Synthesis and Analytical Evaluation of a CCB-4-*N*-Methylglucamine Complex with Enhanced Cellular Uptake in Breast Cancer Cells

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Abstract

Context: Boronic acid as a medicinal agent, owing to low aqueous solubility and cellular uptake has been restricted in many curcumin analogues with potential anticancer activity.

Aims: This study aimed to evaluate the cytotoxicity effect of CCB-4 [a curcumin derivative contain boron with a methylpiperidone core], CCB-4-*N*-methylglucamine (CCB-4 MG) complex in breast cancer cell lines, and also to enhance the aqueous solubility of CCB-4 by complexation with *N*-methylglucamine (MG) and then to evaluate its cellular uptake and microdistribution in MCF-7 cells.

Methods and Material: CCB-4 was synthesized using Claisen–Schmidt condensation and was fully characterized by IR, HR-MS, 1D/2D NMR. Complex formation of CCB-4 with MG were achieved through boronic acid–diol interaction. The physicochemical properties, aqueous solubility, cytotoxicity, inhibitions of colony formation and cellular uptake, retention boron and intracellular microdistribution were determined in MCF-7 breast cancer cells. The collected data were quantified using one-way ANOVA

Results: CCB-4 was synthesized with 70% yield and confirmed structural integrity. The CCB-4 MG complex provided a homogeneous system with enhanced colloidal stability and 25.14-fold increase in aqueous solubility over free CCB-4. The CCB-4 MG complex had greater cytotoxic activity, substantial inhibition of colony formation, high cellular boron uptake and long-term retention in the intracellular environment and was predominantly located in the cytoplasm of MCF-7 cells.

Conclusions: MG significantly increased the solubility, cellular uptake and anticancer efficacy of CCB-4, suggesting its potential to optimize therapeutic performance of curcumin analogues based on boronic acid in breast cancer therapy.

Key-words: CCB-4, breast cancer, *N*-methylglucamine, cellular uptake, MCF-7

Introduction

Boronic acid-functionalized curcumin analogs have attracted greater attention as potential anticancer agents due to their flexible structures, enhanced stability, and ability to modulate various cancer-related signalling pathways.[1,2] Several new curcumin monoketone derivatives bearing boron were designed and synthesized in recent years, and they showed better activity against breast cancer cells than free curcumin, demonstrating that it is necessary to modify the reactive site of the central keto-moiety to achieve good biological activity.[3-8] Of these, CCB-4, which contains a central methylpiperidone ketone and a boronic acid functional group, has been shown to exhibit potent anticancer activity by inhibiting proteasome–dependent apoptosis.[9-11]

Although CCB-4 is an effective agent, its clinical advancement is limited by its low water solubility and inadequate cell permeability, which are standard features of boronic acid-based compounds. Complexation with *N*-methylglucamine (MG), a hydrophilic amino sugar that forms stable boron–sugar complexes, has been identified as an efficient approach to increase the solubility of boronic acid compounds.[12-14] Whereas *N*-methylglucamine has been utilized previously for the enhancement of aqueous solubility in a variety of boronic acid containing agents, the physicochemical and biological implications of this approach are remarkably scaffold dependent. Fundamentally, CCB-4 was shown to possess a unique structure compared to previously reported boronic acid compounds containing a diboronic curcumin framework with a methylpiperidone core. Hence it cannot be inferred that complexation with MG would provide comparable enhancement in solubility, boron transport across cell membranes and hence anticancer activity. These aspects remain unexplored. The possibility whether could MG complexation simultaneously enhance the physicochemical properties, intracellular boron accumulation and anticancer activity of CCB-4 has not yet been explored. In addition, although CCB-4 is known as an active anticancer compound, no report has described its complete structural characterization or assessed its improved physicochemical properties through complexation techniques. This is a bottleneck that warrants further development and optimization of the compound.

Herein, we resynthesized CCB-4, fully analytically characterized it, and prepared a CCB-4 MG complex to address its poor solubility and cellular uptake. Anticancer activity of the complex was further studied in breast cancer (MCF-7) cells as a model and demonstrated to be superior. The results will be useful to obtain boron–containing curcumin analogues with improved physicochemical and biological properties and rationally design more effective boron–based anticancer agents in the future.

Material And Methods

All reagents and solvents utilized in this study were of analytical grade unless stated otherwise. NMR spectra were acquired using a JEOL JNM-ECZR (500 MHz for ^{1}H and 125 MHz for ^{13}C). The HRMS data were recorded on Bruker ESI-TOF-MS micrOTOF II instrument with sodium formate as the calibration standard. Purity analysis of compound CCB-4 and CCB-4 MG were determined through

HPLC analysis utilizing a COSMOSIL C_{18}-MS-II column (5µm, 4.6 x 250 mm). A linear gradient of water and acetonitrile was employed, with detection carried out using a UV lamp over a 35 minute runtime.

Synthesis of CCB-4

The synthesis of CCB-4 followed the approach described by previous publication with slight modification (9). 1-methyl-4-piperidone (0.005 mol) and 4-formylphenyl boronic acid (0.01 mol) were dissolved in 10 mL ethanol, followed by the addition of 2.5 mL NaOH 5 N solution, which was then stirred at RT overnight. The reaction mixture was quenched with HCl 1 N until pH 7, and the solid product was filtered. After washing by ethanol, the final product was dried and collected without further purification. Complete characterization details, as well as full analytical data, were presented in the Supplementary Information.

Preparation of CCB-4 in Complex with N-methylglucamine (CCB-4 MG)

Complexation of CCB-4 with *N*-methylglucamine was prepared following the previous method with slight modification.[5] The *N*-methylglucamine (0.03 mol) and CCB-4 (0.01 mol) was dissolved in water. The mixture was stirred for 1 hour until solution of CCB-4 MG complex forms.

Physicochemical and stability evaluation of the CCB-4 MG

The physicochemical evaluation of CCB-4 and CCB-4 MG in water solution consist of particle size measurement (PSA), zeta potential (ZP), and polydispersity index (PDI) using Malvern Zetasizer Nano ZS90. Samples were analyzed in triplicate at 25 ºC. The morphology of CCB-4 and CCB-4 MG in water solution was further examined using transmission electron microscopy (TEM). The solubility of CCB-4 was compared with that of the CCB-4 MG complex. A sample (10 mg) was dissolved in 1 mL water by stirring at 200 rpm for 24 h. The samples were centrifuged and the supernatant read at the maximum wavelength (λ) for each (CCB-4 and CCB-4 MG, 336 nm and 345 nm, respectively) using a UV–Visible spectrophotometer (Shimadzu 1201). The interaction between CCB-4 and *N*-methylglucamine was also investigated using Fourier Transform Infrared (FTIR) spectroscopy for functional group analysis. FTIR spectra of free CCB-4 and the CCB-4 MG complex were taken between 4000 and 400 cm^{-1} by the KBr pellet method. Comparison of the spectra was made to visualize the variation in characteristic absorption bands after complexation, especially within O-H stretching region as well as fingerprint region due to B-O, B-O-C and C-O and C-N vibrations.

To evaluate the stability of the CCB-4 MG complex under physiological conditions, PBS (pH 7.4) was used to incubate the CCB-4 MG complex at 37 °C for 0, 1, 2, 4, 8, 12, 24, 48 and 72 hours, and then measured by UV–Vis spectra absorbance at λmax as a function of time to monitor spectral stability. See, Supplementary Information for complete data.

Culture of cancer cells

MCF-7 cells were maintained at 37 ° C in a CO_2 incubator using DMEM supplemented with 10% FBS, along with 150 µg/mL penicillin and 150 µg/mL streptomycin. For passage, the cells were detached using trypsin-EDTA.

Cytotoxicity evaluation

Cytotoxicity was evaluated using the MTT assay following previous protocol. MCF-7 cells ($7x10^3$ cells/well) were plated in 96-well plate. After overnight, the cells were exposed to varying concentrations of CCB-4 and CCB-4 MG for 24 hours. Subsequently, 100 µL of MTT reagent was added to each well and incubated for 3 hours. After adding SDS stopper reagents, the absorbance was recorded using a microplate reader and converted into cell viability to determine IC_{50} value. GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) was used to analyze the IC_{50} values using nonlinear regression analysis. Normalized cell viability (%) and log (compound concentration) were plotted and fitted using the log[inhibitor] vs. normalized response–Variable slope model.

Analysis of boron uptake

MCF-7 cells were plated on 24-well plates (2×10^5 cells/well) and pre-cultured for 24 h before CCB-4 or CCB-4 MG treatment in serum-containing medium for 24 h. Post intervention, cells were washed with PBS, aspiration of the medium was conducted and boron extraction was performed using lysis buffer (0.05% Tween-20). The content of total protein was measured by BCA assay. For boron detection, cell lysates and standard solutions of boron were combined with solution of 2-(2-hydroxyphenyl)pyridine (boron sensor), followed by incubation at 37 °C for 1 h. Fluorescence was measured with a microplate reader at Ex 360 nm/Em 480 nm. Data were presented as protein-normalized fluorescence intensity of cellular uptake.

Study of Boron efflux

MCF-7 cells were seeded 2×10^5 cells/well in a 24 well plate. The medium was then replaced with CCB-4–MG in fresh medium after incubation overnight. After 24 h incubation, the medium was changed to fresh medium containing no compound, then the cells were collected after 1, 2, 3 and 4 h of medium replacement with 0.05% Tween 20. Normalization was performed using a BCA protein assay to determine the total concentration of protein. The intracellular boron levels were subsequently measured by fluorescence microplate reader using 2-(2-hydroxyphenyl)pyridine (HPP), a fluorescent boron sensor. Next, cell lysates were combined to the HPP boron sensor and incubated at 37 °C for 1 h when fluorescence intensity was recorded via a Fluoroskan Ascent fluorescence microplate reader (Thermo Scientific, USA). Concentrations of boron were determined according to the calibration curve prepared for standard solutions.

Evaluation of boron microdistribution

Cells were plated at a density of 1.0 x 10^5 cells in 35-mm glass-bottom dishes and cultured for overnight. The medium was then replaced with fresh medium containing 3.75 µM CCB-4 and CCB-4 MG for 24 hours. On the following day, DRAQ5 solution was introduced to the cells at a final

concentration of 5 µM in DMEM, and 2-(2-hydroxyphenyl) pyridine (boron sensor) solution was added at a final concentration of 0.1 mM in DMEM and incubated for 20 minutes. The intracellular distribution of the boron compound was examined using BZ-X810 Keyence.

Statistical analysis

All experiments were independently performed three times, and data are presented as mean ± standard deviation (SD). Statistical comparisons between two groups were performed using Student's *t*-test. IC_{50} values were determined by nonlinear regression analysis using GraphPad Prism version 9.0. A p-value < 0.05 was considered statistically significant.

Results

Synthesis of CCB-4 and preparation of its complex with N-methyl glucamine

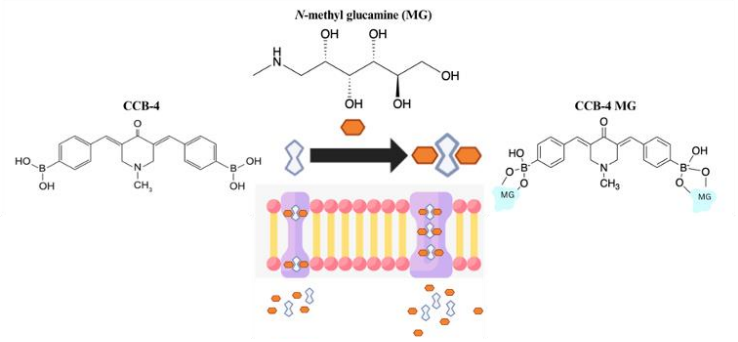
CCB-4 was prepared by Claisen–Schmidt condensation between 4-formylphenyl boronic acid and 1-methylpiperidin-4-one under basic conditions to obtain yellow powder (70%). HPLC examination detected a purity of 97.91%, retention time was 18.87 min (Supplementary file 1). The molecular formula, $C_{20}H_{21}B_2NO_5$, was established by ESI-HRMS (m/z $[M+H]^+$ = 378.1681 (calcd for $C_{20}H_{21}B_2NO_5$: 378.1679). FT-IR spectra The typical absorption bands of O-H, C=O and aromatic C=C in FT-IR spectra for B-O and C-N are shown emphasized (Supplementary file 2). ^{1}H- and ^{13}C-NMR The symmetrical nature of the molecule was confirmed by ^{1}H- and ^{13}C-NMR spectra, which displayed signals typical of a symmetrical structure. Corroborative proton and carbon assignments were provided by HSQC, COSY and HMBC analyses (Table 1) (Supplementary file 3 – file 7).

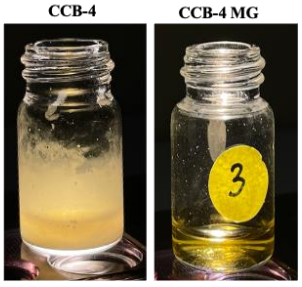
CCB-4 complexed with *N*-methylglucamine (MG) in a molar ratio of 1:3 yielded clear homogeneous solution. The CCB-4–MG complex had a small particle size, relatively low polydispersity index (PdI= 0.19 ± 0.01), and negative zeta potential (-43.58 ± 4.94 mV), implying the high colloidal stability[15-18] (Figure 1E). The TEM images showed that particles were uniformly distributed without aggregate formation (Figure 1D). *N*-methylglucamine complexation greatly enhanced the aqueous solubility. The solubility of CCB-4 MG complex was increased by 25.14 ± 1.02 fold relative to that of native CCB-4 (Figure 1E). Changes of functional groups characteristics CCB-4 by FTIR analysis after complexations with *N*-methylglucamine (Figure 1F). The FTIR spectrum of the CCB-4 MG complex exhibited multiple changes in the characteristic absorption bands as compared with those of free CCB-4, especially in the O-H stretching region and the fingerprint region (1500-400 cm^{-1}). The O-H stretching band shifted from **3351 cm^{-1}** in free CCB-4 to **3233 cm^{-1}** in the CCB-4 MG complex. A similar shift toward a lower wavenumber was also observed for the C=O stretching vibration, from **1677 cm^{-1}** to **1659 cm^{-1}**. In contrast, the aromatic C=C stretching band shifted from **1579 cm^{-1}** to **1595 cm^{-1}**. Furthermore, the absorption bands assigned to the boronic acid B-O vibration in free CCB-4 (1350 **cm^{-1}**) exhibited changes in the CCB-4 MG complex, giving rise to a more complex absorption pattern at **1312, 1283, and 1212 cm^{-1}** within the fingerprint region.

Table 1. 2D-NMR spectra data of CCB-4

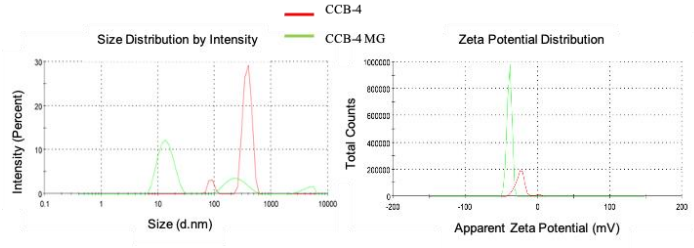
Posisi	^{13}C-NMR δ_H (ppm)	^{1}H-NMR δ_H (Integral,mult., *J* Hz)	COSY	^{1}H-^{13}C HSQC	^{1}H-^{13}C HMBC
N-CH_3	42.370	2.989 (3H, s)	-	N-CH_3	N-CH_3-C2/6
OH	-	3.424 (4H, s, overlapping with residual H_2O, B-OH)	-	-	-
2/6	53.332	4.677 (4H, s)	-	H2/6-C2/6	H2/6-C3/5; C4
3/5	127.405	-	-	-	-
4	181.513	-	-	-	-
7	139.624	7.894 (2H, s)	-	H7-C7	H7-C3/5; C1`; C2`/6`; C4
1`	136.457	-	-	-	-
2`/6`	129.776	7.506 (4H, d, 7.5)	H2`/6`-H3`/5`	H2`/6`-C2`/6`	H2`/6`-C3`/5`; C1`; C4`
3`/5`	134.575	7.931 (4H, d, 7.5)	H3`/5`-H2`/6`	H3`/5`-C3`/5`	H3`/5`-C2`/6`; C1`; C4`
4`	134.911	-	-	-	-

A

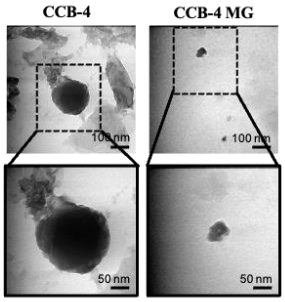




C



D



E

Compound	Characterization				
	Particle size (nm ± SD)	PdI (PdI ± SD)	Zeta potential (mV± SD)	Mean solubility (mg ± SD)	Fold solubility increase (FI ± SD))
CCB-4	890.04 ± 26.45	0.71 ± 0.04	-24.08 ± 0.66	0.26 ± 0.05	-
CCB-4 MG	27.57 ± 2.13	0.19 ± 0.01	-43.58 ± 4.94	6536 ± 0.123	25.14 x ± 1.02

F

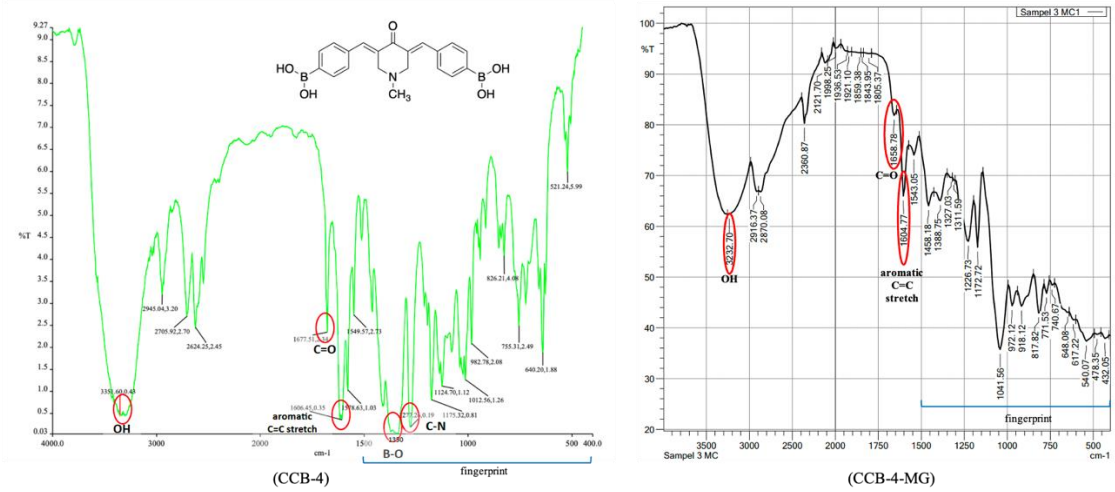


Figure 1. Physicochemical properties of CCB-4 MG. (A) Schematic concept of CCB-4 complexation with *N*-methylglucamine and its cell uptake mechanism. (B) CCB-4 and CCB-4 MG under visible light in water solution. (C) Left:Particle size distribution, Right: Zeta potential of CCB-4 and CCB-4 MG evaluated by dynamic light scattering (n=3). (D) TEM image of CCB-4 and CCB-4 MG. (E) Particle size profile and solubility of CCB-4 and its complexes using *N*-methylglucamine (CCB-4 MG). (F) Comparative FTIR spectra of free CCB-4 and the CCB-4 MG complex, showing changes in the characteristic absorption bands following complexation.

Cytotoxicity of CCB-4 MG toward MCF-7

Our compounds have been investigated for effectiveness as boron carriers and/or anticancer therapies through assessing their cytotoxic effects. When tested against MCF-7, both CCB-4 and CCB-4 MG showed a cytotoxic concentration-dependent impact; nevertheless, CCB-4 MG revealed more effective cytotoxicity than CCB-4 (Figure 2A) (Table 2). Additionally, both compounds demonstrated low selectivity index (SI) and cytotoxicity against Vero cells, indicating selective cytotoxicity[19] (Figure 2A) (Table 2). At concentrations ranging from 0.5-1 µM, CCB-4 showed a progressive increase in cell viability after treatment withdrawal in the washout trials (Figure 2B). Interestingly, even at a lower dose of 0.5 µM, CCB-4 MG maintained its irreversibility and showed a more powerful prolonged cytotoxic effect (Figure 2B). The clonogenic test provides additional support for the findings, demonstrating that CCB-4 MG therapy was superior to CCB-4 due to a significant decrease in colony formation (Figure 2C). Even at low doses, CCB-4 MG seemed to have more cytotoxicity overall, indicating that complexation with *N*-methylglucamine increases cytotoxic efficacy against MCF-7 cells.

Boron uptake, efflux, and microdistribution of CCB-4 in MCF-7

Boron uptake, efflux and intracellular distribution of CCB-4 MG were determined following assessment of the cytotoxicity to assess cellular transport and retention. The detection of intracellular boron was performed using the fluorescent boron sensor HPP and measured with a fluorescence microplate reader

after the exposure to CCB-4 MG. The cellular boron uptake by MCF-7 cells linearly increased with a 20 min incubation, as compared to the control, decelerated by *N*-methylglucamine (Figure 3A). The efflux studies showed that intracellular boron content was maintained up to 4 h after the preincubation of 24 h (Figure 3B). Fluorescence imaging revealed that CCB-4 MG showed similar preferential cytoplasmic localization with L-BPA (Figure 3C).

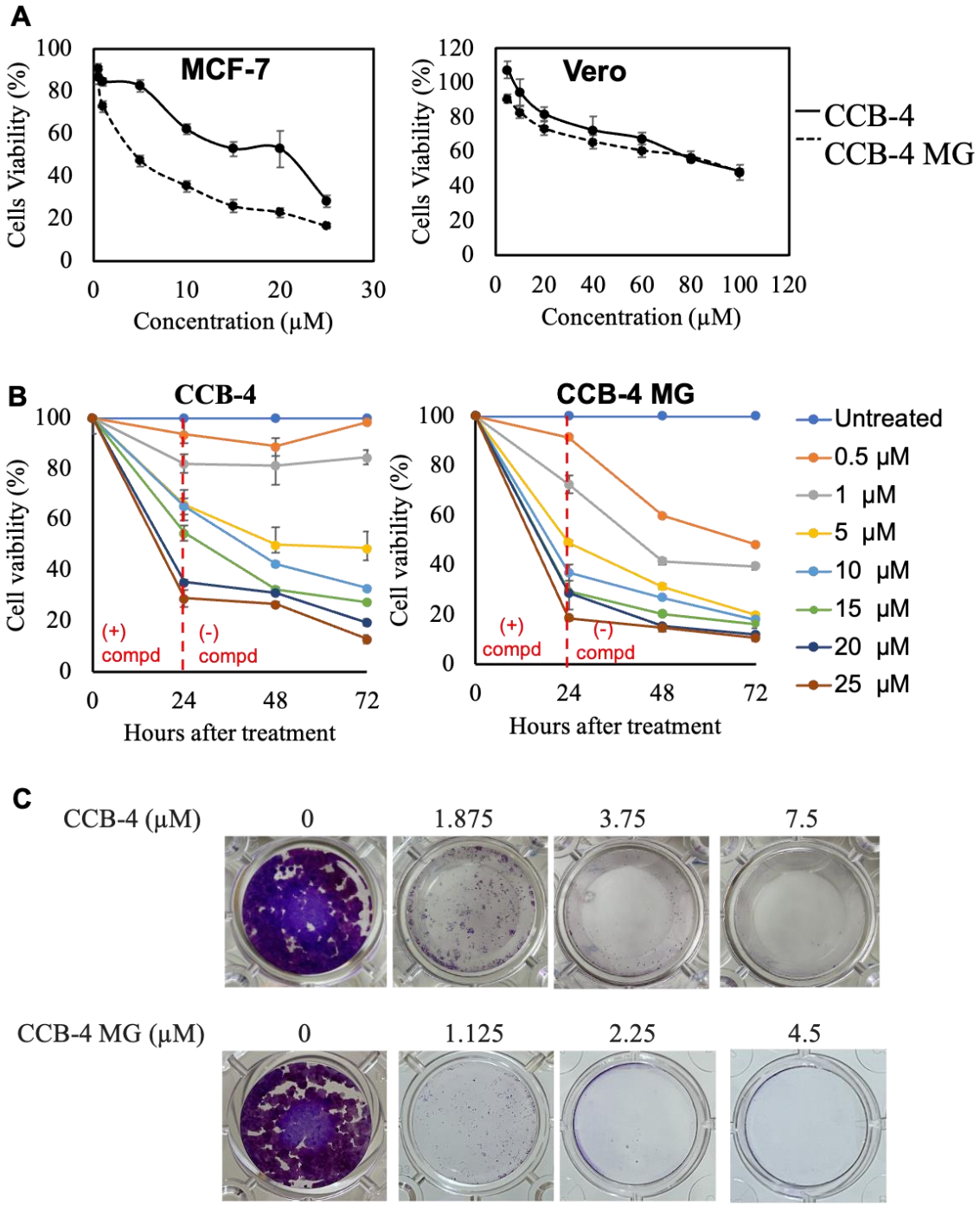


Figure 2. Cytotoxic profile of CCB-4 MG. (a) Dose-dependent cytotoxicity of CCB-4 MG toward MCF-7 and Vero. (b) Irreversible cytotoxic effect of CCB-4 MG toward MCF-7 cells upon withdrawal after 24 hours. (c) Inhibition of colony formation by CCB-4 MG toward MCF-7 cells. All data were expressed as mean ± SD (n=3).

Table 2. IC_{50} value and selectivity index of CCB-4 MG toward MCF-7 and Vero Cells

Compound	IC_{50} (μM) ± SD		Selectivity Index
	MCF-7	Vero	
CCB-4	15.35 ± 1.52	85.93 ± 5.21	5.60 ± 1.18
CCB-4 MG	9.13 ± 0.39	90.85 ± 1.39	9.95 ± 2.79

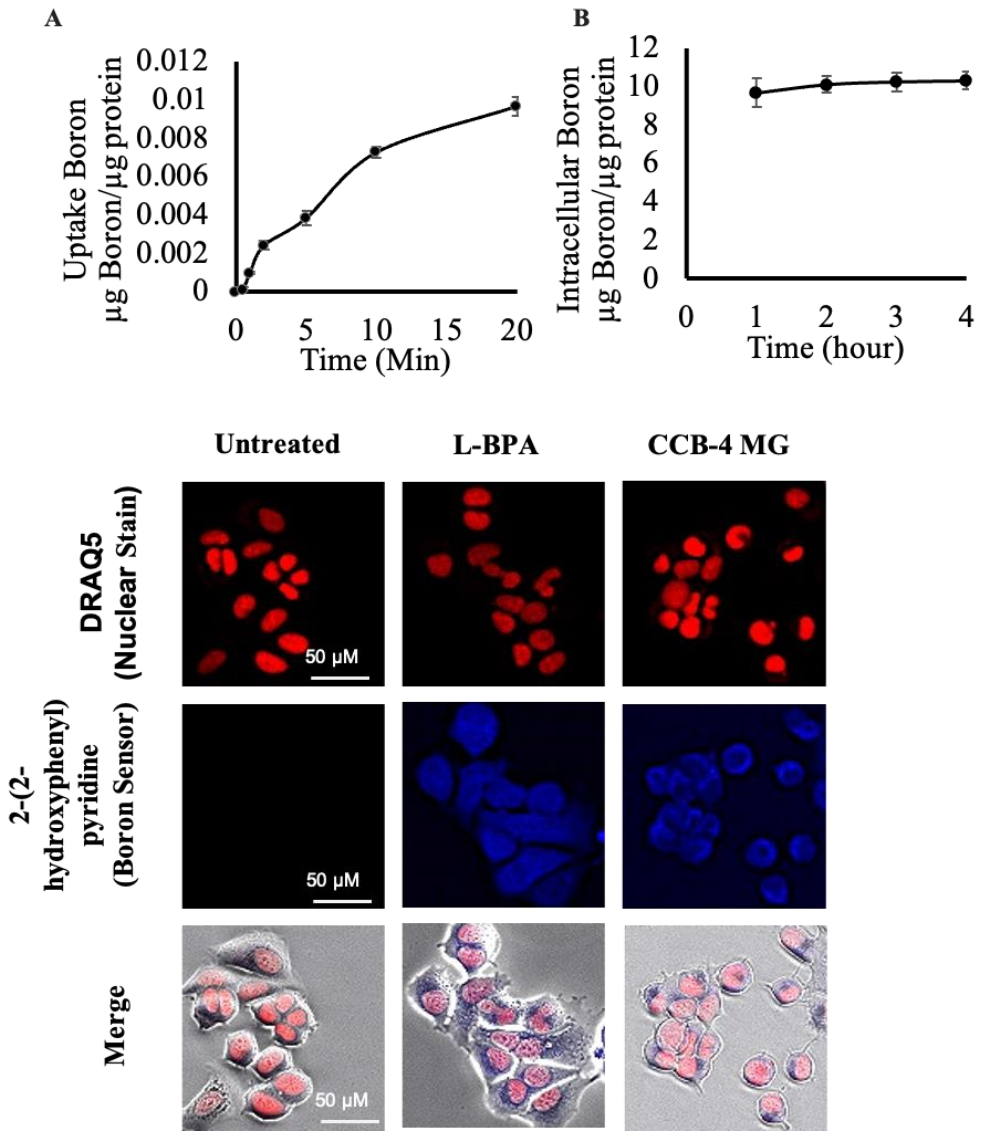


Figure 3. Boron accumulation of CCB-4 MG in MCF-7. (a) Boron uptake profile of CCB-4 MG after incubation for 0.5, 1, 2, 5, 10, and 20 minutes. (b) Efflux study in MCF-7 after treatment with CCB-4 MG evaluated by the fluorescent boron sensor HPP and measured with a fluorescence microplate reader. All data were expressed as mean ± SD (n=3). (c) The microdistribution profile of CCB-4 MG in MCF-7 cells.

Discussion

Particularly in treating breast cancer, the discovery of curcumin analogues with boronic acid has gained significant interest as possible anticancer drugs. The use of CCB-4 is limited by its poor water solubility, despite its intense cytotoxic action.[9-11, 20] Researchers investigated the solubility and cellular absorption of CCB-4 by complexing it with MG to address these challenges.[13-14] This study developed and assessed CCB-4 MG, focusing on its cytotoxicity, cellular uptake, microdistribution, and physicochemical characteristics in MCF-7 breast cancer cells.

In this study, we successfully synthesized and manufactured CCB-4 MG, which has better physicochemical properties than CCB-4. Similar to PGB-0-sor, CCB-4 MG appeared as a transparent yellow aqueous solution, but it had a smaller particle size, reduced PdI, and a higher zeta potential.[5] The results showed that MG improved the solubility and dispersion of boronic acid-containing curcumin analogues more effectively than fructose and sorbitol. Additionally, FTIR analysis supported the formation of the CCB-4 MG complex by indicating changes in the fingerprint region along with O-H, C=O and aromatic C=C stretching bands as proof of changes in chemical environment around the boronic acid moieties post-complexation. Compared to CCB-4, the B-O stretching bands of free CCB-4 were broadened and decreased in intensity in the CCB-4-MG complex, indicating that boronate ester (B-O-C) bonds form through interactions between CCB-4 and *N*-methylglucamine. This type of spectral overlap between B-O, B-O-C, C-O and C-N vibrational modes account for more complicated spectral profiles in the fingerprint region. Altogether, the spectral changes support further evidence for forming CCB-4 MG complex.

The stability of complex CCB-4 MG was further investigated under physiological conditions, because of the boronic acid-diol interaction is reversible. We found that incubation in PBS (pH 7.4) at 37 °C resulted in no appreciable change in the UV-Vis spectral profile or absorbance at λmax (Supplementary file 8-file 10), indicating that throughout an incubation period of up to 72 h, the complex was stable enough. This validates the use of this CCB-4 MG complex for further biological testing.

Regarding cytotoxicity, CCB-4 MG was more effective than free CCB-4 in MCF-7 cells. Comparative examination of cytotoxic activities revealed that CCB-4 MG had the highest cytotoxicity among the studied curcumin derivatives with boronic acid.[21–25] CCB-4 MG exhibited an irreversible cytotoxic effect, shown by the cells' inability to recover even after the compound was withdrawn, while CCB-4 did not have this effect. The significant inhibition of colony formation further demonstrated CCB-4 MG's ability to prevent cancer cell growth and long-term survival. The survival and adaptability of any remaining tumor cells after treatment may lead to colony formation and potential cancer recurrence.[26] Interestingly, chemotherapy may promote metastatic progression and local tumor recurrence by increasing the tumorigenic potential of near-death cells (NDC).[27] Therefore, the persistent cytotoxic effect of CCB-4 MG may be crucial for achieving optimal anticancer activity.

Fluorescence-based boron analysis using HPP and a fluorescence microplate reader showed enhanced cellular absorption of boron and the long-term stability of CCB-4 MG. Compared to PGB-0-F and PGB-0-Sor, CCB-4 MG had a more linear and prolonged uptake profile in MCF-7 cells, with better retention efficiency over time.[28] CCB-4 MG maintained a consistent level of intracellular boron even after medium replacement, unlike PGB-0-Sor, which showed a rapid initial absorption followed by a decline in intracellular boron concentration.[28] Fluorescence imaging supported these results, demonstrating that CCB-4 MG primarily localized in the cytoplasm like L-BPA.[28] This finding showed that MG complexation significantly improves boron retention, which is key to maximizing therapeutic benefits. The increased cytotoxic activity of CCB-4–MG complex as observed in this study can be attributed to high aqueous solubility, cellular uptake and retention time inside the cell. Thus our current study cannot ascertain whether MG complexation also affects the cell-intrinsic pharmacodynamic properties of CCB-4, for example its molecular target interactions or proteasome inhibitory activity. Future studies will be necessary to fill in these details.

Preparation of the complexation process is simple and feasible for scale-up. However, additional studies are needed to elucidate if the MG complexation impacts solely on cell uptake or further also on the epigenetics and molecular mechanisms leading to CCB-4 intracellular pharmacodynamics. These analyses will further facilitate the clinical implementation of CCB-4 MG as a novel boron-containing compound for anticancer treatment.

CONCLUSIONS

CCB-4 MG showed significantly enhanced water solubility, better cellular uptake, and long-term retention in MCF-7 cells compared with the parent CCB-4, leading to a more potent cytotoxic concentration effect and inhibition of colony formation. These results indicate that *N*-methylglucamine complexation is a compatible approach to enhance the physicochemical properties and anticancer activity of boronic acid–containing curcumin analogues.

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Conceptualization: RA Susidarti, E Meiyanto, RI Jenie, E Lukitaningsih, M Kirihata; methodology: RY Utomo, RI Jenie, E Lukitaningsih, M Kirihata; data curation: NPL Laksmiani, RY Utomo, RI Jenie;

formal analysis: NPL Laksmiani, RY Utomo, RI Jenie, E Lukitaningsih, M Kirihata; investigation: NPL Laksmiani, RY Utomo, RI Jenie; supervision: RA Susidarti, E Meiyanto, RI Jenie, RY Utomo, E Lukitaningsih, M Kirihata; validation: RA Susidarti, RI Jenie, Y Suenaga, RY Utomo, NPL Laksmiani; writing-original draft: NPL Laksmiani; writing-review and editing: RA Susidarti, RY Utomo, RI Jenie, E Lukitaningsih, M Kirihata.

Conflict of interest

All authors declare no conflict of interest

AI declaration

This manuscript was prepared without the aid of any AI-assisted technologies.

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