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Enhanced Intracellular Delivery and Improved Antitumor Efficacy of Menaquinone-4

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Abstract

Hepatocellular carcinoma (HCC) is a major malignant tumor type that occurs globally. HCC incidence is increasing, especially in Asian countries. Despite many therapeutic approaches, the long-term prognosis of HCC remains poor because of frequent recurrence due to intrahepatic metastasis or multicentric carcinogenesis. Therefore, it is necessary to develop effective and safe chemopreventive agents to improve the prognosis of HCC. Menaquinone-4 (MK-4) has a suppressive effect on HCC, but cellular delivery is poor. We hypothesized that effective cellular delivery of menahydroquinone-4 (MKH), a fully reduced form of MK-4, would regulate HCC growth and metastasis. We developed a bioreductive activation-independent delivery system with the *N,N*-dimethylglycine ester of MKH (MKH-bis-DMG) to deliver MKH to HCC cells without any bioreductive processing of MK-4. MKH-bis-DMG inhibited the proliferation of both DCP-positive and DCP-negative HCC cell lines in a time- and dose-dependent manner via G1/S cell-cycle arrest. We assessed the effect of MKH-derivatives on HCC metastasis using a mouse model of spleen-liver metastasis. The mean tumor hepatic replacement area of MKH-bis-DMG treated mice was significantly less than that of untreated mice. In conclusion, MKH-bis-DMG may be beneficial as a chemopreventive agent for recurrent HCC.

Keywords: menahydroquinone-4, antitumor agent, prodrug, drug delivery, hepatocellular carcinoma

1. Introduction

Vitamin K has two types of molecular homologues: phylloquinone (vitamin K1, PK) and menaquinone (vitamin K2, MK-n). These homologues have the same aromatic naphthoquinone

“head” but different hydrocarbon “tails”: PK with a phytyl tail and MK-n with an unsaturated isoprenoid chain. MK-n can be classified into 14 types based on the length of the unsaturated isoprenoid chain, where “n” quantifies the repeating isoprenyl units. MK-4 is normally synthesized from PK in certain animal tissues by removal of the phytyl tail of PK to produce menadiol (vitamin K3, MD) as an intermediate. The intermediate is then condensed with a geranylgeranyl tail. UbiA prenyltransferase domain-containing protein 1 (UBIAD1) converts MD to MK-4 with geranylgeranyl diphosphate [1]. For this reason, MK-4 appears to be the most important form of vitamin K.

Vitamin K (PK and MK-n) plays a major role in the clotting cascade by acting as a coenzyme for a vitamin K-dependent carboxylase. The carboxylase catalyzes the carboxylation of glutamic acid (Glu) residues to produce γ -carboxyglutamic acid (Gla). Vitamin K also appears to play a role in the regulation of bone metabolism through a similar mechanism that involves γ -carboxylation of pro-osteocalcin. Interestingly, MK-4 intake seems to be associated with greater effects of reduced bone resorption compared with PK consumption [2]. Clinically, high doses (45 mg daily) of MK-4 have been used as an approved treatment for osteoporosis in Japan since 1995. Therefore, the safety of long-term administration of MK-4 has been established in Japanese patients with osteoporosis.

Over the last decade, many reports have shown that MK-4 has antioncogenic effects within various cancer cell lines, including leukemia, lung cancer, ovarian cancer, prostate cancer, and hepatocellular carcinoma (HCC) [3–6]. Specifically, numerous articles describe the effects of MK-4 against HCC. This is because des- γ -carboxy prothrombin (DCP, PIVKA-II), an abnormal prothrombin that is not completely carboxylated, is a well-recognized HCC-specific tumor marker, and a predictor of vascular invasion, metastasis, and tumor recurrence [7]. It has been reported that apoptosis, cell-cycle arrest, and autophagy are involved in the antitumor activity of MK-4 [8–10]. Although the possible mechanisms of the antitumor effect of MK-4 have been investigated previously, they remain unclear.

2. Mechanisms of growth inhibition of HCC cells by MK-4

Otsuka et al. reported that MK-4 inhibited the growth and invasion of HCC via activation of protein kinase A (PKA) and the subsequent inhibition of Rho activation. It has been reported that PKA induces cell-cycle arrest at the G1 phase and the G2/M phase [11]. Hitomi et al. [12] reported that MK-4-induced G1 arrest of the cell cycle via significantly reduced protein expression of cyclin D1 and cyclin-dependent kinase 4 (Cdk4), but not the p16INK4a Cdk inhibitor in PLC/PRF/5 HCC cells *in vivo*. Cyclin D1 promotes the G1/S phase of the cell cycle and is frequently overexpressed in many human cancers. Matsumoto et al. [13] reported that MK-4-induced arrest of the G1 phase of the cell cycle and apoptosis via activated extracellular signal-regulated kinase $\frac{1}{2}$ (ERK1/2) in a mitogen-activated ERK-regulating kinase-dependent manner in Hep3B HCC cells. Ozaki et al. [10] reported that MK-4 inhibited the growth of HCC cells via suppression of cyclin D1 expression through inhibited I κ B kinase activity, and therefore suppressed I κ B phosphorylation and NF- κ B activation.

In addition, Xia et al. [14] reported that the inhibitory effect on NF- κ B activity by MK-4 is mediated through the inhibition of protein kinase C α and ϵ kinase activities, as well as subsequent inhibition of protein kinase D1 activation. Kaneda et al. proposed that MK-4 suppressed HuH7 HCC tumor malignancy via induced Cx32 expression through the reduction of Cx43 expression. Consequently, gap junctional intercellular communication through Cx32 is activated. Normal hepatocytes communicate with neighboring cells via Cx32-containing gap junction communication, a process essential for suppressing tumorigenesis [15]. Yamamoto et al. suggested that regulation of the expression of the hepatoma-derived growth factor gene is one of the crucial mechanisms of MK-4-induced cell growth suppression in HCC. Hepatoma-derived growth factor stimulates the proliferation of HCC cells after its translocation to the nucleus by use of bipartite nuclear localization signals [16]. Azuma et al. [17] suggested that activation of steroid and xenobiotic receptors (SXR) by MK-4 contributes to the tumor suppressive effects on HCC cells. Li et al. suggested that MK-4 inhibited the growth of Smmc-7721 HCC cells by induction of apoptosis involving caspase-8 activation and p53. This apoptotic process was not mediated by the caspase-9 pathway [18]. Yao et al. suggested that the mechanism involved induced p53 and increased p21 levels that eventually lead to cell-cycle arrest in the G2 phase. In addition, they suggested that the antitumor effect of MK-4 may be improved by silencing BCL-2 expression in SMMC-7721 HCC cells [19].

3. Clinical trials of MK-4 to treat HCC

In clinical trials with cirrhotic women, Habu et al. demonstrated that daily doses of 45 mg MK-4 decreased the risk of HCC to about 20% compared with the control group. Twenty-one women were in the treatment group and 19 in the control group [20]. Mizuta et al. reported that a daily dose of 45 mg MK-4 suppressed the recurrence of HCC in HCC patients who had undergone curative resection or percutaneous local ablation therapy. Thirty-two patients were in the treatment group and 29 in the control group [21]. From the results of these small-scale clinical trials, it is expected that MK-4 acts as a chemopreventive agent for HCC. However, a recent larger scale study that enrolled 548 patients at 31 study sites, and included a placebo-controlled, double-blind trial, demonstrated that the efficacy of vitamin K2 in suppressing HCC recurrence could not be confirmed [22]. The poor anticancer activity of MK-4 observed in this Japanese trial may have been a consequence of the large study design and meant that MK-4 could not be developed as an anticancer drug. However, various attempts are being made to try to improve the anticancer effect of MK-4 in HCC.

4. Improvement of the antiproliferative effect of vitamin K2

4.1. A novel chemosynthetic vitamin K derivative

Carr et al. demonstrated that a new, chemically synthesized vitamin K analog, compound 5 (Cpd5), inhibited Cdc25A phosphatase activity and particularly reduced HCC cell growth through arrest of the G1/S phase of the cell cycle. Inhibition of Cdc25A by Cpd5 results in

prolonged tyrosine phosphorylation and activation of ERK1/2, which could be triggered by upstream epidermal growth factor receptor signaling pathway molecules [23]. Suhara et al. synthesized vitamin K2 analogues with hydroxyl or phenyl groups at the ω -terminal of the side chain, and with dual side chains at the C-2 and C-3 positions. They found that modifying the side chain of vitamin K affects the SXR-mediated transcriptional activity [24, 25]. The novel biological activities of MK-4 include tumor suppressive effects related to gene transcription through the SXR. The new derivatives of MK-4 have shown some efficacy against HCC, but a lengthy development process is still necessary to yield safe and effective clinical products.

4.2. Synergistic drug combinations

Yoshiji et al. [26, 27] reported that combined treatment with MK-4 and angiotensin-converting enzyme inhibitor significantly suppressed experimental hepatocarcinogenesis. Further to this, they reported that the combined treatment with MK-4 and angiotensin-converting enzyme inhibitor may suppress the cumulative recurrence of HCC after the curative therapy, at least partly through suppression of the vascular endothelial growth-mediated neovascularization. Kanamori et al. [28] demonstrated that a combination of MK-4 and acyclic retinoid synergistically inhibited the growth of Huh7 HCC cells by increasing apoptosis. When combined with acyclic retinoid, MK-4 synergistically inhibits Ras activation and inhibits phosphorylation of retinoid X receptor α . Zhang H et al. [29] showed that MK-4 enhanced the inhibition of 5-fluorouracil-induced cell growth in HepG2, Huh7, HLE, and Hep3B HCC cells, and via G1 cell-cycle arrest through induced expression of p21 and p27 and inhibited expression of cyclin D1. Zhang et al. reported that a combination of MK-4 and sorafenib work synergistically to inhibit growth of HepG2, Hep3B, and HuH7 HCC cells. They also demonstrated that the levels of cyclin D1 expression are clearly reduced in HepG2 cells treated with a combination treatment of MK-4 and sorafenib [30]. A clinical trial to test the efficacy of the combination of MK-4 and sorafenib in HCC was attempted [31].

4.3. Delivery of menahydroquinone-4, the active form of MK-4

We have previously synthesized the ester derivatives of menahydroquinone-4 (MKH), the fully reduced form of MK-4, and revealed their effective antiproliferative activity against HCC cell lines [32–35]. The menaquinone (MK-4 to MK-10) concentrations were significantly lower in HCC tissues, from patients with or without increased plasma concentrations of DCP, than in the surrounding normal liver tissue. There was no significant difference between PK and PK epoxide concentration in HCC tissues without increased plasma concentrations of DCP and normal liver tissue [36]. Furthermore, the rate of uptake into MH7777 cells (vitamin K2-sensitive HCC) was lower than for normal hepatocytes. In addition, the rate of uptake into H4IIE cells (vitamin K2-resistant HCC) was negligible compared with that for MH7777 cells and normal hepatocytes. Further, hepatocytes from diethylnitrosamine-induced liver nodules exhibited a significantly lower rate of vitamin K2 uptake than that for normal hepatocytes [37].

MKH acts as a cofactor for γ -glutamyl carboxylase (GGCX), which catalyzes the carboxylation of specific Glu residues (γ -carboxylation) of substrate proteins such as prothrombin. Thus, decreased MKH availability in HCC cells is a possible causative mechanism of DCP production

in HCC. MKH is mainly generated by a vitamin K 2, 3-epoxide reductase complex subunit 1-like-1 (VKORC1L1) in mammalian cells [38]. Key reasons for further development are that MK-4 delivery to HCC cells is poor and reductive activation of MK-4 is low in HCC cells. We hypothesized that effective delivery of MKH into HCC cells is essential in regulating HCC growth and metastasis. However, MKH cannot be used as a therapeutic agent, because it is easy to be oxidized and converted to MK-4. In addition, the production of MKH depends on VKORC1L1 activity in HCC cells. As such, MK-4 does not display sufficient antitumor activity even at high doses.

In light of these findings, we synthesized three types of *N,N*-dimethylglycine esters of MKH (MKH-1-DMG, MKH-4-DMG, and MKH-bis-DMG) and assessed their potential as water-soluble prodrugs for a bioreductive activation-independent delivery system of MKH [33–35] (Figure 1).

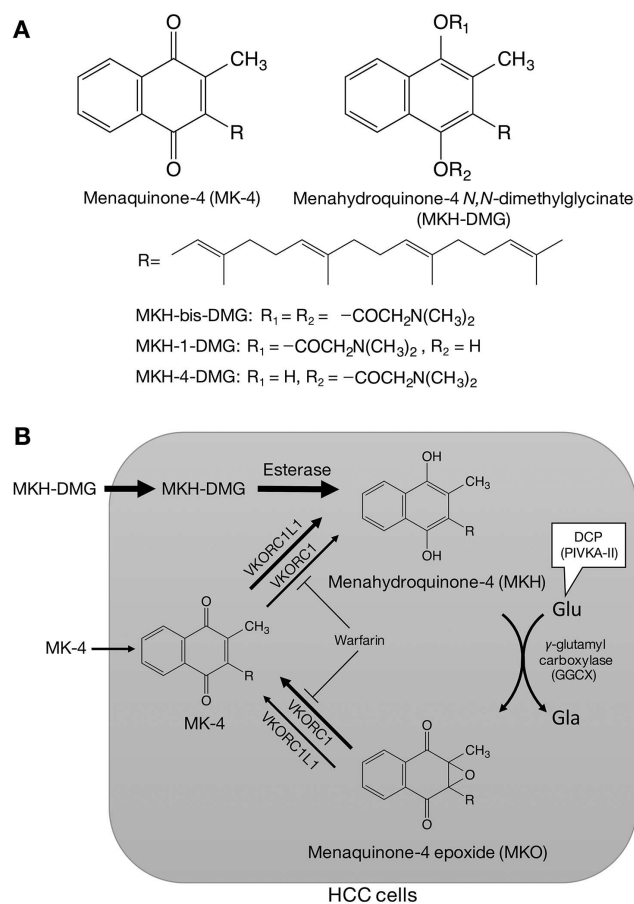


Figure 1. Structure of the MKH derivatives and schematic illustration of the concept of the MKH delivery system. (A) Chemical structures of menaquinone-4 (MK-4), menahydroquinone-4 (MKH) and MKH *N,N*-dimethylglycinate (MKH-DMG). (B) Schematic illustration of the vitamin K cycle and the concept of the MKH delivery system. Adapted from Ref. [32].

5. Water solubility and hydrolysis of the MKH esters

The hydrochloride salts of the esters showed much improved aqueous solubility. The solubilities of MKH-1-DMG and MKH-4-DMG in water were 24 and 5.7 mM respectively and that of MKH-bis-DMG was >50 mM. In contrast, the solubility of MK-4 in water was <2.3 mM. Improving the low water solubility of MK-4 enables a bolus dose necessary for cancer chemotherapy without any surfactant. *In vitro* studies have confirmed that MKH derivatives can be hydrolyzed with esterases located in the rat and human liver and that the resultant MKH acts as cofactor for GGCX without the reductive activation process. The first-order rate constants observed for the hydrolysis of the MKH derivatives in the liver homogenate supernatant are listed in **Table 1**, along with the degradation rate constants of the derivatives in phosphate buffer. The rate of hydrolysis of MKH-1-DMG was about 24-fold and 5.7-fold faster than that of MKH-4-DMG in rat and human liver homogenate supernatant, respectively.

Compound	Without physostigmine ($\times 10^{-2} \text{min}^{-1}$)	With physostigmine ($\times 10^{-2} \text{min}^{-1}$)	Regeneration half-life (min)
Rat liver homogenate			
MKH-1-DMG	27.2	0.261	2.55
MKH-4-DMG	1.14	0.315	60.6
MKH-bis-DMG	17.2 ^a	0.328 ^a	39.7 ^a
Human liver homogenate			
MKH-1-DMG	2.70	0.0714	25.7
MKH-4-DMG	0.476	0.0431	146
MKH-bis-DMG	1.00 ^a	0.117 ^a	227 ^a
Isotonic phosphate buffer of pH7.4			
MKH-1-DMG	0.0203		
MKH-4-DMG	0.0295		
MKH-bis-DMG	0.0500 ^a		

^aDisappearance of MKH-bis-DMG.
Adapted from Refs. [33, 34].

Table 1. Apparent first-order rate constants for the hydrolysis of the MKH derivatives, and regeneration half-lives of MK-4 in human and rat liver homogenate supernatant and phosphate buffer (pH 7.4).

The formation of MK-4 from MKH-bis-DMG should proceed through the intermediates MKH-1-DMG, MKH-4-DMG, and MKH. The pseudo-first-order rate constants for the interconversion of the species are assumed, as shown in **Figure 2**. Detection of MKH was unsuccessful because of its high susceptibility to oxidation.

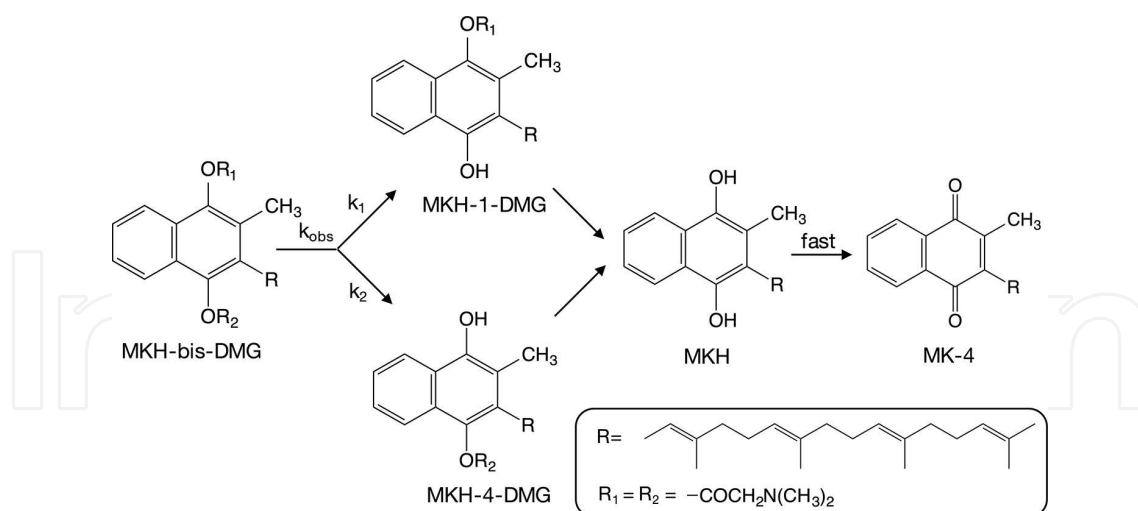


Figure 2. Hydrolytic pathway of MKH-bis-DMG. Adapted from Ref. [34].

As shown in **Table 2**, the rate of hydrolysis of MKH-bis-DMG at the 1-position (k_1) was about 2.6-fold and 5.1-fold faster than that at the 4-position (k_2) in rat and human liver homogenate supernatant, respectively. The rates of hydrolysis of MKH-1-DMG, MKH-4-DMG, and MKH-bis-DMG in the liver homogenate supernatant were significantly reduced in the presence of physostigmine, a liver carboxylesterase inhibitor. Consequently, MKH esters were hydrolyzed by rat and human liver esterases.

Medium	$k_{obs}^a (\times 10^{-2} \text{min}^{-1})$	$k_1 (\times 10^{-2} \text{min}^{-1})$	$k_2 (\times 10^{-2} \text{min}^{-1})$
Rat liver homogenate	17.2 ± 1.0	11.9 ± 0.39	4.39 ± 0.18
Human liver homogenate	1.00	0.794 ± 0.019	0.156 ± 0.008

k_1 and k_2 accordance with **Figure 2**.

^aDisappearance of MKH-bis-DMG.

Adapted from Refs. [33, 34].

Table 2. Rate constants for the hydrolysis of MKH-DMG in human and rat liver homogenate supernatant.

6. Vitamin K-dependent carboxylation *in vitro*

To provide evidence that confirmed the bioreductive activation-independent delivery system of MKH was working properly, carboxylation activity was measured with the incorporation of $^{14}\text{CO}_2$ in the synthetic tripeptide BOC-Glu-Glu-Leu-OMe. The accelerated carboxylation of MK-4 was only observed in the presence of dithiothreitol (DTT), an artificial reducing agent for MK-4, and not in its absence. Conversely, MKH esters stimulated carboxylase activity in the absence of DTT [33], clearly indicating that the MKH esters can stimulate carboxylation without the reductive activation process of MK-4.

7. Evaluation of MKH delivery in HCC cells with MKH esters and the antiproliferative effect

MKH-bis-DMG inhibited the proliferation of both DCP-positive (PLC/PRF/5, Hep3B) and DCP-negative (SK-Hep-1) HCC cell lines in a time- and dose-dependent manner, and exhibited lower IC_{50} values (range from 14–37 $\mu\text{mol/L}$), and a fourfold to 18-fold increase in growth-inhibitory activity compared with MK-4. MKH-bis-DMG showed a rapid and strong growth-inhibitory effect after only 48 h of treatment. In contrast, MK-4 had little inhibitory effect on cell proliferation, and its effects appeared after 72 h of treatment (Figure 3).

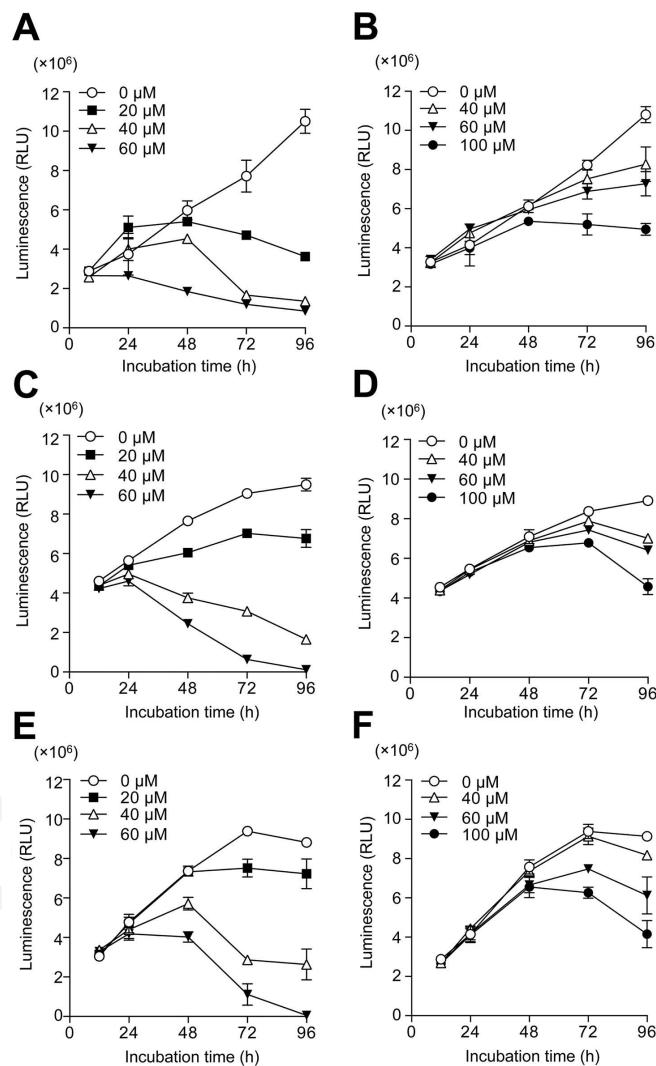


Figure 3. Inhibitory effects of MKH-DMG and MK-4 on DCP-positive and DCP-negative HCC cell proliferation. MKH-DMG treatment of PLC/PRF/5 (A), Hep3B (C), and SK-Hep-1 (E) cell lines, and MK-4 treatment of PLC/PRF/5 (B), Hep3B (D), and SK-Hep-1 (F) cells. Symbols: \circ , 0 μM ; \blacksquare , 20 μM ; \triangle , 40 μM ; \blacktriangledown , 60 μM after MKH-DMG treatment. Symbols: \circ , 0 μM ; \triangle , 40 μM ; \blacktriangledown , 60 μM ; \bullet , 100 μM after MK-4 treatment. Error bars indicate mean \pm SD ($n = 3$). Adapted from Ref. [32].

MKH was susceptible to oxidation, so we measured the levels of menaquinone-4 epoxide (MKO) in HCC cells to assess the function of MKH-bis-DMG as a delivery system for MKH in HCC cells. Concomitant with vitamin K-dependent carboxylation of Glu to Gla by GGcX, MKH is stoichiometrically converted to MKO and that means MKO levels in HCC cells reflect the levels of MKH. The AUC_{MKH} values after MKH–DMG administration in three types of HCC cell lines were 3.5-fold to 15-fold higher than those after MK-4 administration (**Table 3**). Based on these results, it was clearly confirmed that MKH-bis-DMG works as an effective delivery prodrug of MKH into HCC cells. The resultant MKH may exhibit excellent antiproliferative activity against HCC cells despite their DCP-positive and DCP-negative status.

HCC cell line	Compound	AUC _{0–72h} for MKO (nmol·h·mg protein ⁻¹)	AUC _{0–72h} for MK-4 (nmol·h·mg protein ⁻¹)	AUC _{0–72h} for MKH (nmol·h·mg protein ⁻¹)
PLC/PRF/5	MK-4	22.2 ± 3.72	47.7 ± 7.25	22.2 ± 3.72 ^a
	MKH–DMG	193 ± 25.1	143 ± 13.6	336 ± 37.2 ^b
Hep3B	MK-4	113 ± 4.87	74.5 ± 17.0	113 ± 4.87 ^a
	MKH–DMG	371 ± 31.0	25.9 ± 5.04	397 ± 34.2 ^b
SK-Hep-1	MK-4	38.4 ± 4.44	122 ± 19.5	38.4 ± 4.44 ^a
	MKH–DMG	136 ± 14.3	193 ± 21.5	329 ± 35.4 ^b

Doses are 25 μM (at near IC₅₀ value).

^aMKH value after MK-4 administration: MKO.

^bMKH value after MKH–DMG administration: sum of MKO and MK-4.

Adapted from Ref. [32].

Table 3. Area under the curve for intracellular concentration versus time (AUC) after treatment with MK-4 or MKH–DMG in HCC cell lines.

8. Mechanism of growth inhibition of HCC cells by MKH esters

One of the mechanisms of the antiproliferative effect of MK-4 was thought to involve G1/S cell-cycle arrest via reduced protein expression of cyclin D1 and Cdk4, and through suppression of NF-κB activation [10, 12]. We investigated whether the antiproliferative activity of MKH-bis-DMG was via cell-cycle arrest in HCC cells using flow cytometry and Western blotting [32]. MKH-bis-DMG-treated PLC/PRF/5 cells showed an increase in G1 phase cells and a decrease in S phase cells in flow cytometric analysis. Treatment of both DCP-positive and DCP-negative HCC cells with MKH-bis-DMG downregulated cyclin D1, cyclin D3, and Cdk4 expression after 24 h, and almost completely removed expression after 48 h. In comparison, the modest downregulation of cyclin D1, cyclin D3, and Cdk4 expression was observed after 48 h of MK-4 treatment in all tested HCC cell lines. NF-κB was downregulated after MKH-bis-DMG treatment in all tested HCC cell lines, but no effect was observed after MK-4 treatment in PLC/PRF/5 and SK-Hep-1 cell lines at this dose.

These findings strongly support our hypothesis that the rapid and strong growth-inhibitory effects on cells resulted from the rapid and effective delivery of MKH into HCC cells by an MKH prodrug. The mechanism of the MKH-bis-DMG antiproliferative effect is the same as that of MK-4 and involves cell-cycle arrest. Therefore, MKH-bis-DMG is expected to be a safe antitumor agent and chemopreventive agent.

9. Pharmacokinetics of MKH esters

Plasma MKO levels can reflect the levels of MKH not only *in vitro* but also *in vivo*. This also assists the function of GGCX at the active site. The relative bioavailability for MKH (F_{MKH}), after the parenteral administration of the MKH esters, relative to MK-4 solubilized with HCO-60, was calculated using AUC_{MKO} as in Eq. (1), and is shown in **Table 4**. In Eq. (1), $AUC_{MKO, MKH-DMG}$ and $AUC_{MKO, MK-4}$ are the AUC_{MKO} values after the administration of MKH-DMG and MK-4, respectively. D_{MK-4} and $D_{MKH-DMG}$ are the doses of MK-4 and MKH-DMG, respectively. MKH-1-DMG and MKH-bis-DMG, but not MKH-4-DMG, showed an improvement in bioavailability compared with the MK-4 injection.

$$F_{MKH} = \frac{AUC_{MKO, MKH-DMG} \cdot D_{MK-4}}{AUC_{MKO, MK-4} \cdot D_{MKH-DMG}} \quad (1)$$

	MK-4	MKH-1-DMG	MKH-4-DMG	MKH-bis-DMG
for MK-4				
C_{max} (nmol mL ⁻¹)	107 ± 2.07	2.41 ± 0.826	20.8 ± 5.24	11.1 ± 3.03
t_{max} (h)	0.125	0.125	0.125	0.125
AUC_{MK-4} (nmol h mL ⁻¹)	31.7 ± 0.526	2.52 ± 0.387	6.01 ± 1.26	10.1 ± 1.15
MRT_{MK-4x} (h)	0.338 ± 0.018	2.44 ± 0.032	0.924 ± 0.004	1.32 ± 0.063
for MKO				
C_{max} (nmol mL ⁻¹)	1.92 ± 0.172	0.991 ± 0.131	0.518 ± 0.039	0.968 ± 0.180
t_{max} (h)	1	2	0.5	1
AUC_{MKO} (nmol h mL ⁻¹)	2.37 ± 0.115	4.45 ± 0.510	2.06 ± 0.322	3.20 ± 0.467
MRT_{MKO} (h)	2.14 ± 0.091	3.78 ± 0.404	3.93 ± 0.463	2.80 ± 0.066
F_{MKH}	100	188	87	135

^aThe values are the mean ± S.D. of 3 rats at a dose of 5 mg/kg equivalent for MK-4.

^bCalculated from Eq. (1) using the mean values.

Adapted from Ref. [35].

Table 4. Pharmacokinetic parameters for MKO and MK-4 in plasma after the intravenous administration of the prodrugs, and for MK-4 in vitamin K cycle-inhibited rats.^a

The distribution of MKO in the liver after the injection of MKH esters is the most important indicator for assessing the potential of MKH esters as the MKH delivery system for HCC. The values of AUC_{MKO} and MRT_{MKO} of MKH-1-DMG were larger than that for MK-4, which indicates that the MKH esters distribute successfully and provide prolonged deliver MKH to the liver. For the evaluation of MKH-1-DMG as a liver-specific delivery system for MKH, the selective advantage value was defined as in Eq. (2). In Eq. (2), $AUC_{MKO, MKH-DMG}^{Liver}$ and $AUC_{MKO, MK-4}^{Liver}$ are the AUC_{MKO} values in the liver after the administration of MKH-DMG and MK-4, respectively. $AUC_{MKH-DMG, MKH-DMG}^{Plasma}$ and $AUC_{MK-4, MK-4}^{Plasma}$ are the AUC values of plasma levels of MKH-DMG and MK-4 after the administration of MKH-DMG and MK-4, respectively. Remarkable site-specific delivery of MKH was observed after the intravenous injection of MKH-1-DMG. The selective advantage of MKH-1-DMG was 5.7 [35].

$$\text{Selective advantage} = \frac{\left\{ \frac{AUC_{MKO, MKH-DMG}^{Liver}}{AUC_{MKO, MK-4}^{Liver}} \right\}}{\left\{ \frac{AUC_{MKH-DMG, MKH-DMG}^{Plasma}}{AUC_{MK-4, MK-4}^{Plasma}} \right\}} \quad (2)$$

It is proposed that the intravenous injection of MKH esters is appropriate when a rapid and large quantity is necessary for cancer treatment, whereas oral administration of MKH esters is otherwise appropriate for cancer prevention. We performed a pharmacokinetic study of MKH-bis-DMG after oral administration, and found that MKH-bis-DMG was absorbed in the ester form, distributed to the liver and converted to MKH *in vivo* [32].

10. Antiproliferative effects of MKH-bis-DMG in a spleen–liver metastasis mouse model

To assess the pharmacological effects of the MKH delivery system *in vivo*, we assessed the effects of oral administration of MKH-bis-DMG on hepatic metastasis and proliferation of PLC/PRF/5 cells in a spleen–liver metastasis model [39]. MKH-bis-DMG treatment significantly suppressed the increase of liver weight caused by tumor growth (**Figure 4A**). The percentage surface area of the cancer compared with the total surface area of the liver was significantly lower in the spleen-liver metastasis model treated with the MKH-bis-DMG than with the vehicle (**Figure 4B**).

Plasma DCP production was completely suppressed after MKH-DMG administration, while liver metastasis of HCC was not completely prevented (**Figure 4C**). These results suggest that the level of influence of DCP suppression on the antiproliferative effects of MKH-bis-DMG was severely limited. No obvious side effects, such as body weight loss, were observed in the MKH-di-DMG treated animals.

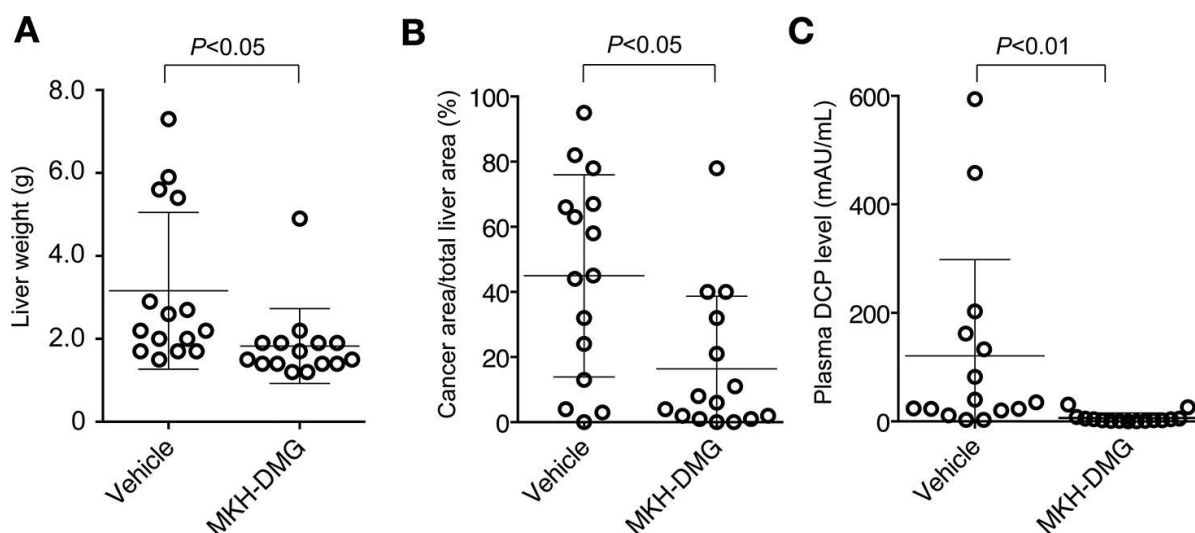


Figure 4. HCC growth inhibitory effects of MKH-DMG in a spleen-liver metastasis mouse model. (A) Total liver weight. (B) Percentage of cancer surface area/total liver surface area. (C) DCP levels in plasma. Central horizontal line, mean; error bar, SD. Vehicle group, $n = 15$; MKH-DMG group, $n = 15$. Doses were $0.2 \mu\text{mol/head/day}$ for 50 days. Adapted from Ref. [32].

11. Conclusion and perspectives

In previous studies, we have indicated that MKH-bis-DMG can be delivered to the liver through intravenous and oral administration and that thereafter it is transported and converted to MKH with enzymatic hydrolysis in HCC cells. Regarding the effective delivery of MKH into the HCC cells, MKH-bis-DMG enhanced the inhibition of HCC cell growth compared with that from MK-4 and suppressed metastasis of HCC. Given these results, we suggest that MKH-bis-DMG is a potential candidate for chemoprevention that can be safely administered over long periods, and can reduce or eliminate the recurrence and metastasis of HCC. This MKH prodrug approach was our original method to improve the antitumor effect of MK-4, and this approach may be applied to improve HCC treatment in the future. However, further studies are required to develop MKH-DMG for use in human HCC treatment.

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