



Cite this: *Chem. Commun.*, 2024, 60, 6427

Received 9th May 2024,
Accepted 25th May 2024

DOI: 10.1039/d4cc02252a

rsc.li/chemcomm

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

Aki Shibata,^a Yoshitaka Koseki,^{*a} Keita Tanita,^a Showa Kitajima,^a Kouki Oka,^a Kiyotaka Maruoka,^a Ryuji Suzuki,^b Anh Thi Ngoc Dao^c and Hitoshi Kasai^{*a}

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 DDSs, 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^- , $\cdot\text{OH}$, 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).

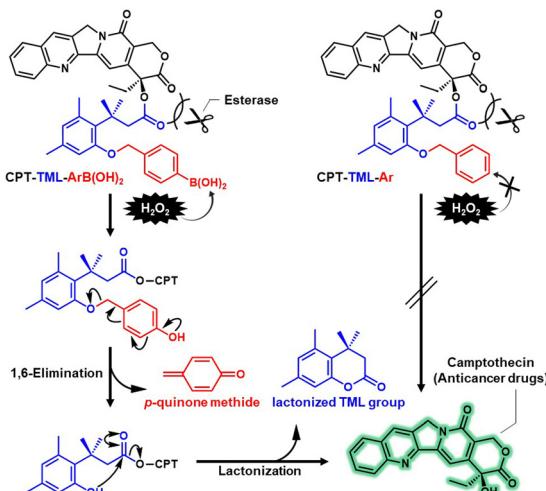
^a Institute of Multidisciplinary Research for Advanced Materials, Tohoku University 2-1-1 Katahira, Aoba-ku, Sendai-shi, Miyagi-ken 980-8577, Japan.
E-mail: koseki@tohoku.ac.jp, kasai@tohoku.ac.jp

^b National Institute of Technology, Sendai College, 48 Nodayama, Medeshima-Shiote, Natori-shi, Miyagi-ken 981-1239, Japan

^c Graduated School of Integrated Science and Technology, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki-shi, Nagasaki-ken 852-8521, Japan

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4cc02252a>

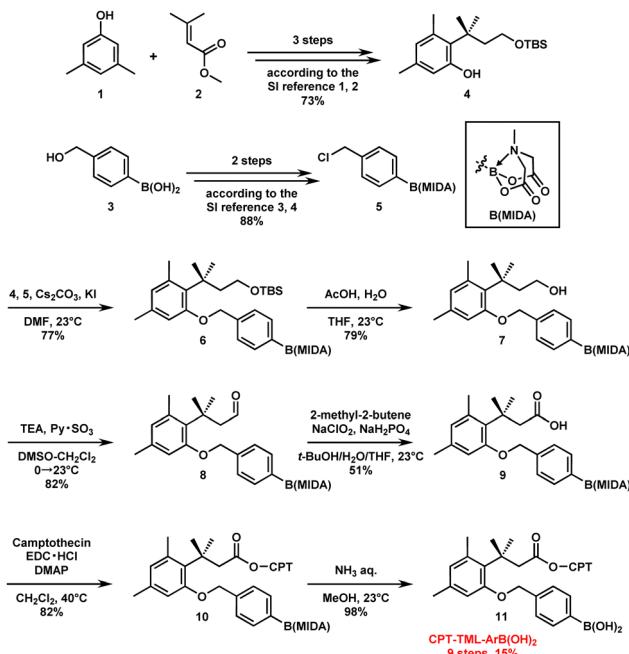
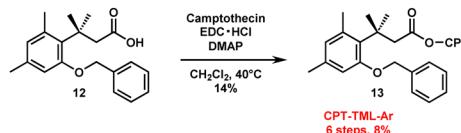




Scheme 1 Proposed CPT release mechanism from CPT-TML.

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 H₂O₂ levels, we quantified the H₂O₂ 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 H₂O₂ assay was employed with the BIOXYTEC® H₂O₂-560™ Assay Kit based on the intracellular Fenton reaction (ESI†). As a result, the H₂O₂ concentration in A549 cells was presented as 0.13 nmol/10⁷ cells. The volume of A549 cells was estimated to be 1670 μm³ per cell,³⁰ which would imply a H₂O₂ concentration of approximately 8.1 μM/10⁷ cells. The H₂O₂ concentration in A549 cells was about 5-fold higher than that in NHDF-Neo cells (0.028 nmol/10⁷ cells) (Fig. 2).

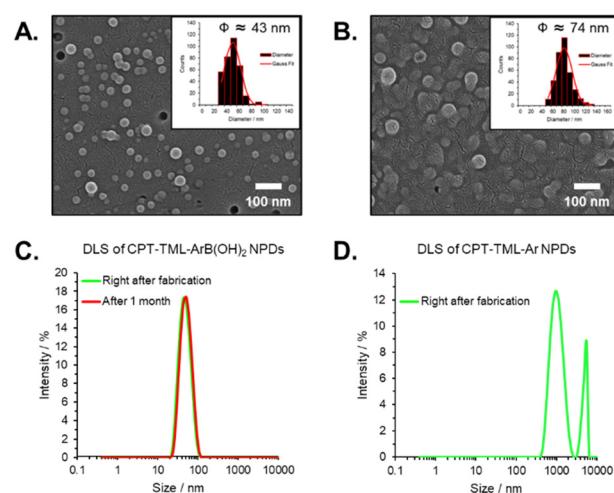
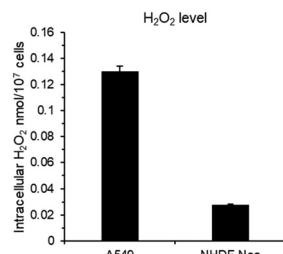


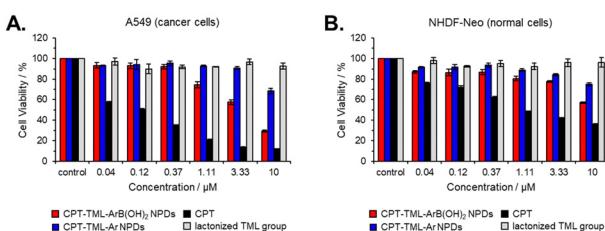
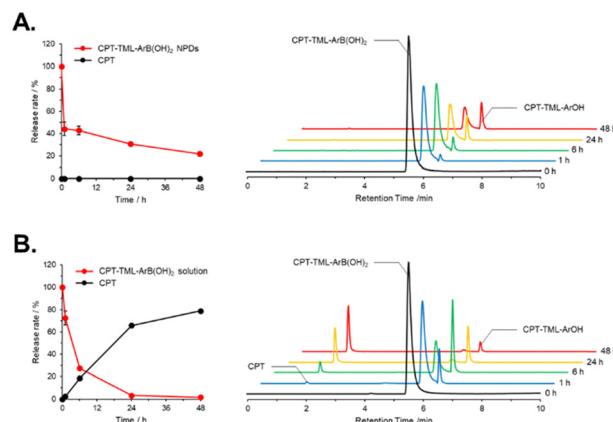
Fig. 1 SEM images of (A) **CPT-TML-ArB(OH)₂** **NPDs** and (B) **CPT-TML-Ar** **NPDs**. Inset: Related diameter distribution (Gaussian fitting in red); dispersion stability of (C) **CPT-TML-ArB(OH)₂** **NPDs** and (D) **CPT-TML-Ar** **NPDs**; DLS measurement immediately after fabrication (green line)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



Fig. 2 Intracellular H₂O₂ level of A549 cells and NHDF-Neo cells.

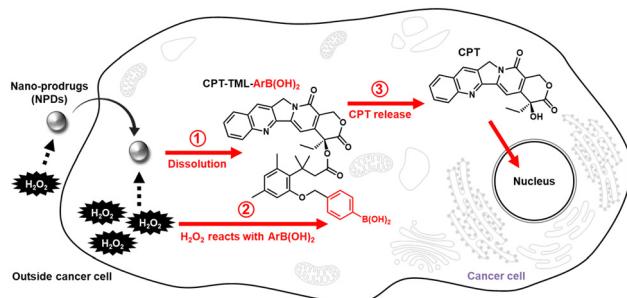
Based on the differences in intracellular H₂O₂ concentrations between cancer and normal cells, we evaluated the *in vitro* cytostatic activities of **CPT-TML-ArB(OH)₂ NPDs** and **CPT-TML-Ar NPDs** in A549 and NHDF-Neo cells. **CPT** and lactonized **TML** groups (substituents after the release of **CPT** 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 μ M 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 H₂O₂. 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 μ M except for **CPT** (IC₅₀ of **CPT** = 0.98 μ M) (Fig. 3B). In particular, the pharmacological effects of the **CPT-TML-ArB(OH)₂ NPDs** were high only in cancer cells treated with high H₂O₂ concentrations. To sum up, the **CPT-TML-ArB(OH)₂ NPDs** are thought to exhibit pharmacological effects in response to intracellular H₂O₂.

Finally, to verify the prodrug-to-drug conversion, **CPT-TML-ArB(OH)₂** and **CPT-TML-Ar** were incubated with 100 μ M H₂O₂ (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 μ M H₂O₂ solution at 37 °C. **CPT-TML-ArOH** is an intermediate in which H₂O₂ 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 μ M H₂O₂ (ESI,† Fig. S3). When the **CPT-TML-ArB(OH)₂ NPDs** were incubated with 100 μ M H₂O₂ 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- μ M H₂O₂ 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 H₂O₂. The results reveal that it is crucial for **CPT-TML-ArB(OH)₂ NPDs** to dissolve in cancer cells before responding to intracellular H₂O₂ 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 H₂O₂. 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 H₂O₂) and NHDF-Neo normal cells (with a low concentration of H₂O₂) exhibited clear cytostatic activity in A549 cells, while NHDF-Neo cells maintained over 50% cell viability even with the addition of 10 μ M of **CPT-TML NPDs**. In the evaluation of the drug release kinetics of the **CPT-TML** to the H₂O₂ 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- μ M H₂O₂ 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.

This work was supported by JSPS Grants-in-Aid for Scientific Research (No. 22H00328, No. 23K14316), Grant-in-Aid for JSPS Fellows (No. 23KJ0161), JST SPRING (Grant Number JPMJSP2114), the Cooperative Research Program of 'Network Joint Research Center for Materials and Devices', and the Research Program of 'Dynamic Alliance for Open Innovation Bridging Human, Environment and Materials' in 'Network Joint Research Center for Materials and Devices'.

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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