Supplementary Data

Material and Methods

Pharmacokinetic study of MKH-DMG after p.o. administration in mice

Prior to the pharmacological (anti-cancer) study, a pharmacokinetic study of MKH-DMG after oral administration was performed in ICR male mice (Kyudo, Tosu, Japan) at 7 weeks of age. Mice were fasted overnight in a coprophagy-preventing cage and had free access to water. For oral administration, 38.6 mg/kg of MKH-DMG, equivalent to 25 mg/kg of MK-4, was used. Under anesthesia, blood was taken from cardiac punctures using heparinized syringes. Mice were killed by cervical dislocation, and livers were isolated at the indicated times. Plasma samples were obtained by blood centrifugation at 1750 × *g* for 10 min. Livers were weighed and homogenized with two volumes of saline using a POLYTRON homogenizer (Kinematica, Lucerne, Switzerland) and were stored at −80°C until analysis. The plasma and liver homogenates were subjected to the extraction procedure described in the intracellular determination of MKH-DMG and MK-4 for HCC cell lines. Plasma and liver concentration versus time data were analyzed using the model independent and

statistical moment method [1, 2]. Both the maximum concentration (C_{max}) and its corresponding time (t_{max}) were directly obtained from the observed data.

Supplementary results

Uptake of MKO and its conversion to MK-4 in HCC cells

MKO uptake into PLC/PRF/5 cells and formation of MK-4 were determined as a function of different initial doses after 24 h of incubation. Intracellular MKO levels increased in a dose-dependent manner (Fig. S1A), although MK-4 increased according to a saturated profile (to hyperbolic curve) (Fig. S1B). Intracellular MK-4 levels were only 0.3–3% those of MKO, indicating that MKO was scarcely reduced to MK-4 under high intracellular concentration of MKO. This low reduction capacity of MKO to MK-4 indicated the low ability of vitamin K epoxide reductase complex 1 (VKORC1) in PLC/PRF/5 cells (Fig. 1B). Thus, the high intracellular MK-4 levels after MKH-DMG administration were regarded as the oxidative product of MKH, which was produced by hydrolysis of MKH-DMG in HCC cells.

Saturation of recycling processes for MK-4 and MKO in HCC cells at

anti-proliferative doses

As seen in Hep3B cells (Fig. 3C), intracellular MKO was quite high compared with MK-4 after MKH-DMG administration, and the AUC_{MKO} value reached 93% of the sum of AUC_{MKO} and $AUC_{MK\text{-}4}$ (Table 1). These results clearly indicated that the majority of the MKH hydrolytically generated from MKH-DMG acted as a cofactor for GGCX and was metabolized to MKO, as shown in Figure 1B, but suggested that the reduction process of MKO to MK-4 with VKORC1 might be saturated. The saturation of VKORC1 was also observed in PLC/PRF/5 cells after MKO treatment (shown in Supplementary Fig. S1). Although the sum values of AUC_{MKO} and $AUC_{MK\text{-}4}$ in the three HCC cell lines after MKH-DMG were same, the AUC_{MKO} values in PLC/PRF/5 and SK-Hep-1 cells were lower than that in Hep3B cells, suggesting that GGCX activities in PLC/PRF/5 and SK-Hep-1 cells were lower than in Hep3B cells. Thus, the sum values of MKO and MK-4 after MKH-DMG administration were regarded as the MKH levels in HCC cell lines. After MK-4 administration in Hep3B, the sum values of AUC_{MKO} and $AUC_{MK\text{-}4}$ were significantly lower than that after MKH-DMG administration, clearly indicating low uptake of MK-4. While MK-4 uptake was low, the intracellular MK-4 levels were higher than that after MKH-DMG administration, indicating that the

reduction process of MK-4 to MKH by VKORC1L1 might be rate limiting compared with the process of MKH to MKO concomitant with carboxylation by GGCX at this MK-4 concentration. Thus, only the MKO value, but not the MK-4 value, was regarded as the MKH value after MK-4 administration. These results indicated that the two reduction processes (VKORC1L1 and VKORC1) in the vitamin K cycle (Fig. 1B) would be saturated at a dose that would confer MK-4 anti-proliferative activity, and that the intracellular delivery of MKH with MK-4 in HCC cells is hampered not only by the MK-4 uptake process [3] but also by two reduction processes in the vitamin K cycle.

Dose effect of MKH-DMG on intracellular delivery of MKH

Initial dose effects of MKH-DMG on intracellular MKO and MK-4 levels in HCC cells after 24 h of incubation were analyzed by the method mentioned in the main text, as shown in Figure S2. The intracellular concentrations of MKH (sum of MKO and MK-4) increased with initial dose, which corresponds to a higher *AUC* value for higher initial dose.

Pharmacokinetic study of MKH-DMG after p.o. administration in mice

To determine whether MKH-DMG could act as a prodrug of MKH for oral use, pharmacokinetic studies were carried out in mice. Mean plasma and liver concentrations versus time profiles of MKH-DMG and MK-4 after oral administration of MKH-DMG in mice are shown in Supplementary Figure S3A and B, respectively. Plasma and liver levels of intrinsic MKH-DMG increased and reached C_{max} at 1 h after MKH-DMG administration. Plasma and liver levels of MK-4 also increased, and exhibited a t_{max} of 1 and 3 h, respectively. These results indicate that MKH-DMG can be absorbed in an ester form, is distributed into liver, and is hydrolyzed to MKH, acting as a prodrug for MKH after oral administration.

References

[1] Yamaoka K, Tanigawara Y, Nakagawa T, Uno T. A pharmacokinetic analysis program (multi) for microcomputer. J Pharmacobiodyn 1981;4:879–885.

[2] Yamaoka K, Nakagawa T, Uno T. Statistical moments in pharmacokinetics. J Pharmacokinet Biopharm 1978;6:547–558.

[3] Li ZQ, He FY, Stehle CJ, Wang Z, Kar S, Finn FM, et al. Vitamin K uptake in hepatocytes and hepatoma cells. Life Sci 2002;70:2085-100.

Supplementary figure legends

Supplementary Figure S1. Intracellular MKO and MK-4 levels after 24-h treatment with MKO in PLC/PRF/5 cells. Symbols: ◇, MKO; ◆, MK-4. Error bars indicate mean ± SD (n = 3).

Supplementary Figure S2. Intracellular MKO and MK-4 levels after 24-h treatment with MKH-DMG or MK-4 in PLC/PRF/5 cells. Symbols: ▲, MK-4; △, MKO after MKH-DMG treatment; ▼, MK-4; ▽, MK-4 after MK-4 treatment.

Supplementary Figure S3. Plasma and liver concentration profiles of MK-4 and MKH-DMG after oral administration of MKH-DMG in mice. (A) Plasma concentration after MKH-DMG administration. Symbols: △, MKH-DMG; ▲, MK-4. (B) Liver concentration after MKH-DMG administration. Symbols: △, MKH-DMG; ▲, MK-4. Each point represents mean ± SD of three mice.