Enhanced Antitumor Effects of Novel Intracellular Delivery of an Active Form of Menaquinone-4, Menahydroquinone-4, into Hepatocellular Carcinoma

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Abstract

Reduced cellular uptake of menaquinone-4 (MK-4), a vitamin K2 homolog, in human hepatocellular carcinoma (HCC) limits its usefulness as a safe long-term antitumor agent for recurrent HCC and produces des-γ-carboxy prothrombin (DCP). We hypothesized that effective delivery of menahydroquinone-4 (MKH), the active form of MK-4 for γ-glutamyl carboxylation, into HCC cells is critical for regulating HCC growth, and may enable it to be applied as a safe antitumor agent. In this study, we verified this hypothesis using menahydroquinone-4 1,4-bis-N,N-dimethylglycinate hydrochloride (MKH-DMG), a prodrug of MKH, and demonstrated its effectiveness. Intracellular delivery of MKH and subsequent growth inhibition of PLC/PRF/5 and Hep3B (DCP-positive) and SK-Hep-1 (DCP-negative) cells after MKH-DMG administration were determined and compared with MK-4. The activity of MKH-DMG against tumor progression in the liver alongside DCP formation was determined in a spleen–liver metastasis mouse model. MKH-DMG exhibited greater intracellular delivery of MKH in vitro (AUC0–72 hour of MKH) and increased growth-inhibitory activity against both DCP-positive and DCP-negative HCC cell lines. The phenomena of MKH delivery into cells in parallel with simultaneous growth inhibition suggested that MKH is the active form for growth inhibition of HCC cells. Cell-cycle arrest was determined to be involved in the growth inhibition mechanisms of MKH-DMG. Furthermore, MKH-DMG showed significant inhibition of tumor progression in the liver, and a substantial decrease in plasma DCP levels in the spleen–liver metastasis mouse model. Our results suggest that MKH-DMG is a promising new candidate antitumor agent for safe long-term treatment of HCC. Cancer Prev Res; 8(2); 1–10. ©2014 AACR.

Introduction

Recent advances in the treatment of hepatocellular carcinoma (HCC) have significantly improved. Nevertheless, the overall survival rate remains unsatisfactory because of the frequent recurrence of HCC even after curative treatment (1). The development of an effective antitumor agent against recurrent HCC is considered to be important for improving HCC prognosis. An alternative approach may be to find a clinically available compound that also exhibits antitumor activity, for which the safety of long-term administration has been demonstrated. Numerous studies have indicated that menaquinone-4 (MK-4), a vitamin K2 homolog, may play a role in controlling the growth of HCC in vitro and in vivo (2–7). MK-4 has been widely used for osteoporosis treatment, and its long-term safety has been confirmed (8–11). In small-scale clinical studies, MK-4 suppresses the de novo development of HCC in cirrhotic patients (12), and suppresses the recurrence of HCC after surgical resection or ablation therapy (13). On the basis of these findings, MK-4 would be an ideal adjuvant agent if it could reduce HCC recurrence by preventing de novo carcinogenesis or suppressing tumor growth. However, a recent larger scale, double-blind, randomized, placebo-controlled trial demonstrated that treatment with MK-4 failed to exert any inhibitory effects on the cumulative recurrence of HCC (14).

The levels of K vitamins in HCC tissues have been found to be consistently lower than those in the surrounding normal liver tissue and, in particular, menaquinone homologs were severely decreased in tumor tissue (15). Furthermore, hepatocytes from diethylnitrosamine-induced liver nodules exhibited a significantly lower rate of MK-4 uptake compared with normal hepatocytes (16). Des-γ-carboxy prothrombin (DCP), an abnormal prothrombin that is not completely carboxylated, is a well-recognized HCC-specific tumor marker (17), and a predictor of vascular invasion, metastasis, and tumor recurrence (18). Recent studies have revealed that DCP functions as a growth and metastasis factor; it may play a significant role in cancer progression (19–23) and partly contributes to the poor prognosis of HCC (24). Previous reports demonstrated that tumor vitamin K content has a critical role in DCP formation (15, 25–27).
Menahydroquinone-4 (MKH), the fully reduced form of MK-4, is a cofactor of γ-glutamyl carboxylase (GGCX), which converts glutamic acid (Glu) residues into γ-carboxyglutamic acid (Gla) residues of vitamin K-dependent proteins, such as prothrombin (28–30). GGCX is required for its activity and depends upon MKH generated mainly by vitamin K 2,3-epoxide reductase complex subunit 1-like-1 (VKORC1L1; ref. 31). A previous study showed that MKH availability regulates the rate of carboxylation (32). Thus, decreased MKH availability in HCC cells is a possible mechanism of DCP production in HCC. Taken together, it appears that deficient MKH utilization may be present in HCC tissues. We hypothesized that effective delivery of MKH into HCC cells is critical in regulating HCC growth and metastasis. However, MKH cannot be used as a therapeutic agent due to its easily oxidizable characteristics. We previously showed that the ester derivative of MKH, menahydroquinone-4 1,4-bis-N,N-dimethylglycinate hydrochloride (MKH-DMG; Fig. 1A), can effectively deliver MKH into normal liver without the reductive activation process of MK-4 to MKH (33, 34).

This study aimed to develop an effective antitumor agent against recurrent HCC, which is important for improving HCC prognosis. In this study, we report that MKH-DMG overcomes the aforementioned problems to achieve effective delivery of MKH into HCC cells and effective antiproliferative activity against both DCP-positive and DCP-negative HCC cell lines. Furthermore, we demonstrated the growth-inhibitory effects of MKH prodrg against HCC in vivo using a spleen–liver metastasis mouse model. Our results suggest that MKH-DMG plays an important role in intracellular delivery of MKH and growth inhibition of HCC cells and represents a promising new approach for suppression of HCC recurrence.

Materials and Methods

Chemicals

MK-4 and menaquinone-4 epoxide (MKO) were kindly provided by Eisai Co., Ltd. MKH-DMG was synthesized in our laboratory using a previously reported method (33). Solvents used for extraction and chromatography were high-performance liquid chromatography grade (Wako Pure Chemical Industries).

Cell lines

The DCP-positive HCC cell lines PLC/PRF/5 and Hep3B were obtained from JCRB Cell Bank and ECACC (DS Pharma Biomedical), respectively. The DCP-negative HCC cell line SK-Hep-1 was obtained from ECACC (DS Pharma Biomedical). All used cell lines were authenticated by short-tandem repeat profiling and tested negatively for mycoplasma by the vendor, and passaged for fewer than 6 months before experiments. All tested cell lines were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Life Technologies Corp.) and 1% penicillin/streptomycin (Life Technologies Corp.) at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Animals

Male Balb/c nu/nu mice were purchased from Japan SLC, Inc. All animal care and use procedures were performed in compliance with the regulations established by the Experimental Animal Care and Use Committee of Fukuoka University (Fukuoka, Japan), which are in accordance with the universal principles of laboratory animal care.

Cell viability assays

Cell viability was determined using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega Corp.). HCC cells were seeded at a density of 5.0 × 10⁴ cells/well in flat bottom 6-well plates and were allowed to adhere for 48 hours. MK-4 and MKO were dissolved in 99.5% ethanol. MKH-DMG was dissolved in sterilized water. Stock solutions of each drug (50 mmol/L) were diluted to the intended final concentrations with medium. The final ethanol concentration was <0.2%. Solvent tolerance testing up to 0.2% under identical conditions confirmed that the proliferation of all cell lines was unaffected. Then, cells were treated with MK-4 or MKH-DMG for up to 96 hours, and the cell viability was determined according to the manufacturer’s instructions. Luminescence was determined with a Veritas Microplate Luminometer (Turner Biosystems). All cell viability assays were performed in triplicate. The experimental luminescence of cell viability was plotted versus time. IC50 values were determined using a log (drug) versus normalized response-variable slope analysis in GraphPad Prism, version 6.0 (GraphPad Software).

Determination of intracellular MKH-DMG, MK-4, and MKO after drug treatment

HCC cells were seeded at a density of 1.5 × 10⁵ cells/well in flat bottom 6-well plates and were allowed to adhere for 48 hours. Then, cells were treated with medium containing MK-4, MKH-DMG, or MKO for various intervals. After drug treatment, media were aspirated and cells were rinsed three times with PBS. Cells were scraped from wells, collected in 1 mL of PBS, and sonicated. Aliquots of cell homogenates were added to an equal volume of methanol and three times volume of n-hexane containing an internal standard of γ-tocotrienol (γ-T3), vortexed for 2 minutes, and centrifuged at 1750 g for 10 minutes. The upper layer (n-hexane) was collected and evaporated under nitrogen. The residue was reconstituted with 100 μL of methanol and subjected to LC/MS/MS analysis. Protein concentration of the cell homogenate was determined using a BCA protein assay kit (PIERE/Thermo Fisher Scientific Inc.).

LC/MS-MS analysis

LC/MS-MS was performed with a 4000 QTRAP LC-MS/MS system (AB Sciex) equipped with a Prominence UFLC system (Shimadzu Corp.). Separations were performed on a CAPCELL PAK C18 MG-II (3 μm, 2.0 mm × 100 mm, Shiseido) using an isocratic solvent consisting of 10 mmol/L ammonium acetate and 0.1% acetic acid in methanol at a flow rate of 0.4 mL/minute. Column temperature was maintained at 40°C. The mass spectrometer was equipped with an atmospheric pressure chemical ionization interface and was run in positive ion mode. Identification and quantitation were based on MS/MS-multiple reaction monitoring mode using the transition ion as follows: m/z 445 → 187 was the [M+H]⁺ MK-4 adduct, m/z 461 → 161 was the [M+H]⁺ MKO adduct, m/z 481 → 181 was the [M+H]⁺ MKH-DMG adduct, and m/z 461 → 151 was the [M+H]⁺ γ-T3 adduct. Retention times were: MK-4, 2.5 minute; MKO, 1.9 minute; MKH-DMG, 1.4 minute; and γ-T3, 1.7 minute.

Cell-cycle analysis by flow cytometry

PLC/PRF/5 cells were plated at 1.5 × 10⁵ cells/well in flat bottom 6-well plates and allowed to adhere for 48 hours. Then, cells were treated with MK-4 or MKH-DMG (at near IC50 concentration) for 12 or 48 hours. Cells were collected and washed
twice with chilled PBS. The supernatant was removed after centrifugation and the cell pellet was fixed with 70% ethanol for 1 hour at 4°C. After two washes with chilled PBS, the cell pellet was resuspended with RNase (4 mg/mL) in BSA-PBS (Sigma-Aldrich) and incubated at 37°C for 50 minutes. The cell solution was stained with propidium iodide solution (1 mg/mL in PBS; Sigma-Aldrich) and incubated on ice for 20 minutes. Cell samples were analyzed by an EPICS flow cytometer (Beckman-Coulter) and the percentage of cells in each phase of the cell cycle was determined by Multicycle AV software (Phoenix Flow Systems).

Western blotting
HCC cells were seeded at a density of 1.5 × 10^5 cells/well in flat bottom 6-well plates and allowed to adhere for 48 hours. Then, cells were treated with 60 μmol/L MK-4 or MKH-DMG for 24, 48, 72, or 96 hours. Cells were lysed with RIPA buffer (0.5% NP-40, 0.25% sodium deoxycholate, 0.05% SDS, 150 mmol/L NaCl, 50 mmol/L HEPES, pH 7.4) containing a protease inhibitor cocktail (Nacalai Tesque). Protein concentrations in cell lysates were determined using the BCA protein assay kit. Cell lysates were electrophoresed on 12% SDS-PAGE gels, and transferred onto polyvinylidene difluoride membranes. After blocking with Blocking One buffer (Nacalai Tesque), membranes were incubated with the following primary antibodies: mouse anti-CDK4 (1:2000), mouse anti-cyclin D1 (1:2000), mouse anti-cyclin D3 (1:2000), rabbit anti-NF-κB (1:10000; Cell Signaling Technology), and mouse anti-GAPDH antibody (1:40000; Sigma-Aldrich). After the primary antibodies were washed, the samples were detected with peroxidase-conjugated goat anti-mouse or -rabbit IgG (γ-chain specific; 1:20000; Cell Signaling Technology), and were visualized using an Immunostar LD (Wako Pure Chemical Ind.).

DCP analysis
DCP levels in PLC/PRF/5 cell culture medium after treatment with MKH-DMG or MK-4, and plasma DCP levels in the spleen-liver metastasis mouse model were determined by electrochemiluminescence immunoassay (ECLIA; Picolumi PIVKA-II; Sanko Junyaku Co. Ltd.). Cell culture medium and mice plasma samples

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Figure 1.
Structure and concept of MKH delivery system. A, chemical structures of MK-4, MKH, and MKH-DMG. B, schematic illustration of the vitamin K cycle and concept of MKH delivery system.
were stored at −80°C until use. ECLIA uses a mouse monoclonal anti-DCP antibody coated on solid-phase beads and a ruthenylated rabbit polyclonal anti-prothrombin antibody. The electrochemically triggered light reaction was quantified by an electrochemiluminescence detection system (Roche Diagnostics).

*In vivo* HCC growth inhibition effects of MKH-DMG in a murine spleen–liver metastasis model

Male Balb/c nu/nu mice (SLC Japan) ages 5 weeks were used. Thirty-nine nude mice were divided into three groups: (i) vehicle group (n = 15); (ii) MKH-DMG group (n = 15); and (iii) sham group (n = 9). After mice were under anesthesia with isoflurane, PLC/PRF/5 cells (1.0 × 10^6) suspended in 0.05 mL of PBS were injected into the spleens of nude mice using Fidler method (35), and these mice were used for the vehicle and MKH-DMG groups. Sham group mice were treated with surgery alone. The drug was dissolved in drinking water at 40 μmol/L and provided *ad libitum*, and the dose of MKH-DMG was approximately 0.2 μmol/head/day, as calculated from consumption of drinking water. Oral drug administration was commenced 6 days before splenic

Figure 2. Inhibitory effects of MKH-DMG and MK-4 on DCP-positive and DCP-negative HCC cell proliferation. MKH-DMG treatment of PLC/PRF/5 (A), Hep3B (C), and SK-Hep-1 (E) cell lines. MK-4 treatment of PLC/PRF/5 (B), Hep3B (D), and SK-Hep-1 (F) cells. PLC/PRF/5 and Hep3B cells are DCP positive, and SK-Hep-1 cells are DCP negative. Symbols: ○, 0 μmol/L; , 20 μmol/L; □, 40 μmol/L; ▼, 60 μmol/L after MKH-DMG treatment. ○, 0 μmol/L; △, 40 μmol/L; ▼, 60 μmol/L after MK-4 treatment. Error bars mean ± SD (n = 3).
implantation. Water bottles were covered with aluminum foil to block out light and changed every 2 days. At 50 days after implantation, mice were anesthetized and blood was collected by cardiac puncture using a heparinized syringe, and then mice were killed by decapitation. Plasma samples were obtained by blood centrifugation. Livers were collected and weighed, and images of both sides (diaphragm and abdominal cavity) were obtained for calculation of cancer area using ImageJ software. The cancer area is given as a percentage of total liver surface area. Samples were processed for routine histology and the results confirmed the presence of premalignant nodules and neoplastic changes in the tissue.

Statistical analysis
Statistical analysis was performed using JMP 8 (SAS Institute Inc.). Significance of changes was tested by Tukey–Kramer test using one-way ANOVA. P values of <0.05 were considered to be statistically significant.

Results and Discussion
Inhibition of HCC cell growth by MKH-DMG
To assess the effects of MKH-DMG on HCC cell proliferation, DCP-positive (PLC/PRF/5, Hep3B) and DCP-negative (SK-Hep-1) HCC cell lines were treated with MKH-DMG or MK-4 and cell viability was determined. The HCC cell lines were treated with different concentrations of the study agents for up to 96 hours. As shown in Fig. 2A, C, and E, MKH-DMG inhibited the proliferation of all tested HCC cell lines in a time- and dose-dependent manner. Although MK-4 had little inhibitory effect on cell proliferation and its effects appeared after 72 hours of treatment (Fig. 2B, D, F), MKH-DMG showed rapid and strong growth-inhibitory effects.

Figure 3.
MKH delivery via MKH-DMG into HCC cell lines. Intracellular MK-4 and MKO concentration-time profiles following 25 μmol/L MKH-DMG treatment of PLC/PRF/5 (A), Hep3B (C), and SK-Hep-1 (E) cells. Intracellular MK-4 and MKO concentration-time profiles following 25 μmol/L MK-4 treatment of PLC/PRF/5 (B), Hep3B (D), and SK-Hep-1 (F) cell lines. Symbols: ■, MK-4; ▲, MK-4+MKO after MKH-DMG treatment. □, MK-4; △, MKO; ◆, MK-4+MKO after MK-4 treatment. Error bars, mean ± SD (n = 3).
after only 48 hours of treatment. The IC_{50} values are summarized in Supplementary Table S1. MKH-DMG effectively inhibited the proliferation of both DCP-positive and DCP-negative HCC cell lines, and exhibited lower IC_{50} values and a 4- to 16-fold increase in growth-inhibitory activity compared with MK-4. We noted that MKH-DMG exhibited antiproliferation activity regardless of DCP formation; the effects of MKH-DMG on the formation of DCP and other vitamin K-dependent proteins aside from DCP are discussed below. The cytotoxicity of MKH-DMG itself was assessed on the basis of lactate dehydrogenase release from the cells. Within the range of MKH-DMG doses used in this assay, cell injury was not observed after 48 hours of treatment (data not shown).

The IC_{50} values of MKH-DMG for the HCC cell lines ranged from 14 to 37 μmol/L. MK-4 is used for osteoporosis at a dose of 45 mg/day as three daily doses of 15 mg, and its long-term safety has been confirmed. At this dosage regimen, the maximum blood level of MK-4 is 1 μmol/L (36). In a distribution study in rats, MK-4 distribution in the liver was at least 10 times greater than that in plasma after oral administration (37). This liver MK-4 level is comparable with the IC_{50} values of MKH-DMG for HCC growth inhibition. Thus, these results strongly suggest that MKH-DMG could act as an antitumor agent with a long-term safety profile.

Effective delivery of MKH into HCC cells with MKH-DMG

To assess MKH-DMG as a delivery system for MKH in HCC cells, the hydroquinone MKH levels in HCC cells must be measured. However, accurate determination of MKH is difficult because of its highly oxidative characteristics to MK-4. Concomitant with vitamin K-dependent carboxylation of Glu to Glal by the vitamin K-dependent GGCX, MKH is stoichiometrically converted to MKO (Fig. 1B). It thus appears that MKO levels in HCC cells can reflect the levels of MKH, which functions as a GGCX cofactor. The intracellular concentrations of MKH-DMG, MK-4, and MKO in DCP-positive (PLC/PRF/5, Hep3B) and DCP-negative (SK-Hep-1) HCC cell lines were determined by LC/MS-MS after MKH-DMG administration and compared with those after MK-4 administration.

MKH-DMG caused a significant reduction in cell proliferation; hence, MKH delivery into HCC cells with MKH-DMG was determined in a time-dependent manner for up to 72 hours at a dose of 25 μmol/L, equivalent to the IC_{50} value. MKH-DMG levels in all of the tested HCC cell lines increased sharply and reached the maximal amount equivalent to the media concentrations within 24 hours. After MKH-DMG administration, MKO and MK-4 levels increased for up to 72 hours, and the sum of MKO and MK-4 was greater than that after MK-4 administration (25 μmol/L; Fig. 3), clearly indicating that MKH-DMG was hydrolyzed to parental MKH effectively in all of HCC cell lines. As shown in the supplementary document, the intracellular MK-4 in HCC cells after MKH-DMG administration was the oxidative product of MKH, which was generated by hydrolysis of MKH-DMG in HCC cells. Thus, the sum of MKO and MK-4 levels after MKH-DMG administration was regarded as the MKH levels delivered into HCC cells. In MK-4 administration, MKH level was assessed by MKO. The AUC of the intracellular concentration versus time profile can be used to determine the extent of drug delivery from the administration formulation. The AUC_{0-72 h} values for MKH (AUC_{MKH}), MK-4 (AUC_{MK-4}), and MKO (AUC_{MKO}) were calculated using the trapezoidal rule (Table 1). The AUC_{MKH} values after MKH-DMG administration were 3.5- to 15-fold higher than those after MK-4 administration. MKH-DMG exhibited increased MKH delivery into HCC cell lines compared with MK-4 administration. On the basis of these results, MKH-DMG was clearly confirmed as a beneficial procedure for effective delivery of MKH into HCC cells, and the resultant MKH might exhibit excellent antiproliferative activity against HCC cells despite their DCP-positive and DCP-negative status. Further discussion of the contribution of the recycling process (vitamin K cycle) of MKH in MKH delivery is included in the Supplementary Documents.

These findings strongly support our hypothesis that the rapid and strong growth-inhibitory effects resulted from the rapid and effective delivery of MKH into HCC cells by MKH prodrg, which was designed to be more membrane permeable and to undergo efficient hydrolytic activation by esterase to release MKH without the reductive activation process in HCC cells (Fig. 1B). A previous study showed that the uptake rate of MK-4 into hepatoma cell lines was less than the uptake rate in normal hepatocytes (16). Thus, the excellent uptake of MKH-DMG compared with MK-4 suggests that the MKH-DMG uptake process might be different from that of MK-4. The uptake mechanism of MKH-DMG including uptake and efflux transporter contribution warrants further research.

Effects of MKH-DMG on DCP formation in HCC cells

Recent studies have revealed that DCP functions as a growth and metastasis factor and partly contributes to the poor prognosis of HCC (24). It has been proposed that inhibition of DCP formation represents a potential target for drug discovery to establish new chemotherapeutic strategies against DCP-positive HCC. MKH-DMG is a promising method for delivery of MKH into HCC cells and satisfies the new strategy aims. Hence, the effect of MKH-DMG on DCP formation in PLC/PRF-5 cells (DCP positive) was assessed by determining DCP concentration in the medium after 72 hours of incubation. DCP levels in nontreated PLC/PRF/5 cells were 43 ± 3.6 mAU/μL, whereas they were significantly decreased after 10 μmol/L MKH-DMG or MK-4 treatment, to 2.0 ± 0.0 and 1.3 ± 0.6 mAU/μL, respectively. However, PLC/PRF/5 proliferation was not inhibited with this

<table>
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<th>HCC cell line</th>
<th>Test drug</th>
<th>AUC_{0-72 h} for MKO (nmol/L/mg protein)</th>
<th>AUC_{0-72 h} for MK-4 (nmol/L/mg protein)</th>
<th>AUC_{0-72 h} for MKH* (nmol/L/mg protein)</th>
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<td>PLC/PRF/5</td>
<td>MK-4</td>
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<td>MKH-DMG</td>
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<tr>
<td>Hep3B</td>
<td>MK-4</td>
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<tr>
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<td>MKH-DMG</td>
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<tr>
<td>SK-Hep-1</td>
<td>MK-4</td>
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<tr>
<td></td>
<td>MKH-DMG</td>
<td>136 ± 14.3</td>
<td>193 ± 21.5</td>
<td>329 ± 35.4</td>
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NOTE: Doses are 25 μmol/L (at near IC_{50} value). PLC/PRF-5 and Hep3B, DCP-positive cells; SK-Hep-1, DCP-negative cells.

*MKH value after MKH-DMG administration: sum of MKO and MK-4. MKH value after MK-4 administration: MKO.
dose of MK-4 (10 μmol/L). MKH-DMG also showed antiproliferative activity against DCP-negative HCC (SK-Hep-1; Fig. 2C). Thus, these results indicate that inhibition of DCP formation did not assume a large role in the antiproliferative effect of MKH.

Although DCP formation in PLC/PRF/5 cells was inhibited by low doses of MKH-DMG, intracellular MKO levels increased according to dose of MKH-DMG (Supplementary Fig. S2), indicating that a large amount of GGCX was expressed and was able to work in a wide range of MKH concentrations in PLC/PRF/5 cells. Thus, these results suggested the possibility of another vitamin K-dependent protein aside from DCP that exhibits a potential impact on the antiproliferative activity of MKH-DMG. Further study is required to identify other potential participating proteins.

Effects of MKH-DMG on cell-cycle arrest in HCC cells

MKH-DMG caused a significant reduction in cell proliferation, thus, the underlying mechanisms were investigated. Cell-cycle arrest is believed to be involved in the antiproliferative action of MK-4 (2–4, 6, 37, 38). Aberrant expression of NF-κB is linked to cyclin D1 (5), and to the onset and progression of HCC.

![Figure 4](https://example.com/figure4.png)

*Figure 4.* Effect of MKH-DMG on cell cycle and expression of cell-cycle-related proteins in HCC cells. A, flow cytograms of MKH-DMG–treated cells. Indicated percentage values represent DNA content in G1- and S-phase per number of whole DNA. B, effects of MKH prodrug on expression of cell-cycle regulatory proteins and NF-κB.
tumorigenesis (38), and both cyclin D1 and NF-κB regulate cellular migration (39, 40). The inhibition of both NF-κB and cyclin D1 may contribute to the suppression of HCC cell proliferation and invasion. Thus, to clarify the mechanisms of MKH-DMG-induced growth inhibition in HCC cells, we analyzed the cell cycle using flow cytometry, and cell-cycle–related proteins were determined by Western blotting after drug treatment.

As shown in Fig. 4A, flow-cytometric analysis of MKH-DMG–treated PLC/PRF/5 cells showed an increase in G1 phase cells and a decrease in S phase cells. Treatment of cells with MKH-DMG downregulated cyclin D1, cyclin D3, and CDK4 expression after 24 hours, and completely suppressed their expression after 48 hours in both DCP-positive (PLC/PRF/5 and Hep3B) and DCP-negative (SK-Hep-1) HCC cells (Fig. 4B). In contrast, slight downregulation of cyclin D1, cyclin D3, and CDK4 expression was observed after 48 hours of MK-4 treatment in all tested HCC cell lines (Fig. 4B). The cyclin D-CDK4 complex is the primary regulator of the G1–S phase checkpoint, and flow-cytometric analysis of MKH-DMG–treated cells also indicated an increased proportion of cells in G1 phase. Thus, these results indicated that G1–S arrest is one of the mechanisms of MKH-DMG–induced HCC cell proliferation inhibition as well as those reported for MK-4. NF-κB was downregulated after MKH-DMG treatment in all tested HCC cell lines, but no effect was observed after MK-4 treatment at this dose (Fig. 4B). These reductions in NF-κB could also be regarded as the results of effective intracellular delivery of MKH in HCC cells with MKH-DMG. Because NF-κB and cyclin D1 are known to regulate cellular migration, inhibition of both NF-κB and cyclin D1 in HCC cell lines by MKH-DMG might contribute to the suppression of HCC cell proliferation and invasion.

A recent report suggested that human VKORC1L1 promotes the reduction of MK-4 to MKH and supports vitamin K hydroquinone-mediated intracellular antioxidation, which is critical for cell survival (31). In our study, MKH-DMG effectively provided intracellular MKH to HCC cells without reductive activation. The effects of intracellular MKH produced by MKH-DMG on HCC cell survival remain to be clarified.

In vivo HCC growth-inhibitory effects of MKH-DMG in a spleen–liver metastasis mouse model

Before the pharmacologic (anticancer) study, we conducted a pharmacokinetic study of MKH-DMG after oral administration in mice, and found that MKH-DMG was absorbed in the ester form (MKH-DMG), distributed to liver and converted MKH in vivo (Supplementary Fig. S3 and Supplementary Table S2). Thus, to assess the pharmacologic effects of the MKH delivery system, we examined the effects of MKH-DMG against hepatic metastasis and proliferation of PLC/PRF/5 cells in a spleen–liver metastasis model. Hitomi and colleagues (7) previously showed a significant antiproliferative effect of orally used MK-4 against xenografted PLC/PRF/5 cells in nude mice after 53 days of treatment. Therefore, the endpoint of the study was set at day 50. Fifty days after transplantation, macroscopic findings of the developed liver tumors were compared between the MKH-DMG treatment and vehicle groups (Fig. 5A). MKH-DMG treatment significantly suppressed the increase of liver weight caused by tumor growth (Fig. 5B). The percentage of cancer surface area to total liver surface area was significantly lower in mice treated with MKH-DMG (Fig. 5C). Plasma DCP production was completely inhibited by MKH-DMG administration (Fig. 5D). The numerical data of vehicle, MKH-DMG, and sham groups are summarized in Supplementary Table S3. No significant difference was observed in liver weight and plasma DCP levels between the MKH-DMG and sham groups. The volume of drinking water was almost similar among three groups, and daily administration of MKH-DMG was well tolerated because no overt signs of toxicity and weight loss were observed (data not shown).

Figure 5.
HCC growth-inhibitory effects of MKH-DMG in a splenic-liver metastasis mouse model. A, macroscopic appearance of mouse liver. B, total liver weight. C, percentage of cancer surface area/total liver surface area. D, DCP levels in plasma. Central horizontal line, mean; error bar, SD. Vehicle group, n = 15; MKH-DMG group, n = 15. Doses are 0.2 mmol/head/day for 50 days.
Inoue T, Fujita T, Kishimoto H, Makino T, Nakamura T, Nakamura T, et al. In vitro and in vivo administration. The beneficial effects of MKH-DMG were confirmed in xenografted human HCC, suggesting that MKH-DMG can act as an antitumor agent for HCC. However, to ultimately translate the effect of MKH-DMG to human HCC treatment, further study is required.

Conclusions

The development of tumor-specific, cell penetrating molecules remains a primary focus in cancer research because reduced drug uptake is an important mechanism of chemoresistance. In the present study, we confirmed our hypothesis that effective delivery of MKH, the active form of MK-4, into HCC cells is critical for regulating HCC growth and for the generation of a long-term safe antitumor agent, and found that an ester-type produg of MKH (MKH-DMG) was able to effectively deliver substantial amounts of MKH into HCC cells and effectively inhibit proliferation of HCC cells in vitro. Cell-cycle arrest and downregulation of cyclin D1 and NF-kB expression were involved in the growth inhibition mechanism of MKH-DMG. Furthermore, we demonstrated the growth-inhibitory effects of MKH-DMG on HCC in vivo. These results suggest that effective MKH delivery has an important role in MKH-DMG-mediated HCC growth inhibition. As a result, we believe that MKH-DMG is a promising new candidate for an antitumor agent to suppress HCC recurrence that fulfills long-term safety requirements.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Enhanced Antitumor Effects of Novel Intracellular Delivery of an Active Form of Menaquinone-4, Menahydroquinone-4, into Hepatocellular Carcinoma

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