An anticancer nanodrug with few side effects that does not require the use of a nanocarrier, polyethylene glycol, or other additives has been developed. We have fabricated nano-prodrugs (NPDs) composed only of homodimeric prodrugs of the anticancer agent SN-38, which contains a disulfide bond. The prodrugs are stable against hydrolysis but selectively release SN-38 when the disulfide bond is cleaved by glutathione, which is present in high concentrations in cancer cells. The best-performing NPDs showed good dispersion stability in nanoparticle form, and animal experiments revealed that they possess much higher antitumor activity than irinotecan, a clinically applied prodrug of SN-38. This performance was achieved by improving tumor accumulation due to the size effect and targeted drug release mechanism. The present study provides an insight into the development of non-invasive NPDs with high pharmacological activity, and also offers new possibilities for designing prodrug molecules that can release drugs in response to various kinds of triggers.

Various approaches for the delivery of small-molecule drugs for anticancer treatments have been investigated (e.g., prodrugs, intracellular synthesis, nanoparticles), but there are still very few approaches that combine high pharmacological activity and low side effects while possessing promising potential for practical applications. For instance, nanodrugs have attracted attention due to their size-selective ability to accumulate in tumors, also known as the enhanced permeability and retention (EPR) effect. However, the clinical application of nanodrugs is hampered by the side effects and the high stability of the carriers themselves. The concentration of various factors (e.g., receptors, enzymes, and small molecules) is higher in the tumor microenvironment than in normal tissue. Many researchers have reported the selective metabolism of prodrug molecules by cancer cells and drug release via the selective dissociation of carriers in cancer cells using the above factors as stimuli. Importantly, these improvements using triggers specific to cancer cells do not resolve the intrinsic challenges of using prodrugs and nanocarriers, such as the size-dependent systemic diffusion of prodrugs, the low drug loading ratios, and the toxicity of the carriers themselves.

Recently, a new form of nanodrug that produces low molecular weight aggregate of prodrug molecules has gained substantial attention. While these nanodrugs avoid employing nanocarriers, most use polyethylene glycol (PEG) or other polymers to improve their dispersion stability. Generally, the cellu-
lar uptake of PEGylated nanoparticles is very low due to their low affinity towards the cell membrane, and the process of endosomal escape is significantly suppressed due to their excessive stability in vivo (known as the PEG dilemma). These nanodrugs also induce an immune response against PEG that results in rapid clearance via a complementary activation process called the accelerated blood clearance (ABC) phenomenon (Fig. 1a). Ultimately, it can be argued that the price of having improved dispersion stability from the use of polymeric materials is very high. Considering the disadvantages related to the use of polymer-based prodrug nanoparticles, completely polymer-free prodrug nanoparticles that consist exclusively of pharmacologically active compounds would be very attractive. However, the following two problems remain to be resolved to develop an optimal prodrug nanoparticle that can reliably migrate to cancer cells and exert its effects with little or no side effects. Firstly, the use of PEG or any other polymeric materials must be eliminated, and secondly, a drug release mechanism that is specific to cancer cells should be incorporated into the molecular design.

Our group has already reported methods to fabricate nanodrugs without using carriers. These produce hydrophobic prodrug nanoparticles called nano-prodrugs (NPDs) that exhibit a high cell uptake efficiency, suggesting that they are subject to a cell uptake mechanism different from that of conventional polymer-based prodrug nanoparticles (Fig. 1b). In this study, we designed a novel derivative of the anticancer reagent SN-38 with a cancer cell-specific prodrug activation/drug-release mechanism mediated by glutathione (GSH) (Fig. 1c). Depending on the location of the tumor and malignancy in cancers, the intracellular GSH concentration in cancer cells (1–10 mM) is several times higher than the concentration in normal cells, and is about 1000 times higher than the concentration in the extracellular compartment (2–20 μM). Several studies have been reported that incorporate the cleavage of an S–S bond by GSH into their strategies. The results of those studies support the possibility of the cancer cell-selective release of drug molecules, albeit many still rely on modifications with polymers or PEG. Our reported NPDs, which combine high pharmacological activity with extremely low side effects, disperse very well without additives other than the prodrug molecules.

Results and discussion
Search for an optimal molecular design
As with generally water-soluble prodrugs, most hydrophobic prodrugs that are composed of NPDs are designed with substituents connected via ester bonds at the primary, secondary, and phenolic hydroxy groups. There is a risk of non-targeted release of the drug triggered by esterases, which are ubiquitous in the body. Conversely, ester bonds that involve tertiary hydroxy groups do not always release the drug in cancer cells due to high levels of steric hindrance. In this study, SN-38 prodrugs (SNCnDC) were designed by dimerization of linear carboxylic acid-conjugated SN-38 (SN-38 × Cn) molecules through a linker formed from carbonate groups joined by an S–S bond (Fig. 2a). The molecular design of SNCnDC allows the release of SN-38 via an intramolecular cyclization driven by

Fig. 1 General drug delivery systems and the system used in this study. (a) General prodrug NPs. Such nano drugs, which often exhibit high dispersion stability because they are coated with a hydrophilic polymer, are often too stable, even within cells, making drug release difficult. Moreover, such nano drugs are not easily taken up by cells due to their low affinity with cell membranes. (b) Previously reported NPDs. These NPDs are easily internalized by cells owing to their hydrophobic and negatively charged surface. However, enzymes commonly found in vivo may non-selectively metabolize the NPDs at non-cancerous sites within the body. (c) The system used in this study. The release of the active drug from these NPDs is not triggered by esterases, which are ubiquitous in the body. After being taken up by the cancer cells, the drug is released via reductive cleavage of disulfide bonds by glutathione, which is present in high concentration in cancer cells.
the reductive cleavage of the S–S bond by GSH, which is abundant in cancer cells (Fig. 2b).

First, the potential of a hexanoic acid-conjugated SN-38 dimer (SNC6DC) was evaluated as a model compound. To analyze the effects of differences in bonding on anticancer activity, hexanoic acid-conjugated SN-38 (SN-38 × C6), a SN-38 × C6 dimer with an ester bond in its linker (SNC6DE), and SNC6DC with a methylene group instead of the disulfide bridge (SNC6DC (without S–S)) were prepared.

SN-38 × C6 showed cancer cell growth inhibitory activity comparable to that of SN-38. SNC6DC showed much higher pharmacological activity than SNC6DE and SNC6DC (without S–S) (Fig. 2c). These results indicate that steric hindrance around the tertiary hydroxy group prevents the approach of esterases and hinders the intramolecular cyclization of SNC6DE required for drug release (Fig. 2d). One of the most crucial differences in the cyclization process is that the release of SN-38 × C6 may be driven by the formation of ethylene sulfide (a three-membered ring) after the metabolism of SNC6DC. This intramolecular cyclization proceeds away from the tertiary hydroxy group of SN-38. Furthermore, SNC6DC (without S–S), which does not trigger the drug release in response to GSH, was found to be pharmacologically inactive. In summary, the carbonate bond was considered able to alleviate the steric hindrance associated with the intramolecular cyclization process, and thus SNC0DC was selected as the optimal prodrug design.

The nanoparticles were formed by hydrophobically driven self-assembly using the reprecipitation method.34 Since the hydrophobicity and flexibility of prodrug molecules are closely related to the size and dispersion stability of the NPDs, it is very important to study the molecular design to obtain optimal nanoparticles. Hydrophilic prodrugs such as irinotecan cannot form nanoparticles in water, and NPDs with less hydrophobic prodrugs cannot be fabricated without the use of polymeric materials. In contrast, it can be predicted empirically that NPDs composed of prodrugs that are too hydrophobic likely inhibit the dissociation of the NPDs in cells, thus reducing their pharmacological activity.25

To optimize the characteristics of the NPDs, various substituents were chosen to conjugate to the SN-38 phenolic hydroxy group for the synthesis of the different SN-38 dimers (SNCnDC, n = 0, 2, 4, 6, 8, 10). Following optimization of the synthetic route and conditions, the key building block, SNC0DC, was synthesized and subsequently converted into the various SNCnDC via esterification (Fig. S1 and Table S1, see the ESI†). The three steps of the synthesis of SNC0DC do not involve complicated purification procedures, and the target product can be obtained in high yields by recrystallization only. Although this reaction is a circuitous synthetic route compared to the direct dimerization from SN-38 × Cn, this method avoids conversion to various byproducts through the formation of chloroformate and by transesterification (confirmed by ESI-MS) (Fig. S2, see the ESI†). The hydroxy group of
the intermediate in the direct dimerization is more nucleophilic than the phenolic hydroxy groups of the SN-38 × Cn derivatives, and thus undesired reactions proceed. These are irreversible and the SN-38 × Cn derivatives are rapidly depleted from the reaction system. When the reprecipitation method was applied to SNC0DC or SNC2DC, the formation of aggregates was observed immediately in the dispersion solution (Fig. 3a and b). It is possible that these compounds still have a high affinity for the poor solvent water. In contrast, when the reprecipitation method was applied to the other SNCnDC prodrugs \((n = 4, 6, 8, 10)\), the formation of aggregates was not observed, not even after a week (Fig. 3c–f). This is possibly due to the relatively low affinity of these compounds for water. The sizes of these NPDs were found to be in the effective size range (60–130 nm) required for the EPR effect. Under conditions where these NPDs do not degrade in the bloodstream, they can efficiently accumulate in tumor tissue. In a previous study, an “SN-38 dimer” linked via its phenolic hydroxy group was synthesized, albeit the low hydrophobicity and high intramolecular forces arising from the \(\pi-\pi\) interactions of the dimer made it difficult to fabricate NPDs using the reprecipitation method.\(^{23}\) Various NPDs that are derivatives of the SN-38 monomer have also been reported, but these do not represent truly optimal designs due to their low drug loading ratios and high hydrophobicity.\(^{24}\) In contrast, the SNCnDC without any substituents, SNC0DC, possesses a high drug loading ratio of 79% and a very high Clog \(P\) value of 5.7 (Table 1). In brief, the SNCnDC prodrugs that allow the fabrication of NPDs were achieved by the introduction of smaller substituents. Compared to previous studies, the major difference is the hydrophobicity of these prodrugs, and the dimer prodrugs proposed in this study can be expected to break the trade-off between hydrophobicity and pharmacological activity.

**In vitro cell growth inhibitory activity of SNCnDC**

To clarify the potential pharmacological activity, the cancer cell growth inhibitory activity of the SNCnDC \((n = 0, 2, 4, 6, 8, 10)\) was evaluated in the range of 0.04–10 \(\mu\)M against KPL-4 and HCT-116 cells. An important trend in the inhibitory activity of each SNCnDC at an equal dose of SN-38 was observed (Table 1). Overall, SNCnDC whose substituents contain shorter carbon chains exhibit higher anticancer activity and lower IC\(_{50}\) values. If the same amount of SN-38 is released in the cancer cells, the pharmacological activity of each SNCnDC should be equal. The following discussion of this trend is made with reference to previous papers about the intracellular dynamics of prodrug nanoparticles inside cancer cells.\(^{27}\) In the case of SNCnDC, the hydrophobicity of the substituents may mediate (1) the internalization in the cells, and (2) the degradation/dissociation inside the cells. The rate of degradation/dissolution is particularly important for anticancer activity and could be the rate-limiting factor for the release of SN-38. The degradation/dissociation inside the cell, and thus the lower anticancer activity, decelerates with increasing length of the carbon chain of the substituent. SNC4DC was selected as the optimal compound for animal experiments as it has the highest anticancer activity among the prodrug molecules that can be fabricated into NPDs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Loading capacity(^a) [%]</th>
<th>Clog (P) value(^b)</th>
<th>IC(_{50}) against KPL-4 cell [(\mu)M]</th>
<th>IC(_{50}) against HCT-116 cell [(\mu)M]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNC0DC</td>
<td>79</td>
<td>5.7</td>
<td>0.37</td>
<td>0.18</td>
</tr>
<tr>
<td>SNC2DC</td>
<td>73</td>
<td>5.0</td>
<td>0.36</td>
<td>0.28</td>
</tr>
<tr>
<td>SNC4DC</td>
<td>70</td>
<td>7.1</td>
<td>0.41</td>
<td>0.06</td>
</tr>
<tr>
<td>SNC6DC</td>
<td>66</td>
<td>9.2</td>
<td>1.66</td>
<td>0.33</td>
</tr>
<tr>
<td>SNC8DC</td>
<td>63</td>
<td>11.3</td>
<td>9.67</td>
<td>0.68</td>
</tr>
<tr>
<td>SNC10DC</td>
<td>60</td>
<td>13.4</td>
<td>&gt;10</td>
<td>0.96</td>
</tr>
</tbody>
</table>

\(^a\) The drug loading efficiency is expressed as a percentage of the active body (SN-38) in the prodrug molecule, based on molecular weight.\(^b\) The Clog \(P\) value is a calculated value related to the partition coefficient \(P\) in \(\pi\)-octanol/water, whereby a higher number represents a more hydrophobic molecule. The Clog \(P\) values were calculated using the function “Chemical Properties” in ChemDraw Professional 20.1.

Fig. 3 Characterization of the SNCnDC \((n = 0, 2, 4, 6, 8, 10)\) NPDs. (a)–(f) Scanning electron microscopy (SEM) images and size distribution profiles of the SNCnDC NPDs formed using the reprecipitation method: SNC0DC (a), SNC2DC (b), SNC4DC (75 nm) (c), SNC6DC (60 nm) (d), SNC8DC (65 nm) (e), and SNC10DC (130 nm) (f) (solid line: after fabrication; dashed line: after 7 days).
and their in vitro cell growth inhibitory activity was evaluated. SNC4DC NPD and SN-38 showed higher cell growth inhibitory activity compared to irinotecan, because irinotecan, a water-soluble prodrug, is more hydrophilic and has less affinity for cell membranes (Fig. S3, see the ESI†). Given that SN-38 is itself the active form of the drug, it will exert pharmacological activity once it has been taken up into the cells. In contrast, the SNC4DC NPDs are internalized into the cells and dissociate, and the active SN-38 is only released after metabolism. These differences in inhibitory activity are suggested to be due to the number of steps in the release of the active form.

**Intracellular dynamics of the SNC4DC NPDs**

The cellular uptake of NPDs by cancer cells is also one of the driving forces behind drug delivery and therapeutic efficiency. The SNC4DC NPDs were not observed in the intracellular matrix or on the cell membrane when cells were pre-incubated at 4 °C (Fig. S4, see the ESI†). This result suggests that the uptake of the nanoparticles relies on energy-dependent endocytosis pathways such as clathrin-dependent endocytosis, caveolae-dependent endocytosis, and macropinocytosis. A number of endocytosis inhibitors were then applied to investigate the pathway by which SNC4DC is taken up into the cells. Chlorpromazine showed inhibition activity against cellular uptake, and although SNC4DC NPDs were found on the cell membrane, the internalization was inhibited. As chlorpromazine inhibits clathrin-dependent endocytosis, it was concluded that clathrin-dependent endocytosis is the major pathway for cellular uptake of the SNC4DC NPDs.

The Förster resonance energy transfer (FRET) principle was used for the investigation of the intracellular dynamics, and FRET-SNC4DC NPDs composed of SNC4DC (FRET donor) and the dye bodipy FL (BPFL)–cholesterol (FRET acceptor) were fabricated (Fig. S5a and S5b†) in reference to our previous research. The nanoparticles produced in this study are internalized into cells in the nanoparticle state. Subsequently, the dissolution of compounds from particulate states occurs spontaneously. The decrease in intensity of the fluorescence signal derived from both the FRET-SNC4DC NPDs and the SNC4DC monomer is related to the quenching of the SNC4DC fluorescence due to the hydrolysis of the ester bond at the phenol hydroxy group. The general consensus is that SN-38 is gradually released in the cells. After 48 hours, the morphology of the HeLa cells changes and only a small number of cells are left attached to the dishes, indicating a decreased viability of cells treated with SNC4DC NPDs. These time-dependent observational results are shown in Fig. S5c and S5d (see the ESI†).

**Hydrolysis resistance of SNC4DC and drug release behavior under S–S bond reducing conditions**

The drug release behavior of SNC4DC under S–S bond reducing conditions was evaluated by incubating a mixture of the SNC4DC NPDs and GSH at 37 °C. SN-38 was gradually released, and the NPDs degraded under these conditions (Fig. 4a). Drug release was also observed when SNC0DC and SN-38 × C4 were incubated with GSH. The difference in the drug release rates of SNC4DC and SNC0DC was attributed to their different hydrophobicities (Fig. S6, see the ESI†). As mentioned in the section on molecular design, the phenyl esters of SN-38 × Cn derivatives are very susceptible to nucleophilic attacks, and thiols are nucleophiles that are known to attack phenyl esters. There still remain two possible mechanisms for intracellular drug release, i.e., (1) intramolecular cyclization triggered by GSH and (2) hydrolysis by esterase. To confirm the mechanism via which SNC4DC releases the active drug, the hydrolysis resistance of SNC4DC was evaluated. The hydrolysis resistance of NPDs fabricated from SNC4DC and some degradants of SNC4DC was examined by incubating a mixture of the NPDs and porcine liver esterase (PLE) at 37 °C. In a hydrolysis test using PLE, SNC4DC and SNC0DC were found to be stable for more than 6 hours, whereas SNC4M was hydrolyzed within 60 minutes. These results indicate high hydrolysis resistance for the homodimeric prodrugs (Fig. 4b). Importantly, the amount of SNC4DC was found to decrease slightly over time, but only SNC0DC was released and remained inactive (Fig. S7, see the ESI†). The in vitro cell experiment results and the drug release behavior studies suggest that SNC4DC converts to SN-38 inside the cells and that this process is driven by the cleavage of the S–S bond by GSH (Fig. 4c).

**In vivo experiments and side effects**

**Preparation of high-concentration NPDs dispersion for in vivo experiment.** With reference to the administration method and clinical regimen, a highly concentrated SNC4DC NPD dispersion was prepared for the in vivo experiments.
Although the dispersion of highly concentrated NPDs appears to be cloudy, Tyndall scattering can be observed after diluting the solution to a concentration of 0.1 mM (Fig. 5a). The SEM images indicate that the fabricated NPDs have a spherical morphology with a diameter of 150 nm (Fig. 5b). The size distribution profile obtained from dynamic light scattering (DLS) shows that the SNC4DC NPDs are stably dispersed in saline for more than one month at 50 °C without any aggregation (Fig. 5c). The size of these NPDs is in the effective range for the EPR effect, which promises high in vivo antitumor activity. HPLC analysis and DLS measurements of the dispersions stored for long periods of time corroborated the good dispersion stability for the high-concentration SNC4DC NPDs (Fig. S8, see the ESI†).

**Antitumor activity of SNC4DC NPDs.** Notably, SNC4DC NPDs suppress tumor growth more strongly than irinotecan, even when the same amount of SN-38 was applied (Fig. 5d). This result should probably be ascribed to the fact that irinotecan is a water-soluble drug that diffuses throughout the body. In contrast, SNC4DC circulates in the blood for a long time in its nanoparticulate state, thus accumulating in the tumor site due to the EPR effect. A tumor removed 48 hours after drug administration was found to still contain a small amount of the drug derived from the NPDs (Fig. S9a, see the ESI†). It is therefore possible that SNC4DC NPDs accumulate in tumor tissue in the nanoparticle state and selectively release SN-38 only in the tumor tissue.

**Evaluation of side effects.** Although SNC4DC NPDs possess low toxicity and high pharmacological activity against cancer cells, their potential side effects still require investigation. One of the known side effects of chemotherapy is extreme loss of weight and premature death. In our experiments, premature death and abnormal weight loss over time were not observed (Fig. 5e). Despite possessing very high antitumor activity, damage to intestinal tissue, which is a major side effect of camptothecin derivatives, was not observed (Fig. S9b, see the ESI†). In addition, SNC4DC, irinotecan, and saline were intravenously injected into mice and their white and red blood cells were examined, which showed a smaller decrease in their number compared to irinotecan (Fig. 5f and g). The above side effects are closely related to toxicity against normal cells. It is therefore feasible to conclude that the SNC4DC NPDs possess both higher pharmacological activity and lower side effects compared to irinotecan.

**Conclusions**

Here, novel prodrugs were developed based on a chemical structure that allows release of the anticancer agent specifically...
in cancer cells. The prodrugs were fabricated into nanoparticles without the use of nanocarriers or PEG. These nano-prodrugs (NPDs), which consist of two molecules of the anticancer agent SN-38 connected via a linker with a S–S bond, are stable toward hydrolysis by esterases, and selectively release SN-38 in environments with high concentrations of GSH such as cancer cells. The best performing NPDs, SNC4DC NPDs, exhibited high antitumor activity in vivo, and severe side effects were not observed. The results indicate that at the same dosage, SNC4DC NPDs exhibit a much better pharmacological potential than irinotecan, a commercially available water-soluble prodrug of SN-38. SNC4DC can be fabricated into drug nanoparticles that have not only both high pharmacological activity and low side effects but also the high dispersion stability at high concentrations that is required for clinical applications. In the field of prodrug molecular design for NPDs, this research has opened up the new possibility of designing prodrug molecules where drug release can be triggered by various stimuli, and not only GSH. Additional research on the pharmacokinetics and a strategy for the mass production of these nanoparticles can be expected to lead to very safe nanodrugs in the near future.

Author contributions

K. T. and Y. K. designed the study and the main conceptual ideas. K. T. and S. K. performed all synthesis. K. T., A. M. and H. N. collected data. F. T. performed the intracellular dynamics experiment and the analysis, and drafted the manuscript. F. F. provided data on the evaluation of toxicity to the small intestinal tissue from mouse samples. H. T., T. I., K. S. and C. I. aided in interpreting the results and worked on the manuscript. Y. K. and H. K. supervised the project. K. T. and S. K. wrote the paper with support from R. S. and A. T. N. D. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The data supporting the findings of this study are included within the article and its ESI† files. Further data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts to declare.

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