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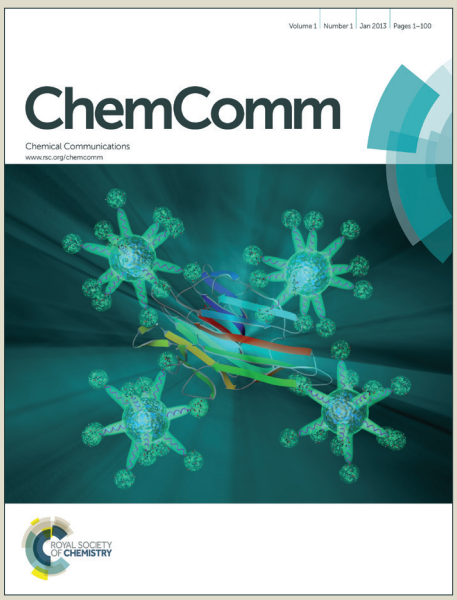

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COMMUNICATION

Effect of molecular structure on anticancer drug release rate from prodrug nanoparticles†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Controlled release of anticancer agent from drug nanoparticles could be achieved by varying the linker length of dimeric compounds as prodrug. Notably, the cytotoxicities of cancer cells were closely related to the release rate of drug compounds. This strategy will lead to establishing the novel delivery system using drug nanoparticles.

The development of cancer therapeutics that are both effective and safe is an important, yet challenging, task. One possible solution is the use of drug delivery systems that take an advantage of the enhanced permeation and retention (EPR) effect so as to deliver anticancer agents to tumors efficiently and without causing side effects.[1] The most widely studied drug delivery systems are nano-sized carriers such as polymer micelles and hydrogels,[2-10] liposomes,[11-13] proteins,[14] dendrimers,[15] solid lipid nanoparticles,[16,17] and combinations of these.[18,19] Importantly, these carriers can be also engineered in such a way that they control the release of the active agent by responding to the differences in pH or ion concentration between tumors and healthy tissues.[7,8] However, nano-sized carriers are not without limitations, including immunogenic effects, low drug loading, and short retention time in blood.[20]

We previously reported that nanoparticles formed from dimeric prodrugs of the commercial anticancer agents, *i.e.*, SN-38 and podophyllotoxin (PPT), were effective, even without nano-sized carriers, by reference to comparable, clinically relevant water-soluble prodrugs.[21,22] These nanoparticles were obtained using our own organic nanocrystal fabrication technique, which is known as the reprecipitation method.[23] Our method involves rapidly injecting a solution of the target compound into a poor solvent so as to reprecipitate the nanocrystals. Many kinds of organic nanocrystals have been prepared using the reprecipitation method, including polydiacetylene,[24,25] perylene,[26] a pigment molecule,[27] and an organometallic compound.[28]

The reprecipitation method is a useful technique for preparation of aqueous dispersions of nanoparticles of anticancer agents. Generally, nanoparticles of physiologically active compounds are fabricated using top-down methods such as wet milling with bead mills or homogenization.[29-31] However, the resulting nanoparticles still need to be dispersed in aqueous media, and the high pressure or heat required can lead to degradation of the molecules. The reprecipitation method has no such drawbacks and allows to provide aqueous dispersions of nanoparticles directly and rapidly prepared under mild conditions.

The relationship between the anticancer activity of our dimeric prodrug nanoparticles and their physical properties has not yet been investigated. The cytotoxicity, release rate, and size of the nanoparticles are all and closely related to the design of the prodrug. Therefore, in this study, we synthesized four PPT dimer compounds to evaluate the relationship between chemical structure, anticancer activity, and hydrolysis rate. PPT was selected, because it is a well-known anticancer agent,[32-34] its derivatives are easily synthesized,[35-37] and it is often used in the study of nanomedicines.[17,38,39]

A PPT dimer with a four-carbon linker (PPT dimer C4) was synthesized according to a previously published method.[22] All PPT dimers were prepared by a condensation reaction between two molecules of PPT and a dicarboxylic acid (Scheme 1). PPT dimers were characterized using IR spectroscopy, ^{1}H and ^{13}C NMR spectroscopy, and high-resolution mass spectrometry (HRMS). The ester bonds were incorporated to allow PPT dimers so as to be hydrolyzed by an enzyme that is expressed in cancer cells. Additionally, PPT dimer SS contained a disulfide (S-S) bond to enable further degradation to take place in the reducing environment of the cell.[5,9,40] The linker itself was the same length as that of PPT dimer C10.

All aqueous dispersions of PPT dimer nanoparticles were obtained using the reprecipitation method. The zeta potentials of PPT dimer C4, C10, and C18 nanoparticles were all in the range –15 to –25 mV (Fig. S1). Scanning electron microscopy (SEM) images of

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†Electronic Supplementary Information (ESI) available: Experimental details, NMR, HRMS, zeta potential data, XRD patterns, and dimer hydrolysis data are given. See DOI: 10.1039/x0xx00000x

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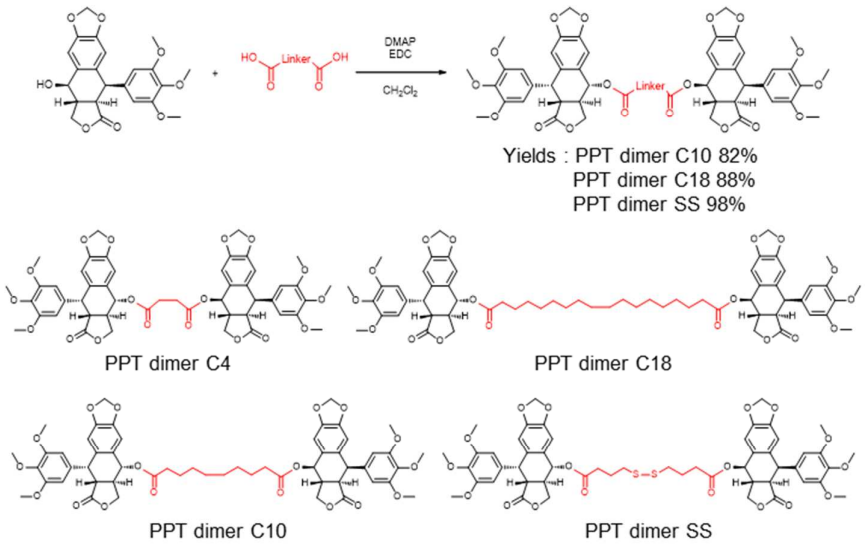

Scheme 1 Preparation of podophyllotoxin (PPT) dimers C10, C18 and SS and chemical structures of the four types of PPT dimers described in this article.

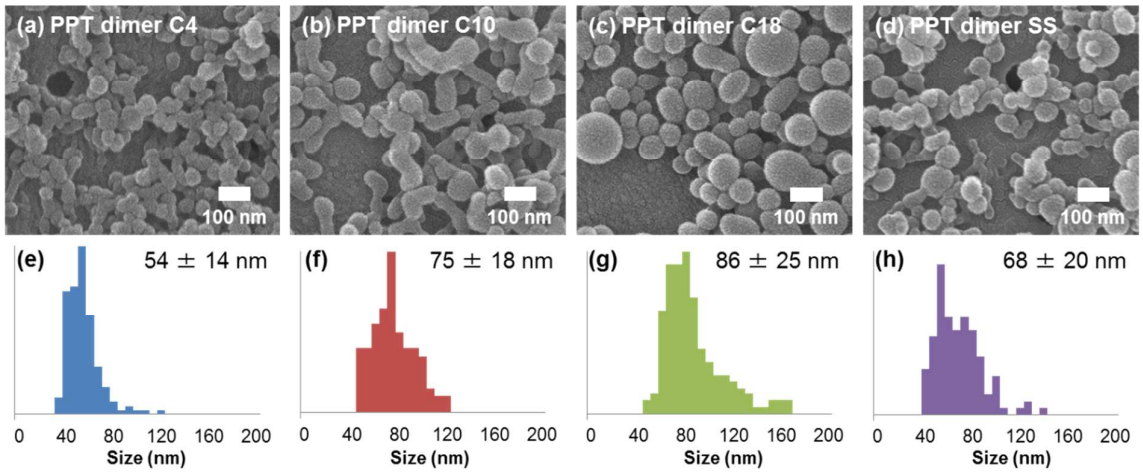

Fig. 1 SEM images of podophyllotoxin (PPT) dimer C4 (a), C10 (b), C18 (c) and SS (d) nanoparticles, and size distribution of PPT dimer C4 (e), C10 (f), C18 (g) and SS (h) nanoparticles. Data show means ± SD. The size of PPT dimer nanoparticles increased with the linker length. PPT dimer C10 nanoparticles are similar to PPT dimer SS nanoparticles in size.

the PPT dimer nanoparticles are shown in Fig. 1a–d. All PPT dimer nanoparticles had a spherical shape. The average size of PPT dimer nanoparticles increased from 54 to 86 nm as the linker was lengthened. PPT dimer C10 and SS nanoparticles were both approximately 70 nm (Fig. 1e–h). As evidenced by the lack of an observed diffraction peak in the powder X-ray diffraction (XRD) pattern, the PPT dimer C4, C10, C18, and SS nanoparticles were in an amorphous phase (Fig. S2).

KPL-4 human breast cancer cells were treated with aqueous dispersions of PPT dimer nanoparticles. The concentration of PPT dimer nanoparticles was 0.1–5 μM based on PPT monomer concentration. After 48 h, PPT dimer C4 nanoparticles had shown the strongest anticancer activity (Fig. 2a). In addition, cytotoxicity increased as the length of the linker got shorter. PPT dimer SS nanoparticles had a higher anticancer activity than PPT dimer C10 nanoparticles, and were equally as effective as PPT dimer C4 nanoparticles (Fig. 2b).

In this study, it was assumed that anticancer activity depended on both the cellular uptake of the nanoparticles and the release rate of the active agent. When the cellular uptake rate of PPT dimer nanoparticles to KPL-4 cells was evaluated, it was found that after 3 h, the extent of cellular uptake was similar regardless of the linker used (Table 1). Cellular uptake is determined by several factors, for example, size,[3,7] zeta potential,[41,42] and the morphology of particles.[43,44] In this study, it was to be expected that the uptake of the different nanoparticles would be similar because the size (50–90 nm), zeta potential (approximately –20 mV), and shape of the different nanoparticles were largely unaffected by the choice of dimer.

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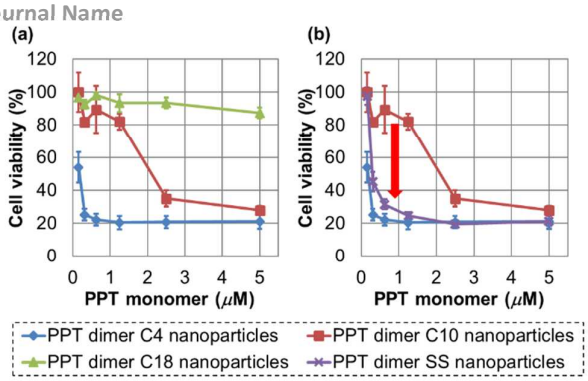

Fig. 2 (a) Cell viability of KPL-4 human breast cancer cells cultured for 2 days in the presence of PPT dimer C4, C10 and C18 nanoparticles. PPT dimer C4 nanoparticles had the highest anticancer activity. (b) PPT dimer SS nanoparticles had higher anticancer activity than PPT dimer C10 nanoparticles, and similar activity to PPT dimer C4 nanoparticles, Data show means ± SE.

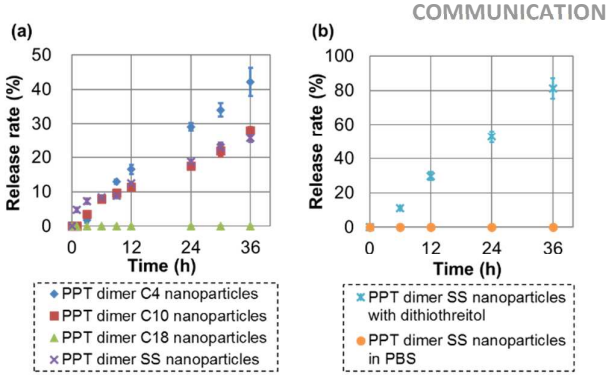

Fig. 3 (a) Esterase-mediated release of podophyllotoxin (PPT) from PPT dimer C4, C10, C18 and SS. The hydrolysis rate of PPT dimer C4 nanoparticles was the fastest. PPT dimer C10 nanoparticles had a released PPT at a similar rate to PPT dimer SS nanoparticles under these conditions. (b) Release of PPT from PPT dimer SS nanoparticles with or without dithiothreitol (DTT). PPT dimer SS nanoparticles also underwent DTT-mediated degradation. Data show means ± SE

Table 1 Cellular uptake after 3 h in the presence of podophyllotoxin (PPT) dimer nanoparticles as determined by LC-MS/MS. Data show means ± SE.

Nanoparticles	Amount of dimer compound (pmol/well)
PPT dimer C4	8.24 ± 1.76
PPT dimer C10	7.49 ± 2.87
PPT dimer C18	8.81 ± 3.30
PPT dimer SS	10.66 ± 3.13

Since the cellular uptake of the nanoparticles was almost similar between the different types, it was thought that the difference in cytotoxicity was caused by differences in drug release rate. Fig. 3a shows the release rate of the different PPT dimer nanoparticles as a result of hydrolysis by porcine liver-derived esterase. The release rate of PPT from PPT dimer C4 nanoparticles was the fastest, and the hydrolysis rate of PPT dimer nanoparticles decreased as linker length increased. This result corresponds with the anticancer activities of PPT dimer C4, C10 and C18 nanoparticles. PPT dimer nanoparticles were not hydrolyzed in esterase-free phosphate-buffered saline (PBS) (Fig. S3) and acidic condition. Therefore, it can be concluded that the observed hydrolysis was esterase-mediated and not a result of heat or pH. Additionally, PPT dimer SS nanoparticles could undergo degradation by the reducing agent dithiothreitol (DTT),[40] as shown in Fig. 3b. Therefore, it was found that release rate of PPT was the key factor for anti-cancer activity of PPT dimer nanoparticles. And then, it is considered that both the degradation of disulfide bonds and hydrolysis resulted in the drug release from nanoparticles in the cells.

Hydrolysis of the PPT dimers must occur to enter the nucleus of a cancer cell and cause apoptosis.[45] Therefore, the hydrolysis mechanism of the PPT dimers should be clearly evaluate. It is unlikely that esterase is able to penetrate the nanoparticles due to their hydrophobic nature, and also esterase is larger than the PPT dimer molecules. It is also unlikely that sufficient is not released from the nanoparticles, because the concentration of free PPT dimer C4 (6.05 nM) and C18 (< 1 nM) in aqueous media is less than 0.1% of the nanoparticle concentration (10 μM).

The alternative, and most reasonable, explanation is that PPT dimers are hydrolyzed on the surface of nanoparticles. This also explains the difference in hydrolysis rate. In the case of PPT dimer C4 nanoparticles, a greater number of ester bonds are likely to exist at the nanoparticle surface, due to less hydrophobic short linker. The accessible ester bonds are then degraded and the particle is gradually decomposed. For comparison, the ester bonds of PPT dimer C18 are part of longer, more hydrophobic chains, and are therefore likely to be forced into the interior of the nanoparticle by the hydrophobic effect. The ability to control drug release by changing dimer design is particularly exciting from a drug delivery point of view, as it would allow drug release to be tuned depending on therapeutic need. We need to further investigate the details according to cellular uptake route and anticancer mechanism of PPT dimer nanoparticles in order to optimize the prodrug design.

In summary, four types of PPT dimer were prepared with different linker lengths and degradation triggers. The dimers were synthesized in high yields, and aqueous dispersions of dimer nanoparticles (50–90 nm diameter) were fabricated using the reprecipitation method. The anticancer activity of a PPT dimer nanoparticle was found to decrease as the length of the linker increased. Additionally, the incorporation of a disulfide bond was found to increase anticancer activity. Investigations into the uptake and release behavior of the nanoparticles indicated that their anticancer activity was entirely dependent on their drug release characteristics. In the future, this discovery will enable improved drug delivery vehicles to be developed.

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This study was greatly supported by The Asahi Glass Foundation and the JSPS programs of Scientific Research (A), Cooperative Research Program of "Network Joint Research Center for Materials and Devices" and Research fellow. We are grateful to Prof. Noriaki Ohuchi, Prof. Kohsuke Gonda, Dr. Hiroshi Tada, and Dr. Liman Cong of the Graduate School of Medicine, Tohoku University, for provision of KPL-4 human breast cancer cells and fruitful talks. We would like to thank Dr. Tatsuya Murakami of the Institute for Integrated Cell-Material Sciences, Kyoto University, who provided fruitful discussion. We also thank to Dr. Hiroshi Yabu of Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, who setup the *in vitro* experiment.

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