPT and its derivatives such as topotecan (TPT) and 10-hydrocamptothecin (HCPT) are one famous category of topoisomerase I inhibitors and have been applied in tumor treatments [16–25]. Among them, two water-soluble analogs, TPT and irrinotecan, have been approved by Food and Drug Administration (FDA). Nevertheless, all of the members in the CPT family are faced with a severe “stability” problem. The hydrolysis of the active lactone ring to the inactive carboxylate reduces the drug efficacy, and meanwhile the inactive form can lead to side effects [26–28]. The transformation between the two forms is pH dependent. Unfortunately, the equilibrium favors the inactive form at physiological pH. For instance, the equilibrium lactone fraction of TPT at neutral pH is only a bit more than 10% [29]. While many efforts have been made to overcome this problem [30–37], the approach in this report might be the simplest, based upon the phenomenon schematically presented in Fig. 1. The present paper is aimed to report this novel phenomenon and reveal the underlying reason.

2.2. HPLC analysis of TPT and HCPT

The lactone and carboxylate forms of CPT analogs were separated and quantified with high performance liquid chromatography (HPLC) equipped with Waters Separation Module e2695 and Waters
UV/visible detector 2486. For analysis of TPT, the separation process was accomplished by the column of 5 μm C18 reverse phase column (150 mm × 4.6 mm, SunFire™) with the mobile phase of 89% phosphate buffer (pH 6.5, 25 mM), 6% methanol, and 5% tetrahydrofuran at the flow rate of 1 ml/min. The detection wavelength was 381 nm and the column temperature was 25 °C. For analysis of HCPT, the mobile phase was 70% phosphate buffer (pH 6.5, 25 mM), 23% methanol, and 7% tetrahydrofuran with the detection wavelength of 382 nm. The other conditions were the same as those of TPT. The lactone fraction was calculated by the equation of \( f_{\text{lactone}} = \frac{A_{\text{lactone}}}{A_{\text{total}}} \), in which \( A_{\text{lactone}} \) refers to the area of the lactone peak and is positively related to the lactone content; and \( A_{\text{total}} \) is the peak area of the lactone form after acidiﬁcation, which is positively related to the total content of the lactone and carboxylate forms.

2.3. Ring-opening and ring-closing kinetics of TPT

6 mg TPT powder was first dissolved in 1 ml acidic (pH<4.0) or basic (pH>9.0) medium to ensure that all the drug molecules were in the lactone or carboxylate form. Then 0.2 ml of the stock solutions was added into 10 ml phosphate buffer saline (PBS) or 40 wt.% PEG solution (with PBS as medium). After being quickly adjusted to pH 7.4, specimens were equilibrated in a 37 °C water bath. Samples were then taken out for HPLC analysis to detect the ring-opening or ring-closing kinetics of TPT.

2.4. Lactone fractions of TPT before and after addition of PEG

TPT solutions with or without 40 wt.% PEG in PBS were adjusted to preset pH values in the range of 4–10. All the pH values were detected in the same pH meter (Aurora Scientiﬁc Instruments (Shanghai) Co., Ltd). After equilibrating at 37 °C for 12 h, samples were taken out for HPLC analysis. The pH dependence of the lactone fraction of TPT was ﬁtted by

\[
f(pH) = f_{\text{f}} + \frac{f_{\text{f}} - f_{\text{b}}}{1 + 10^{(pH - pH_{1/2})}}.
\]

Here we employed Henderson–Hasselbalch (HH) equation, which has been successfully applied in data ﬁtting of pH titration in other biological experiments [38,39], to obtain the transition pH, pH_{1/2}.

2.5. PEG-concentration dependence of lactone fraction of CPT analogs

Drug aqueous solutions with a series of PEG contents (10 wt.%, 20 wt.%, 30 wt.%, 40 wt.%) were prepared and adjusted to pH 7.4 with drug concentrations of 120 μg/ml for TPT and 50 μg/ml for HCPT, respectively. After equilibrating at 37 °C for 12 h, samples were taken out for HPLC analysis.

2.6. Lactone fraction of TPT in mixture solvents

Organic/aqueous mixture solvents of ethanol/PBS, ethylene glycol/PBS and glycerol/PBS with 40 wt.% organic phase were prepared. A TPT stock solution was added into the above mixtures, leaving a ﬁnal TPT concentration of 0.12 mg/ml. After equilibrating at 37 °C for 12 h, samples were taken out for HPLC analysis.

2.7. Solubilities of HCPT in PEG solutions

Overly amounts of HCPT powder were added into series of PEG/PBS solutions (10 wt.%, 20 wt.%, 30 wt.%, 40 wt.%). After equilibrating in 37 °C water bath for 3 days under 100 rpm, solutions were ﬁltered with 0.45 μm Millipore to remove the insoluble HCPT powder. The HCPT content in the ﬁltrate was determined through HPLC analysis.

2.8. Fluorescence spectra of HCPT in PEG solutions

40 μl HCPT stock solution (5 mg/ml) and 100 μl concentrated hydrochloric acid were added to 40 ml PEG solutions (0 wt.%, 40 wt.%, 80 wt.%, and 100 wt.%) with PBS as the medium. After equilibrating at room temperature for 5 h, 3 ml samples were taken out for ﬂuorescence emission detection, which was conducted in the ﬂuorescence spectrophotometer FLS920 (Edinburgh Instrument) with an excitation wavelength of 380 nm.

2.9. In vivo antitumor efficacy against S180 solid tumor in mice

An S180 solid tumor model was used to examine the in vivo antitumor efficacy. All animal tests obeyed the “Principles of Laboratory Animal Care” (NIH publication #85-23, revised 1985) and were approved by the Institute Animal Care and Use Committee at Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Briefly, Kunming mice were inoculated 5180 cells in the right armpit, and 24 h later sample...
solutions were injected. The drug injection day was defined as day 0. At day 5, the mice were sacrificed and the solid tumors were taken out for photographing and weighing. The tumor inhibition ratio was calculated by

\[
\text{tumor inhibition ratio} \%(%) = \frac{W_1 - W_2}{W_1} \times 100.
\]

Here, \(W_1\) and \(W_2\) refer to the mean tumor weights of the negative control group and the drug-treated group, respectively. A ratio over 40% was regarded as effective, if no significant loss of body weight was found meanwhile.

3. Results

3.1. Ring-opening and ring-closing kinetics of TPT with and without PEG

The lactone and carboxylate forms of TPT could be well separated and characterized by HPLC. Some typical chromatograms are shown in Fig. 2a. The retention times for the lactone and carboxylate forms were about 16 min and 4 min, respectively. The content of drug is linearly related to the peak area, and a standard curve is presented in Supplementary Fig. S1. The changes of the lactone fraction upon pH jumps are presented in Fig. 2b. The presence of PEG not only slowed down the ring-opening or closing kinetics, but also shifted the equilibrium significantly. The lactone fraction of TPT in the 40 wt.% PEG solution at 37 °C and pH 7.4 was about 50%, which is almost triple to that in PBS at the same temperature and medium pH.

3.2. The pH dependence of equilibrium lactone fractions of TPT with and without PEG

The equilibrium lactone fractions of TPT in a wide pH range were determined. The full titration data are presented in Fig. 3. The value of \(pH_{1/2}\) increased significantly from 6.3 in PBS to 7.4 in 40 wt.% PEG solution.

3.3. Effects of PEG molecular weight and concentration on lactone fraction of TPT

The equilibrium lactone fraction of TPT increased with the PEG concentration, as shown in Fig. 4. We also examined the effect of molecule weight of PEG under a given concentration (40 wt.%). The results are shown in Fig. 5, and the extreme case of the small molecule ethylene glycol (EG) was used as control. In 40 wt.% EG/PBS solutions, the lactone fraction of TPT was 28.1% at pH 7.4 and 37 °C, about twice of that in PBS.

3.4. Effect of PEG additives on HCPT

We also examined another CPT family member besides TPT. Different from TPT, HCPT is not water-soluble (or of low solubility) in its lactone form. Yet, the PEG additives increased its solubility significantly, as shown in Fig. 6a. At pH 1.8 and 37 °C, the solubility of HCPT was only 2.58 ± 0.14 μg/ml in PBS solution, and the value increased to 19 times in 40 wt.% PEG400 solution and 26 times in 40 wt.% MPEG5000 solution. We further examined the equilibrium lactone fraction of HCPT at pH 7.4 in a series of PEG solutions, and found that the fraction of the active lactone form was changed from about 13% to 46% after addition of 40 wt.% PEG, as indicated in Fig. 6b.

3.5. Organic property of PEG

Some of CPT family drugs are fluorophores, and 10-hydroxy in the HCPT molecule is sensitive to the microenvironment polarity [40]. So, HCPT was employed as a hydrophobic probe to characterize the possible change of the medium polarity after addition of PEG. The fluorescence spectra of HCPT in a series of PEG solutions (0–100 wt.%) were detected, and the corresponding molecular fluorescence emission intensity appeared as a blue-shifting with the largest emission peak changed from 550–600 nm to 400–450 nm, as shown in Fig. 7 for PEG400.
the bulk state of PEG5000 is solid, its liquid state of 100% concentration at room temperature is not available; nevertheless, the increase of fluorescence in the range of 400–450 nm was also well repeated with the concentration of MPEG5000 from 0 to 40 wt.%, as illustrated in Supplementary Fig. S2.

3.6. Lactone fractions of TPT in mixtures of organic solvents and water

The PEG effect on the equilibrium lactone fraction was anticipated to be related to the change of the solvent polarity, and thus several solvents of varied dielectric constants were tried. According to literature [41], the constants are 25.0 (20 °C) for ethanol, 38.7 (20 °C) for ethylene glycol, 41.1 (20 °C) for glycerol, and 80.4 (20 °C) for water. Those dielectric constants were determined in the “pure” state. Those of the mixtures with PBS were, however, hard to be measured due to difficulty to decouple the dielectric constants with electroconductivities in the presence of plenty of ions in liquids. Nevertheless, the effective polarities of the mixture solvents might follow the same sequence as that of the dielectric constants in the pure state. The equilibrium lactone fractions of TPT showed negative correlation with the polarity of the mixture solvent. For instance, in 40 wt.% ethanol/PBS mixture solvent, the lactone fraction was 59% compared with that of 21% in 40 wt.% glycerol/PBS solution, as shown in Fig. 8.

3.7. In vivo antitumor efficacy against S180 solid tumor in mice

The images of the tumors extracted from mice are shown in Fig. 9, and the detailed conditions and resultant tumor inhibition ratios are listed in Table 1. The addition of PEG was confirmed to enhance the in vivo drug efficacy. For instance, a significant difference was found between the group of PEG/TPT (intra) and the group of TPT (intra) with $p = 0.02$.

![Fig. 4. Lactone fraction of TPT as a function of concentration of MPEG5000 at pH 7.4 and 37 °C in PBS.](image4)

![Fig. 5. Equilibrium lactone fractions of TPT in PBS solutions of 40 wt.% PEG of indicated molecular weights. The extreme case of the mixture solvent of 40 wt.% EG and 60 wt.% PBS was also examined, marked as the group of “EG”.](image5)

![Fig. 6. a) HCPT lactone solubilities in indicated solutions at 37 °C. The medium pH was 1.8 to ensure the lactone form of all the drug molecules. After equilibrating for 3 days, samples were filtered and analyzed by HPLC. b) Equilibrium lactone fraction of HCPT (50 μg/ml) in solutions of MPEG5000 of indicated polymer concentrations at pH 7.4 and 37 °C. Here, 0% in the horizontal coordinate indicates merely the PBS solution.](image6)

![Fig. 7. Relative fluorescence intensity of HCPT in PEG solutions. The HCPT concentration was 5 μg/ml. Indicated are the concentrations of PEG400.](image7)
4. Discussion

PEG is well known as an agent to increase the solubility of some insoluble drugs or avoid absorption of various proteins to achieve long circulation of drugs in blood. These goals are mostly based on some chemical techniques such as PEGylation or physical methods such as using liquid PEG as solvent. We here discover that in some cases a physical additive of PEG could change the chemical property of drug molecules.

The equilibrium lactone fractions of TPT and HCPT in 40 wt.% PEG/PBS solutions were both increased to around 50% at pH 7.4 and 37 °C, as shown in Figs. 4 and 6, compared with that of only about 10% in PBS at the same pH and temperature. This phenomenon was a real thermodynamic shift, for the final equilibrium lactone fractions of TPT in both ring-opening and ring-closing kinetic tests merged together, as shown in Fig. 2b. The in vivo antitumor experiments also confirmed the PEG effect on the lactone fraction of TPT. The presence of PEG enhanced the efficacy of the drug TPT irrespective of intratumor or extratumor injection, as shown in Fig. 9 and Table 1.

A question arises: why does this common polymer have this marvelous effect on CPT analogs? We anticipated that the organic property of PEG might account for the effect. Two pertinent organic small molecules ethylene glycol (EG) and ethanol were thus figured out. The equilibrium lactone fractions of TPT in 40 wt.% EG/PBS and ethanol/PBS solutions were 28% and 59%, and the group of 40 wt.% PEG solution lied between these two extreme cases, as shown in Fig. 8. Meanwhile, the change of EG to glycerol with the same weight fraction of 40 wt.% led to a lower lactone fraction of TPT of 21%. Hence, the addition of PEG is similar to that of a low dielectric constant organic phase and thus makes the medium more “organic” compared to PBS.

In order to certify this assumption, we detected the fluorescence emission of HCPT, which is very sensitive to the microenvironment polarity, in a series of PEG solutions of different polymer concentrations. As shown in Fig. 7, obvious blue-shifting of the emission spectra appeared with the increase of PEG concentration. A similar blue sifting of HCPT in methanol/H₂O solutions as a function of methanol concentration was found by Mi and Burke early in 1994 [40]; it has also been explicitly indicated by Hirano et al. in 2011 that PEG

Fig. 8. Equilibrium lactone fraction of TPT in mixture solvents at pH 7.4 and 37 °C. The weight fraction of organic solvents (ethanol, ethylene glycol, glycerol) was 40 wt.%, and the other 60 wt.% was PBS. 40 wt.% Milli-Q water (60 wt.% PBS) was also taken as control. The PEG group (MPEG5000, 40 wt.%) was detected under the same pH and temperature as other groups.

Fig. 9. S180 solid tumors extracted from mice at day 5 for all of the groups indicated in Table 1.
“behaves as a weakly organic solvent” [42]. So, our phenomenon of PEG additives to enhance the lactone fraction of CPT family drugs in aqueous solutions is related to the organic property of this super-star molecule as well as its high water solubility.

Then how does the organic property of PEG influence the lactone fractions of camptothecin analogs? The hydrolysis process of the CPT family drugs contains two steps, the ring-opening of the lactone structure and ionization of the carbonyl:

\[ L \rightarrow C + C^- + H^+ \quad (3) \]

\( K_{\text{open}} \) is relatively small with the value of about 0.002 [43]. The global equilibrium constant could be written as \( K = [C^-][H^+]/[L] \), if the difference between activity and concentration is neglected. Since \( K = K_{\text{open}}K_a \), we have \( pK = pK_{\text{open}} + pK_a \).

The form “acid” could be regarded as an “acid”. It has been reported that \( pK_a (=-\log K_a) \) of an acid was increased in organic solvents or organic/water mixtures [44, 45]. It is thus not hard to understand that the \( pK_a \) and thus \( pK \) of CPT analogs would be increased after addition of PEG solutions. Therefore, the equilibrium moves to the left and leads to a higher lactone fraction.

A more quantitative analysis available for comparison between theory and experiment is afforded as follows, based upon the definition of \( pH_{1/2} \) by us. Since [C] is very low in the CPT family [43], we think that \( pK \approx pH_{1/2} \) in the case of equal lactone and carbonyl acid forms. We then obtained the relation

\[ pH_{1/2} = pK_{\text{open}} + pK_a \quad (4) \]

The presence of PEG increased \( pK_a \) and thus \( pH_{1/2} \), which has been convinced in our pH titration of the equilibrium lactone fraction (Fig. 3). Here, it seems necessary to note that the actual proton activity \( c_{H^+} \) might be slightly different from the output in a pH-meter measurement due to an error of the liquid junction potential \( \delta \) of the organic mixture in a glass electrode with \( p_{H^+} = \delta \) [46, 47]. The values of \( \delta \) are 0.051 and 0.086 for 33.3 wt.% methanol and ethanol, 0.130 and 0.221 for 52 wt.% methanol and ethanol [40]. So, the liquid junction potential error is not sufficiently high to interfere our basic conclusion about the PEG effect, since the \( pH_{1/2} \) difference in our experiments is as large as 7.4—6.3 = 1.1 after addition of PEG (Fig. 3).

Finally, the shift of \( pH_{1/2} \) leads to a higher equilibrium lactone fraction in the same proton activity or under a given pH, and such an effect is most significant at neutral and weak acidic conditions (Fig. 3). This is very meaningful for medical applications, because blood pH is normally around pH 7.4 and tumor sites are usually weakly acidic due to heathy metabolism [48, 49].

5. Conclusions

Two CPT analogs, water-soluble TPT and weakly water-soluble HCPT, and six PEG agents of MW 200—5000, were tested. All of the examined cases illustrated the enhancement of the active lactone fraction of the drugs. The phenomenon was interpreted by the organic property of PEG, which was confirmed via blue-shifting of the fluorescence emission of HCPT in PEG solutions. The microenvironmental change led to the increase of \( pH_{1/2} \), and thus the equilibrium lactone fraction under physiological pH. Free of any chemical reaction and special agent as well as extremely easy in preparation, the present approach might be the simplest way among the efforts to increase the lactone fraction of CPT family members. The new insight of this very common medical material and the facile approach to enhance the active drug form might be stimulating for research and development in the fields of Biomaterials and Pharmaceutics.

Acknowledgments

The authors are grateful for the financial supports from NSF of China (grants No. 91127028 and No. 21034002), and Chinese Ministry of Science and Technology (973 program No. 2009CB930000).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2012.12.004.

References
