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Anticancer nano-prodrugs with drug release triggered by intracellular dissolution and hydrogen peroxide response†

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We developed prodrug nanoparticles that release drugs through intracellular dissolution and a cancer-specific hydrogen peroxide response. To reveal the unclear mechanism regarding drug release from nanoparticles by reacting with hydrogen peroxide in cancer cells, this study demonstrates the *in vitro* evaluation of drug release kinetics under conditions simulated in cancer cells.

Various drug delivery systems (DDSS) have been reported to selectively release drugs in response to cancer intracellular ROS to reduce side effects.¹ Among the DDSSs, prodrugs designed to release pharmacologically active drugs following specific chemical transformations within cancer cells have become a key research focus.² In recent years, numerous prodrugs that trigger drug release *via* reactive oxygen species (ROS), which are present in higher concentrations in tumor tissues than in normal tissues, have been developed.^{3,4} ROS include $^1\text{O}_2$, O_2^- , $\bullet\text{OH}$, and H_2O_2 . In particular, the concentration of H_2O_2 in cancer cells (10–100 μM) is significantly higher than that in normal cells (0.0001–0.7 μM).^{5,6} Nevertheless, the selectivity against cancer cells is reduced because ROS are also present in blood and normal cells,⁷ and prodrug molecules can diffuse throughout the body.⁸

Consequently, research has focused on ROS-responsive prodrug nanoparticles to increase their selectivity for cancer cells.^{9–13} Prodrug nanoparticles with a controlled particle size of 10–200 nm are known to selectively accumulate in the abnormal vascular space surrounding tumour tissues through the enhanced permeability and retention (EPR) effect.¹⁴

Conventional prodrug nanoparticles are fabricated by encapsulating the ROS-responsive prodrugs in a nanocarrier,^{15,16} and there are concerns about the low drug loading rate and side effects of carrier materials.¹⁷ The burst release from nanoparticles may also exhibit unexpected pharmacological effects.¹⁸ Therefore, to eliminate these concerns in conventional systems, our group has developed carrier-free prodrug nanoparticles, called nano-prodrugs (NPDs), which are fabricated from only prodrug molecules.^{19–22} These NPDs maintain in the particle state until reaching cancer cells and do not release drugs.²⁰ Additionally, we have reported that the NPDs exhibit pharmacological effects after being taken up by cancer cells as intact particles.²² However, the detailed mechanism by which the NPDs release drugs in response to cancer intracellular ROS remains unclear.

We aimed to develop NPDs that release drugs only after reaching cancer cells by designing prodrugs that are stable against esterases existing abundantly in the body^{23,24} and that only react with cancerous ROS to release drugs. Specifically, we focused on a prodrug (CPT-TML), which combines camptothecin (CPT), an anticancer drug, with a trimethyl lock (TML) group containing H_2O_2 -responsive aryl boronic acid.²⁵ Herein, to accurately reflect the intracellular drug release mechanism of NPDs, we performed *in vitro* assessment and the evaluation of drug release kinetics in the “nanoparticles” or “dissolution” state.

To confirm the drug release kinetics in response to intracellular H_2O_2 , we designed two versions of CPT-TML: one with aryl boronic acid (ArB(OH)₂) or aryl (Ar) on the protecting group (PG) of the TML group. In CPT-TML, we found that esterases cannot directly cleave sterically hindered esters, while the release of CPT through the cyclization reaction of the TML group occurs by deprotection of the PG and conversion to phenol.²⁶ CPT-TML-ArB(OH)₂ is expected to release CPT *via* the 1,6-elimination of *p*-quinone methide and lactonization of the TML group after the conversion of boronic acid to alcohol by H_2O_2 . Conversely, CPT-TML-Ar, which is insensitive to H_2O_2 and esterases, does not release CPT (Scheme 1).

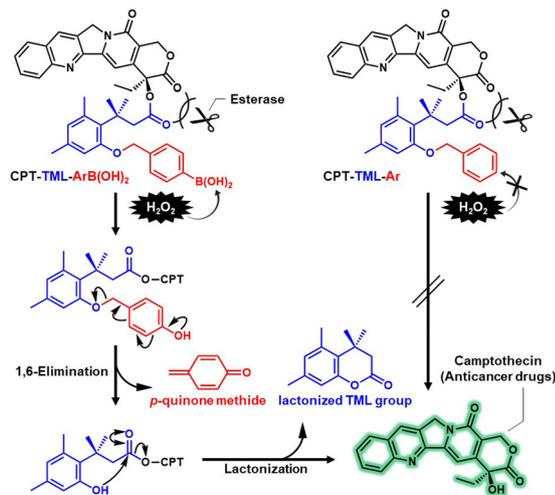
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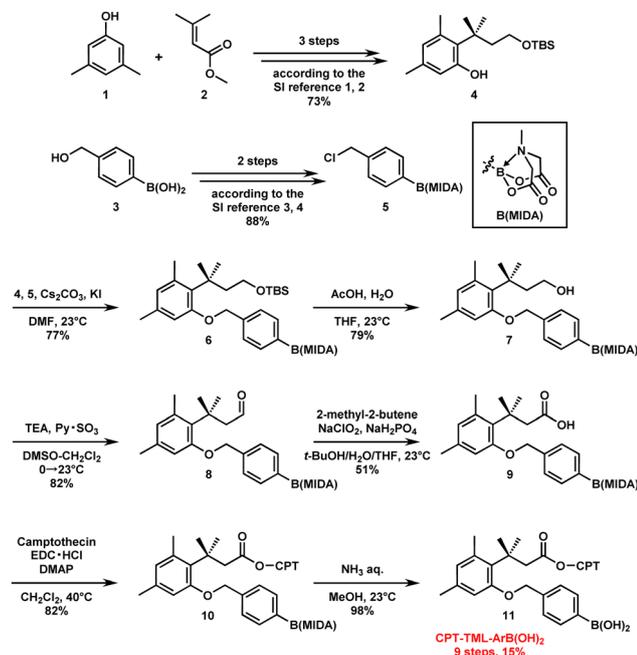
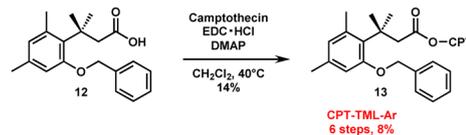




Scheme 1 Proposed CPT release mechanism from CPT-TML.

CPT-TML-ArB(OH)₂ (**11**) was synthesised from 3,5-dimethylphenol (**1**) and methyl 3,3-dimethyl acrylate (**2**) through nine steps shown in Scheme 2, with a total yield of 15% (see the ESI[†]). **CPT-TML-Ar** (**13**) was obtained *via* the esterification of 3-(2-(benzyloxy)-4,6-dimethylphenyl)-3-methylbutanoic acid (**12**) with **CPT** (Scheme 3). Consequently, **CPT-TML-Ar** (**13**) was synthesised from 3,5-dimethylphenol (**1**) through six steps with a total yield of 8% (for details, please see the ESI[†]).

The **NPDs** of the synthesized **CPT-TML-ArB(OH)₂** and **CPT-TML-Ar** were fabricated using a previously reported reprecipitation method.²⁷ A solution of **CPT-TML-ArB(OH)₂** or **CPT-TML-Ar** in tetrahydrofuran (THF) was injected into vigorously stirred deionized water. Scanning electron microscopy (SEM) images showed that each **NPD** appeared as a spherical nanoparticle,

Scheme 2 Synthetic scheme of compound (**11**) (**CPT-TML-ArB(OH)₂**).Scheme 3 Synthetic scheme of compound (**13**) (**CPT-TML-Ar**).

with a mean diameter of approximately 100 nm (Fig. 1A and B). In addition, powder X-ray diffraction (PXRD) analysis showed the amorphousness of the fabricated **NPDs** (ESI[†], Fig. S1). Although the particle sizes and crystal structures were almost identical, two types of **NPDs** exhibited clear differences in their dispersion stability in water. Dynamic light scattering (DLS) of the hydrodynamic size of the **CPT-TML-ArB(OH)₂** **NPDs** revealed no significant change even after one month, whereas the **CPT-TML-Ar** **NPDs** immediately aggregated after reprecipitation (Fig. 1C and D). This may be due to the presence of boronic acid on the surface of the **CPT-TML-ArB(OH)₂** **NPDs**, which generates electrical repulsive forces,²⁸ resulting in a higher dispersion stability in water compared to that of the **CPT-TML-Ar** **NPDs**.

To evaluate the *in vitro* cytostatic activity of the fabricated **NPDs** due to the difference in intracellular H_2O_2 levels, we quantified the H_2O_2 concentration in A549 human lung cancer cells,²² NHDF-Neo normal dermal fibroblasts, and neonatal cells²⁹ used to assess the toxicity of nanomedicines. An intracellular H_2O_2 assay was employed with the BIOXYTEC[®] H_2O_2 -560[™] Assay Kit based on the intracellular Fenton reaction (ESI[†]). As a result, the H_2O_2 concentration in A549 cells was presented as 0.13 nmol/ 10^7 cells. The volume of A549 cells was estimated to be 1670 μm^3 per cell,³⁰ which would imply a H_2O_2 concentration of approximately 8.1 μM / 10^7 cells. The H_2O_2 concentration in A549 cells was about 5-fold higher than that in NHDF-Neo cells (0.028 nmol/ 10^7 cells) (Fig. 2).

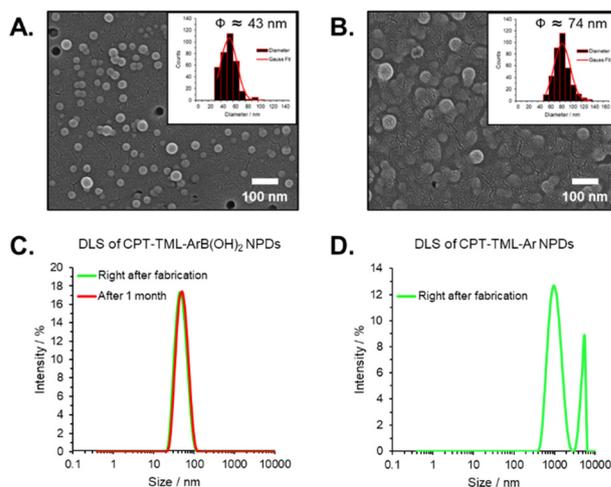


Fig. 1 SEM images of (A) **CPT-TML-ArB(OH)₂** **NPDs** and (B) **CPT-TML-Ar** **NPDs**. Inset: Related diameter distribution (Gaussian fitting in red); dispersion stability of (C) **CPT-TML-ArB(OH)₂** **NPDs** and (D) **CPT-TML-Ar** **NPDs**; DLS measurement immediately after fabrication (green line) and one month (red line).



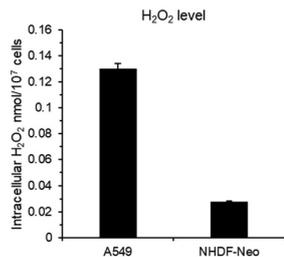


Fig. 2 Intracellular H_2O_2 level of A549 cells and NHDF-Neo cells.

Based on the differences in intracellular H_2O_2 concentrations between cancer and normal cells, we evaluated the *in vitro* cytostatic activities of CPT-TML-ArB(OH)₂ NPDs and CPT-TML-Ar NPDs in A549 and NHDF-Neo cells. CPT and lactonized TML groups (substituents after the release of CPT from CPT-TML-ArB(OH)₂) were also prepared in accordance with a previous report²⁶ for comparison of cytostatic activity. CPT-TML-ArB(OH)₂ NPDs, CPT-TML-Ar NPDs, CPT, and lactonized TML groups in the concentration range of 0.04–10 μM were added to a culture medium comprising A549 cells or NHDF-Neo cells. As shown in Fig. 3A, the CPT-TML-ArB(OH)₂ NPDs exhibited lower cytostatic activity than CPT. Furthermore, the CPT-TML-Ar NPDs and lactonized TML groups did not exhibit any pharmacological effects. These results indicate that a certain amount of time is required for CPT release from CPT-TML-ArB(OH)₂ NPDs in response to intracellular H_2O_2 . Meanwhile, in NHDF-Neo cells, CPT-TML-ArB(OH)₂ NPDs, CPT-TML-Ar NPDs, and lactonized TML groups did not reach IC₅₀ values of 10 μM except for CPT (IC₅₀ of CPT = 0.98 μM) (Fig. 3B). In particular, the pharmacological effects of the CPT-TML-ArB(OH)₂ NPDs were high only in cancer cells treated with high H_2O_2 concentrations. To sum up, the CPT-TML-ArB(OH)₂ NPDs are thought to exhibit pharmacological effects in response to intracellular H_2O_2 .

Finally, to verify the prodrug-to-drug conversion, CPT-TML-ArB(OH)₂ and CPT-TML-Ar were incubated with 100 μM H_2O_2 (the highest concentration of cancer cells) or esterase from porcine liver in phosphate-buffered saline (PBS) (–), respectively. The release of CPT-TML-ArB(OH)₂, CPT-TML-Ar, CPT, and lactonized TML was monitored using HPLC-MS/MS analysis. In the case of incubation with 10 units or 100 units of esterase in PBS (–), the CPT is seldom released from CPT-TML-ArB(OH)₂ NPDs and CPT-TML-Ar NPDs (ESI,† Fig. S2). Similar

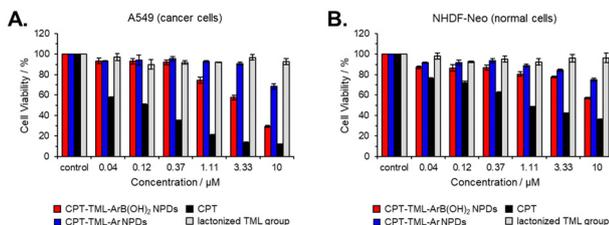


Fig. 3 *In vitro* cytostatic activity of CPT-TML-ArB(OH)₂ NPDs, CPT-TML-Ar NPDs, CPT, and the lactonized TML group with regard to (A) A549 (cancer cells) and (B) NHDF-Neo (normal cells). These results are indicated as the mean \pm standard error ($n = 3$).

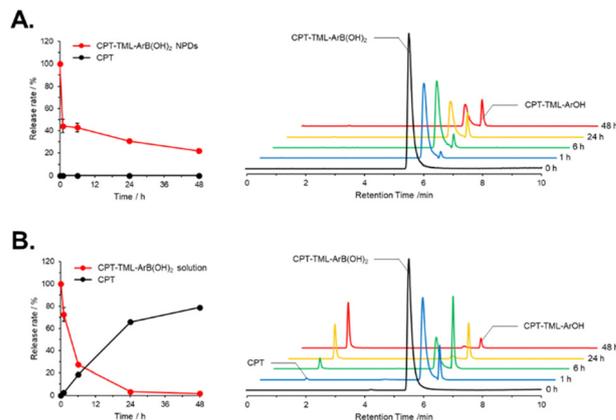
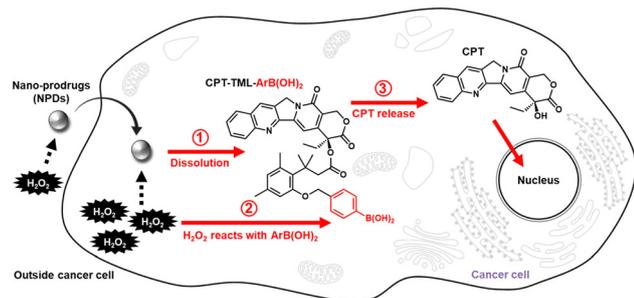


Fig. 4 Release kinetics and HPLC-MS/MS profiles of (A) CPT-TML-ArB(OH)₂ NPDs in water and (B) CPT-TML-ArB(OH)₂ in DMSO incubated with 100 μM H_2O_2 solution at 37 $^\circ\text{C}$. CPT-TML-ArOH is an intermediate in which H_2O_2 converted boronic acid to alcohol. The relative amounts were calculated for each sample based on the concentration of the CPT-TML-ArB(OH)₂ before incubation. These results are indicated as the mean \pm standard error ($n = 3$).

to a previous report,²⁶ esterase-catalysed hydrolysis of sterically hindered esters in CPT-TML was not possible. Moreover, CPT-TML-Ar did not release CPT after incubation with 100 μM H_2O_2 (ESI,† Fig. S3). When the CPT-TML-ArB(OH)₂ NPDs were incubated with 100 μM H_2O_2 solution for 48 h, CPT was not released, even though an intermediate in which boronic acid was converted to alcohol (CPT-TML-ArOH) was gradually detected. In contrast, CPT-TML-ArB(OH)₂ released about 80% CPT after 48 h of incubation of the CPT-TML-ArB(OH)₂ DMSO solution in the presence of a 100- μM H_2O_2 solution (Fig. 4B). Simultaneously, a lactonized TML group was detected (ESI,† Fig. S5), indicating that CPT-TML-ArB(OH)₂ released CPT *via* lactonization of the TML group after the conversion of boronic acid to alcohol by intracellular H_2O_2 . The results reveal that it is crucial for CPT-TML-ArB(OH)₂ NPDs to dissolve in cancer cells before responding to intracellular H_2O_2 for the NPDs to exhibit pharmacological effects (Scheme 4).

In this study, we synthesized a novel prodrug (CPT-TML) consisting of a TML group with ArB(OH)₂ on PG and CPT in a molecular design that releases drugs in response to intracellular H_2O_2 . After reprecipitation of CPT-TML, we fabricated NPDs that were controlled to approximately 100 nm, within the size range relevant to the improvement of tumour accumulation, and stably dispersed for one month in water. The fabricated CPT-TML NPDs with pharmacological effects against A549 cancer cells (with a high concentration of H_2O_2) and NHDF-Neo normal cells (with a low concentration of H_2O_2) exhibited clear cytostatic activity in A549 cells, while NHDF-Neo cells maintained over 50% cell viability even with the addition of 10 μM of CPT-TML NPDs. In the evaluation of the drug release kinetics of the CPT-TML to the H_2O_2 solution, the CPT-TML NPDs in water did not release CPT, whereas CPT-TML dissolved in DMSO could release approximately 80% of CPT after 48 h of incubation with a 100- μM H_2O_2 solution. In summary, to effectively release CPT from CPT-TML NPDs, the NPDs must





Scheme 4 Schematic representation of the proposed cancer intracellular CPT release mechanism from CPT-TML-ArB(OH)₂ NPDs by H₂O₂ in cancer cells; reactivity of H₂O₂-to-dissolved CPT-TML-ArB(OH)₂ (solid red line) is higher than that of H₂O₂-to-NPDs (dotted black line).

dissolve in cancer cells before responding to intracellular H₂O₂. In conclusion, we revealed the process by which NPDs react with H₂O₂ in cancer cells to release previously unknown drugs. For the further development of NPDs with high cancer selectivity, we must consider both the molecular design that can release drugs through tumour-specific triggers and the solubility of NPDs in cancer cells.

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Conflicts of interest

The authors declare that they have no competing financial interest or personal relationships that may have influenced the work reported in this study.

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