Nanotechnology

DOI: 10.1002/anie.201204596

Creation of Pure Nanodrugs and Their Anticancer Properties**

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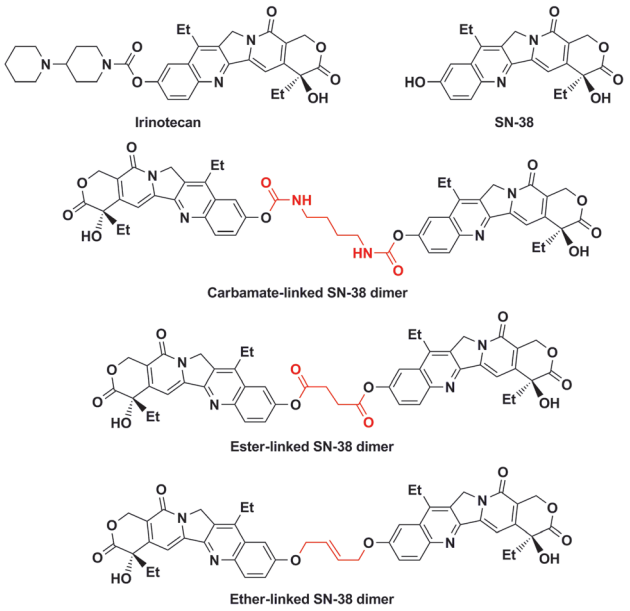
Irinotecan (Scheme 1) is a derivative of camptothecin[1] and is known to be currently used as a major chemotherapeutic agent with favorable water solubility.[2] It is, in fact, a prodrug of the pharmaceutically active moiety of SN-38 (Scheme 1), which is formed as a result of hydrolysis of the carbamate moiety and is active as a DNA repressor.[3,4] It would be ideal if SN-38 could itself be delivered to appropriate locations; however, because of its poor solubility in water, sophisticated formulation technologies need to be used. With regard to drug delivery of SN-38, in the past, some formulation technologies have been reported, such as the enclosure of SN-38 in small (100 nm or less) polymer micelles[5–7] containing hydrophilic polyethylene glycol, so that the particles have a sufficient retention time in the bloodstream to enhance cell permeability and to elicit an efficient retention effect. On the other hand, there is no doubt that carrier-free pure nanodrugs (PNDs) that consist exclusively of pharmacologically active compounds will become candidates for use as the next generation of drugs.[8,9] There have been several reports on the preparation of nanoparticles of drug compounds with poor aqueous solubility, such as steroids, by using various methods.[8,10,11] However, PNDs of a size of between 20 nm and 100 nm that are free of contaminant particles have not yet been prepared by a rapid and low-energy process, and several problems remain with regard to their preparation.



Scheme 1. Camptothecin derivatives used in this study.

Given this background, we discovered about 20 years ago that size-controlled organic nanocrystals can be produced by a simple reprecipitation method in which a solution of a compound (solute) is rapidly injected into a poor solvent (antisolvent) to cause reprecipitation of the solute.[12] Nanocrystals of more than 50 organic dyes,[13] including perylene,[14] polydiacetylene,[15,16] and phthalocyanine[17] have been successfully prepared by the reprecipitation method.

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[**] The Asahi Glass Foundation and the JST programs of PREST "Search for Nanomanufacturing Technology and Its Development" (Supervisor: Dr. Naoki Yokoyama) and Adaptable and Seamless Technology Transfer Program through Target-driven R&D have been greatly supportive in this study. We would also like to thank Dr. Jun Matsui and Dr. Takahiro Muraoka of IMRAM, Tohoku University, who provided fruitful discussion. In addition, we received generous support from Nao Naohara, Sayaka Ishibane, Akie Takanashi, Shoko Yamamoto, and Kenji Sugai in relation to the experimentation performed herein. We are very grateful to Prof. Shigefumi Kuwahara of Faculty of Agriculture, Tohoku University, for help with the organic synthesis and spectroscopic measurements.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201204596.

In the initial stage, we hypothesized that the preparation of aqueous dispersions of nanocrystalline SN-38 by the reprecipitation method might permit the development of a carrier-free drug-delivery system. The experimental trials were performed by reprecipitation through injecting a solution of SN-38 in dimethyl sulfoxide (DMSO) into water. In our previous study, the main experimental conditions that affect the size of the reprecipitated particles have been shown to be the concentration of the injected solution and the temperature of the antisolvent.[18,19] Here, the supersaturation degree of the target organic compounds in poor solvent was critically dependent on the size of the nanoparticles formed, where higher supersaturation degree yield smaller particles.[19,20] However, it was found that changing the concentration from 1 mM to 20 mM in the injected solution or the temperature of the antisolvent led only to the formation of nanofibers of SN-38 with a width of 50–300 nm and a length of 1–10 μm with a high degree of reproducibility (Figure 1a; Supporting Information, S1 and S2). The SN-38 molecule contains two hydroxy groups, and has a slight solubility in water. Therefore, the supersaturation degree of SN-38 molecules in water was not so high that it was difficult to obtain the nanoparticles. The reason that nanofibers were obtained was considered to be that SN-38 is a highly planar molecule, which allows the growth of crystals in one direction.[21]

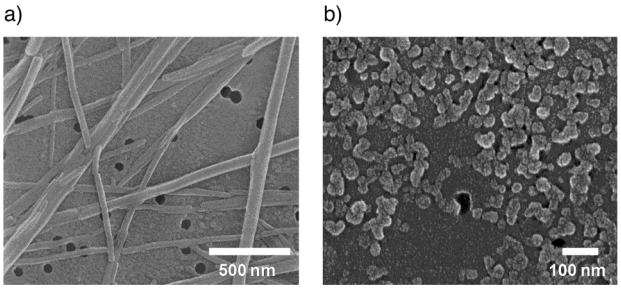

Figure 1. SEM micrographs of a) SN-38 nanofibers and b) carbamate-linked SN-38 dimer nanoparticles with a size of 30–50 nm. Size distribution of carbamate-linked SN-38 dimer nanoparticles dispersed in water measured by DLS is shown in the Supporting Information, Figure S3.

To reduce the aqueous solubility of the SN-38 molecule and make the molecule less planar to prevent crystal growth in one direction, we designed and synthesized three kinds of SN-38 dimers in which the phenolate moieties on two molecules of SN-38 were linked through a butanediylbiscarbamate moiety, a succinate diester moiety, or a (2*E*)-but-2-ene-1,4-diylbis(oxy) diether moiety (Scheme 1). When these compounds were reprecipitated, SN-38 dimer nanoparticles with a size of 30–50 nm were successfully obtained (Figure 1b). Generally, in almost all drug compounds, their supersaturation degree in water are intended to be small, and as a result it has been difficult to prepare nanoparticles of drug compounds with a size of less than 100 nm, even by means of reprecipitation. Herein we introduce the idea of dimerization, and we succeeded in producing aqueous dispersions of SN-38 dimer PNDs with a size of less than 100 nm for the first time. The SN-38 nanofibers showed poor aqueous dispersibility and they aggregated within 30 min of their preparation. On the other hand, the aqueous dispersion of SN-38 dimer nanoparticles was stable and showed no aggregation during the first week of their preparation. Incidentally, when an aqueous dispersion of the SN-38 nanoparticles was left in at −20 °C for more than one month, a clear re-dispersion could still be attained after sonicating the thawed solution. In the case of the water-soluble irinotecan, the preparation of nanoparticles has so far proved elusive, even by using the reprecipitation method.

Regarding the chemical stabilities of the carbamate-linked SN-38 dimer nanoparticles dispersed in water, we performed an ultrahigh-performance liquid chromatography study of the aqueous dispersion over a one-week period of aging, and found no evidence for the existence of the SN-38 monomer during this period (Supporting Information, Figure S4). Furthermore, the aqueous dispersions of the carbamate- and ester-linked SN-38 dimer nanoparticles (concentration, 0.05 mM) showed no clear changes in their physical appearance or in their absorption and fluorescence properties when monitored using SEM (Supporting Information, Figure S5), UV/Vis spectroscopy, and fluorescence spectroscopy, even when the two aqueous dispersions were subjected to aging for one week after their preparation by reprecipitation.

An aqueous solution of irinotecan (**1**), together with aqueous dispersions of the carbamate- (**2**) and ester-linked (**3**) dimer nanoparticles prepared by reprecipitation were individually added to a culture medium of cancer cells (HepG2) after adjusting the concentrations of each pair to the same value (0.1–10 μM). The cells were cultured for a further 48 h and their viability was then examined; the results are shown in Figure 2. The nanoparticles of the SN-38 dimers (**2** and **3**) were found to be more effective in inhibiting proliferation of the cancer cells than irinotecan (**1**). Based on this result, we have found that more molecules of SN-38 with an active site

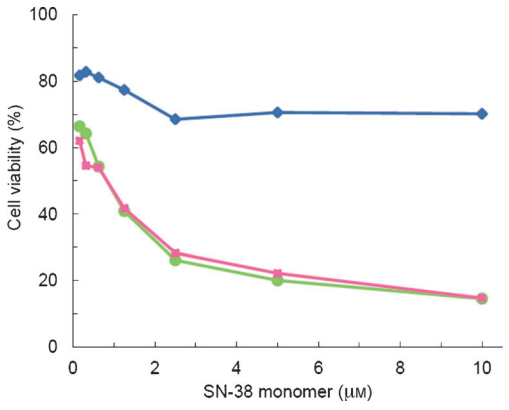

Figure 2. Cell viabilities 48 h after the following solutions were added at varying concentrations (0.1–10 μM) to cultures of cancer cells (HepG2): aqueous irinotecan solution (blue ◆: **1**), aqueous dispersion of carbamate-linked SN-38 dimer nanoparticles (pink ■: **2**), aqueous dispersion of ester-linked SN-38 dimer nanoparticles (green ●: **3**). For **2**, **3**: T test $p < 0.05$ vs. Irinotecan (**1**) for all drug monomer concentrations, except the lowest concentrations of **2** and **3** ($p > 0.05$).

could be introduced into the cancer cells by the carbamate- and ester- linked SN-38 dimer nanoparticles (**2** and **3**) as compared with irinotecan (**1**) owing to the hydrolysis of irinotecan into SN-38. This result indicated that the nanoparticle formation would be more efficient for delivering SN-38 molecules inside cells, as compared to molecule formation (that is, irinotecan). There were no marked differences between the carbamate-linked SN-38 dimer nanoparticles (**2**) and the ester-linked SN-38 dimer nanoparticles (**3**) in terms of their lethal effect on the cancer cells (Figure 2). However, the ether-linked nanoparticles (**B**) showed little inhibitory effect on cancer-cell proliferation, even compared with that of irinotecan (**A**; Supporting Information, Figure S6). We consider that this was because the ether bonds were not hydrolyzed. In other words, we believe that one molecule of the SN-38 dimer with carbamate- or ester-linked nanoparticles is hydrolyzed inside the cancer cell to provide two molecules of SN-38 and two molecules of butane-1,4-diamine and CO_2 or one molecule of succinic acid, which is reported to be almost non-toxic, thereby allowing the released SN-38 to then exert its pharmacological activity.

The estimated amount of SN-38 in the cells, as determined by HPLC analysis, when the irinotecan aqueous solution or the aqueous dispersion of the ester-linked SN-38 dimer nanoparticles was placed into cancer cell cultures are given in the Supporting Information, Table S1. The only compound detected in significant amount by HPLC was the SN-38 monomer, and the amounts of SN-38 monomer were estimated by measuring the area of the corresponding peak in the HPLC plot. The results (Supporting Information, Table S1) are perfectly in agreement with the results shown in Figure 2. If both irinotecan and SN-38 dimer are hydrolyzed and converted into SN-38 out of the cancer cells, the difference of cytotoxicity would be not observed. As SN-38 dimers are insoluble and keep in the form of nanoparticles in the aqueous media, it was considered that the SN-38 dimer nanoparticles themselves, which have high lipophilicity, were easier to penetrate into the cell membrane than irinotecan molecules. Finally, through the hydrolysis that occurred within the cells, they changed into the pharmacologically-active SN-38 monomer. In general, many studies have been made on the penetration of particles through cell membranes, but very few definitive conclusions have emerged regarding the size of the penetrating particles into cells. A recent report described the penetration of 3 μm nanorods into cells,[22] and another report described the preparation of nanoparticles with regularly aligned hydrophobic and hydrophilic parts on their surfaces and discussed the evaluation of the penetrability of such nanoparticles into cells.[23] These reports have attracted considerable attention.

To investigate this cell penetration of nanoparticles, an aqueous dispersion of the ether-linked SN-38 dimer nanoparticles with 1 μM were added into cultures of cancer cells (HepG2) for 20 h. The cells with or without the treatment were observed using confocal fluorescence microscopy (Supporting Information, Figure S7). The fluorescence of the ether-linked SN-38 dimer nanoparticles is much stronger than that of SN-38 with 359 nm excitation (Supporting Information, Figure S8). It is considered that the observed fluorescence (Supporting Information, Figure S7a) originates from the nanoparticles of ether linked SN-38 dimer, because the ether-linked SN-38 dimer nanoparticles are insoluble in aqueous media and have non-hydrolysable linker. In the future, the study of cell permeation of SN-38 dimer nanoparticles will be performed in detail, including the size dependence.

To summarize, we have successfully developed, for the first time, a well-refined process for the formation of SN-38 dimer nanoparticles by the reprecipitation method. The resulting dimer nanoparticles, which can be thought of as carrier-free PNDs, possess a very potent cytotoxic activity. The dimer nanoparticles are expected to combine the following two important properties in relation to their use in drug delivery: good stability of aqueous dispersions and improved cell penetrability. In the near future, on the basis of the concept of PNDs, in which native drugs are converted into nanoparticles, we plan to use the reprecipitation method to convert compounds with anticancer properties into nanoparticles with a controlled size of less than 100 nm with the aim of developing a drug-delivery system based on such nanoparticles.

Received: June 13, 2012
Published online: September 11, 2012

Keywords: cancer · colloids · drug delivery · drug design · nanoparticles

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