Prodrug for Bioreductive Activation-Independent Delivery of Menahydroquinone-4: Human Liver Enzymatic Activation and Its Action in Warfarin-Poisoned Human Liver

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The N,N-dimethylglycine esters of menahydroquinone-4 (1-mono, 1; 4-mono, 2; 1,4-bis, 3) were established in previous reports as prodrugs that could achieve the systemic bioreductive activation-independent delivery of menahydroquinone-4 (MKH), the active form of menaquinone-4 (MK-4), in rat. The present study was undertaken to investigate if the prodrugs could undergo cleavage to parent drug (MKH) by a human tissues enzyme catalyzated hydrolytic pathway, the mechanism of the prodrugs for vitamin K-dependent carboxylation in human liver and their action in the warfarin poisoned human liver. The hydrolysis of the esters was shown to be catalyzed by esterases located in human liver but not in human plasma. The susceptibility of the esters to undergo human liver esterase hydrolysis was affected by the esterified position: 1>2>3. By using a human liver microsomal test system, the stimulation of vitamin K-dependent carboxylation with the prodrugs was determined. The prodrug could stimulate the carboxylation activity in the absence of dithiothreitol, an artificial activator of the reductive activation pathway of MK-4. The carboxylation activity of the prodrug was strongly inhibited in the presence of esterase. The prodrug could also stimulate the carboxylation under warfarin-poisoned conditions, where the vitamin K cycle was strongly inhibited. The results confirmed that the prodrug could generate MKH in human liver (active site), and that the resultant MKH could act as a cofactor for the carboxylase without reductive activation processes of MK-4 to MKH. Such bioreductive activation-independent vitamin K-dependent carboxylation characteristic of the prodrug leads to enhanced pharmacological efficacy in the treatment of hypoprothrombinemia induced in patients with coumarin and cephalosporin therapies.

Key words: human liver esterase; vitamin K-dependent carboxylation; menahydroquinone-4; bioreductive activation; prodrug; warfarin.

The most important condition for the successful therapeutic application of a drug preparation is the bioavailability of the active form of the drug at its site of action. K vitamins (3-substituted 2-methyl-1,4-naphthoquinone) undergo reductive activation during effective processing, and their pharmacological efficacy is expressed upon enzyme-dependent reduction of the quinone to the hydroquinone. Vitamin K hydroquinone, the fully reduced form of vitamin K, is an essential cofactor for vitamin K-dependent carboxylase to catalyze the post-translational carboxylation of the specific glutamic residue (Glu) in precursor proteins to the γ-carboxyglutamyl (Gla) residue in the mature protein (Fig. 1).[1–3] Two bioreductive activation pathways have been described: one is inhibited by coumarins (coumarin-sensitive pathway), and another is unaffected by coumarins (coumarin-insensitive pathway).

Coumarin anticoagulants block the vitamin K cycle by inhibition of the coumarin-sensitive pathway and vitamin K epoxide reductase. However, the anticoagulation can be overcome by administration of a large dose of vitamin K. The antidiuretic effect of the vitamin is due to the formation of vitamin K hydroquinone via the coumarin-insensitive pathway, which has a much lower reduction activity than that of the coumarin-sensitive pathway. Based on these results, the efficacy of the vitamin depends on the pathway and the extent of reductive activation to the hydroquinone at the site of action and especially the use of the quinone form of vitamin K in the treatment of coumarin anticoagulant poisoning which might be inefficient and unreliable. A more effective treatment would be to deliver the hydroquinone to its active site selectively and independently of the bioreductive activation pathways.

Another delivery problem associated with vitamin K hydroquinone arises from the fact that vitamin K is practically insoluble in aqueous media. Intravenous dosing often produces an anaphylactoid reaction in certain individuals. These adverse reactions are believed to be related to the poloxamethylene hydrogenated castor oil (HCO-60) used in the parenteral dosage form.

With the aim of overcoming the delivery problems of vitamin K hydroquinone mentioned above, we recently proposed the ester prodrug concept of vitamin K hydroquinone for systemic bioreductive activation-independent delivery of the hydroquinone (Fig. 1). In earlier papers, the N,N-dimethylglycine esters (1-mono, 1; 4-mono, 2; and 1,4-bis, 3) were prepared, and it was shown that the esters can improve the water solubility and can also generate the hydroquinone using esterase localized in the rat liver and that the resultant hydroquinone can act as cofactor for the γ-glutarylcarboxylase without the bioreductive activation processes of MK-4 to MKH. The water-soluble and liver esterase hydrolyzable derivatives of MKH allow systemic bioreductive activation-independent delivery of the hydroquinone, and these characteristics of the esters lead to enhanced efficacy (coagulation activity) against warfarin-induced hypoprothrombinemia in rats.

For the successful therapeutic application of the prodrug...
candidates, human liver enzymes catalyzed prodrug activation is an important criteria. It has been shown that esterase activities in tissues (e.g., liver, blood etc.) against hydrolysis of ester-type drugs and prodrugs are known to be different among species. Thus, the present study was conducted to determine if the prodrugs could undergo cleavage to the parent drug (MKH) by a human esterase catalyzed hydrolytic pathway, the mechanism of the prodrugs for vitamin K-dependent carboxylation in human liver and their action in the warfarin poisoned human liver.

MATERIALS AND METHODS

Materials The hydrochloride salts of the N,N-dimethylglycine esters of MKH were synthesized in our laboratory using previously reported methods. MK-4 and warfarin (racemate) potassium were generous gifts from Eisai Co., Ltd. (Tokyo, Japan) and used as received. The tripeptide butoxy-carbonyl (BOC)-Glu-Glu-Leu-Me was purchased from BACHEM Feinchemikalien AG (Bubendorf, Switzerland). Eserine (physostigmine sulfate) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The bicinchoninic acid (BCA) protein assay reagent 23225 was purchased from PIERCE (IL, U.S.A.). All other chemicals were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). NaHCO3 (54.0 mCi/mmole) and scintillation cocktail ACS II were obtained from Amersham Japan Co., Ltd. (Tokyo, Japan). The human autopsy liver, without pathosis, was obtained from three autopsies within 12 h of death and stored at −80°C until analysis. The human plasma samples were obtained from volunteers in our laboratories.

Hydrolysis of the Prodrugs in Human Plasma and Human Liver Preparation The hydrolysis of the esters was studied at 37°C in isotonic phosphate buffer (pH 7.4) and human plasma as well as in the 20% human liver homogenate 9000g supernatant fraction. The liver homogenate supernatant was prepared according to the previously reported method for 20% rat liver homogenate 9000g supernatant.

The reactions were initiated by adding 75 μl of an aqueous stock solution of the esters and 75 μl of isotonic phosphate buffer to 1350 μl of a preheated reaction medium in amber test tubes. The initial concentration of the esters was 5.6×10−5 M. The solutions were incubated at 37°C. At appropriate intervals, samples of 100 μl each were withdrawn and 350 μl of methanol–ethyl acetate (4:1, v/v) was added. After 2 min of vortex mixing and centrifugation at 3000 rpm for 5 min, 50 μl of the clear supernatant was analyzed by HPLC. The rates of hydrolysis were generally followed by monitoring the disappearance of the esters and the appearance of MK-4. In the case of 3, the appearance of the metabolites (1 and 2) was also monitored.

The effects of an esterase inhibitor (eserine, 1 mM) on the hydrolysis of the esters were also studied. The liver preparations (1350 μl) were incubated with 75 μl of the inhibitor solution for 15 min at 37°C. Following the initial incubation, 75 μl of the stock solution of the esters was added to the mixture, and the ensuing experimental procedure was the same as that mentioned above.

Vitamin K-Dependent Carboxylation The human liver microsomal test system for assessing the vitamin K-dependent carboxylation mechanism of the prodrugs was prepared.
according to the previously reported method for the rat liver microsome. Vitamin K-dependent carboxylase activity was measured as $^{14}CO_2$ incorporation into the synthetic tripeptide BOC-Glu-Glu-Leu-OMe using the slightly modified method described by Grossman and Suttie. The reaction mixture contained 0.6 ml of microsomal suspension and 0.1 ml of BOC-Glu-Glu-Leu-OMe dissolved in IC buffer (25 mM imidazole, pH 7.2, containing 0.5% CHAPS) containing NaH$^{14}$CO$_3$. The final concentrations of the tripeptide substrate and NaH$^{14}$CO$_3$ were 1 mM and 40 μCi, respectively. Other compounds (dithiothreitol (DTT), warfarin potassium, and eserine) were added in 80 μl of the IC buffer. The final concentration of DTT was 5 mM. The total volume of the reaction mixtures was 0.8 ml. Reactions were started by the addition of 20 μl of stock solution of MK-4 and the prodrugs, and then incubated at 25°C. Aliquots of 200 μl were removed at appropriate times, and 0.5 ml of 10% ice-cold trichloroacetic acid was added. After centrifugation (3000 rpm, 10 min), 200 μl supernatant was transferred to scintillation vials. N$_2$ was bubbled in to remove unreacted $^{14}$CO$_2$, and the solution was mixed with 6 ml of ACS II liquid scintillation cocktail. The sample radioactivity was assayed in a Packard 2250CA Tri-Carb liquid scintillation analyzer.

The hydrolysis of the prodrugs was studied under the same conditions as in the carboxylase study, except that the tripeptide substrate and NaH$^{14}$CO$_3$ were omitted. At appropriate intervals, samples of 100 μl each were withdrawn and 350 μl of methanol–ethanol acetate (4:1, v/v) was added. After 2 min of vortex mixing and centrifugation at 3000 rpm for 5 min, 50 μl of the clear supernatant was analyzed by HPLC.

**HPLC Analysis**

The concentrations of the esters and MK-4 in the reaction media were determined with HPLC according to previously reported methods. Standards were prepared by adding various amounts of the prodrugs and MK-4 to the supernatant of liver homogenate, the pooled plasma and the human liver microsome containing an esterase inhibitor (1 mM eserine). The quantitation of the compounds was achieved using the linear calibration curves of the peak area vs. concentration.

**RESULTS AND DISCUSSION**

**Hydrolysis of the Prodrugs in Human Plasma and Human Liver Preparation**

The prodrugs 1–3 must undergo cleavage to MKH under in vivo conditions to be true prodrugs for parenteral use. The susceptibilities of the prodrugs to hydrolysis were firstly studied in the supernatant fraction of 20% human liver homogenate and human plasma. Significant acceleration of the hydrolytic rates of the prodrugs was found in the human liver homogenate preparation but not in human plasma.

Monoesters 1 and 2 underwent hydrolysis with quantitative formation of MK-4 as revealed by HPLC analysis, and the reaction followed apparent first order kinetics at an initial concentration of $5.6 \times 10^{-5}$ M in all media. An attempt to monitor MKH was unsuccessful because of its high susceptibility to oxidation. The HPLC procedure was able to quantify MK-4 and the monoesters. The mass balance of these compounds in the reaction medium was well maintained (over 95% recovery) throughout each kinetic run; therefore, it was concluded that the monoesters were hydrolyzed to MKH, which was subsequently oxidized to MK-4 quantitatively as shown in Fig. 2. The apparent first order rate constants for the hydrolysis of the compounds in the human liver preparation are listed in Table 1 along with the degradation rate constants of the prodrugs in the phosphate buffer. In liver homogenate, the rate of hydrolysis of 1-monoester 1 was about 5.7-fold faster than that of 4-monoester 2.

Theoretically, bisester 3 can be hydrolyzed in two steps via two routes (Fig. 2). The time courses of the degradation of 3 in the human liver preparation is shown in Fig. 3. The disappearance of 3 followed apparent first order kinetics under the experimental conditions (Table 1) and was accompanied by formation of the monoesters (1 and 2) and MK-4. At any given reaction time, the sum of the concentrations of 3, 1, 2 and MK-4 was over 95% of the initial concentration of 3. The formation of MK-4 from 3 should proceed through the intermediates 1, 2 and MKH, and the pseudo first order rate constants for the interconversion of the species are assumed as shown in Fig. 2. The rate equation corresponding to this model can be integrated by standard integrating factor meth-
Table 1. Apparent First Order Rate Constants for the Hydrolysis of the Esters of MKH and Regeneration Half-Lives to MKH in Human Liver Homogenate Supernatant at 37°C and pH 7.4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Without Eserine ($\times 10^{-2}$ min$^{-1}$)</th>
<th>With Eserine ($\times 10^{-2}$ min$^{-1}$)</th>
<th>Regeneration half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver homogenate$^{a,b}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.70</td>
<td>0.0714</td>
<td>25.7</td>
</tr>
<tr>
<td>2</td>
<td>0.476</td>
<td>0.0431</td>
<td>146</td>
</tr>
<tr>
<td>3</td>
<td>1.00$^c$</td>
<td>0.117$^c$</td>
<td>227$^c$</td>
</tr>
<tr>
<td>Buffer$^{d}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.0203</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.0295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.0500$^d$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Eserine 2.5 mm, $^b$ 20% human liver homogenate 9000g supernatant, $^c$ disappearance of 3, $^d$ calculated from eq. 4 (see text), $^e$ isotonic phosphate buffer of pH 7.4.

Fig. 3. Time Course for the Hydrolysis of 3 (■) and Concomitant Formation of the Monoesters (1 (○) and 2 (■)) and MK-4 (□) in the 20% Human Liver Homogenate 9000g Supernatant at 37°C and pH 7.4

The curves are calculated from Eqs. 1–4.

ods, and the concentrations of the species may be expressed by the following equations:

$$C_1 = C_0 e^{-k_{1}t}$$  
(1)

$$C_2 = C_0 \frac{k_1}{k_5 - k_{1} + k_2} (e^{-(k_{1}+k_2)t} - e^{-k_2t})$$  
(2)

$$C_1 = C_0 \frac{k_2}{k_3 - k_{5} + k_2} (e^{-(k_{1}+k_2)t} - e^{-k_2t})$$  
(3)

$$C_{MKH} = C_0 \left[ 1 - \frac{k_1}{k_4 - (k_{1} + k_2)} (e^{-(k_{1}+k_2)t} - e^{-k_2t}) - \frac{k_2}{k_3 - k_{5} + k_2} (e^{-(k_{1}+k_2)t} - e^{-k_2t}) \right]$$  
(4)

where $C_0$ represents the initial concentration of 3, and $C_1$, $C_2$, $C_1$, and $C_{MKH}$ are the concentrations of 3, intrinsic 2, intrinsic 1, and MKH at time $t$, respectively. The progress curves according to the above equations were analyzed by using the nonlinear regression analysis program MULTI$^{15}$ and gave reasonable fits of these equations to the curves shown in Fig. 3. The estimated kinetic parameters of $k_1$ and $k_2$ are listed in Table 2. In these analyses, the hydrolytic rates of 1 and 2 under the condition shown in Table 1 were used for $k_3$ and $k_4$, respectively, and the concentration of MK-4 was regarded as that of MKH ($C_{MKH}$). The sum of the rate constants ($k_{1} + k_2$) obtained in this way agreed with $k_{obs}$ determined in the hydrolysis experiment of 3 within ±10%.

As can be seen in Table 2, the rate of hydrolysis of 3 at the 1-position ($k_1$) was about 5.1-fold faster than that at the 4-position ($k_2$) in the human liver supernatant preparation, demonstrating that the cleavage of the 1-ester is the dominating hydrolytic route.

To assess whether the observed catalytic regeneration of MKH in the human liver preparation can be attributed to the esterases, the effect of eserine on the hydrolysis was studied. Eserine is an inhibitor of liver carboxylesterase. $^{20}$ The rates of 1, 2, and 3 in the biological medium were significantly reduced in the presence of eserine (Table 1). It is quite evident from the results that human liver esterase significantly catalyze the hydrolysis of the prodrugs.

A significant difference in the human liver esterase-catalyzed hydrolytic rates of ester bonds at positions 1 and 4 of MKH was observed in both the monoesters and the bisester, indicating that liver esterase catalytic hydrolysis depends on the esterified position of MKH. Similar esterified position dependency of the hydrolysis of the prodrugs was found in rat liver esterase catalytic hydrolysis of the prodrugs, $^{10}$ suggesting human and rat liver esterase for the hydrolysis of the prodrugs were similar. It has been shown that the hydrolysis of the prodrugs are catalyzed by rat plasma esterase. $^{10}$ However, there was no significant acceleration of the hydrolytic rates in human plasma whereas the prodrugs appear to be relatively good substrates for the human liver esterase, indicating the absence of the esterase for the prodrugs in human plasma.

Since one of the aims of the present prodrug approach is to overcome the solubility problem in formulating the parenteral solution of MK-4, the facile enzymatic reconversion of the esters should satisfy the criteria as prodrugs for parenteral use.

Vitamin K-Dependent Carboxylation Stimulated with the Prodrugs

For achieving a bioreductive activation-independent delivery system of MKH (another aim of this study), the prodrugs must first undergo cleavage to MKH at the ac-
tive site and the resultant MKH must act as a cofactor for the carboxylase. It has been shown that the rat liver microsomal system can provide the pathways for cleavage of the prod drug and for the reductive activation of MK-4, and the vitamin K-dependent carboxylase can accept the synthetic tripeptide BOC-Glu-Glu-Leu-OMe. A human liver microsomal test system was prepared from human liver using the previously described method for a rat liver microsomal test system, and used for assessing the carboxylation mechanism of the prod drugs. The most successful prod drug for a delivery system is that in which the reconversion of the prod drug to MKH is catalyzed by enzymes located in the liver. Therefore, we selected compound 1 and 2 as candidates for the delivery system because of their high susceptibility to liver esterase catalyzed hydrolysis (Table 1).

To confirm that the human liver microsomal system used in this study can provide the functions of the reductive activation pathway of MK-4 and of the carboxylase, the carboxylation stimulated with MK-4 was determined in the system (Fig. 4). The carboxylation activity was measured with the incorporation of $^{14}$CO$_2$ in the synthetic tripeptide BOC-Glu-Glu-Leu-OMe. The accelerated carboxylation with MK-4 could only be found in the presence of DTT (5 mM) but not in the absence of it. DTT is an artificial activator for the enzymes comprising the K cycle (coumarin-sensitive reduction pathway and vitamin K epoxide reductase) in the microsomal system. These results clearly indicate that the human liver microsomal system can reflect the functions of the coumarin-sensitive reduction pathway of MK-4 and the carboxylase. Using the microsomal test system, the carboxylation stimulated with 1 and 2 was assessed, and the typical $^{14}$CO$_2$ incorporation profiles at an initial concentration of $8.28 \times 10^{-8}$ mol/mg of protein are shown in Fig. 4. Prodrugs stimulated the carboxylase activity in the absence of DTT, clearly indicating that the prod drugs could stimulate carboxylation without the reductive activation process of MK-4. The concentration effect of the prod drugs on the carboxylation was measured in the absence of DTT. Carboxylation of the substrate was linear during 60-min incubation, and the initial slope was regarded as the carboxylation rate. The rate data can be described by simple Michaelis-Menten kinetics. It is difficult to envisage a model for this complex enzyme system which can explain the simple enzyme kinetics observed. This simple kinetics has also been observed when chemically reduced MKH was used as a cofactor for the carboxylase.

Thus, the kinetic data were analyzed according to the method of Larson and Suttie using the Lineweaver-Burk equation (5):

$$\frac{1}{v} = \frac{K_m}{V_{\text{max}}} \frac{1}{S_0} + \frac{1}{V_{\text{max}}}$$

where $v$ was the initial rate of the incorporation of $^{14}$CO$_2$ into the tripeptide substrate, $S_0$ was the initial concentration of the ester, $K_m$ was the Michaelis constant, and $V_{\text{max}}$ was the maximal incorporation rate. Representative Lineweaver-Burk plots are shown in Fig. 5. The apparent kinetic parameters generated from linear regression analysis are listed in Table 3. To assess whether the observed reductive activation-independent stimulation of the carboxylase can be attributed to the MKH that was regenerated from the prod drugs, the regeneration of MKH from the prod drugs was determined in the human microsomal test system under the same conditions as those for the carboxylation study, mentioned above. Significant acceleration of the hydrolytic rates of the prod drugs was found in the liver microsome preparation and produced MK-4 in a quantitative amount as revealed by HPLC. An attempt to monitor the MKH was unsuccessful because of its high

![Fig. 4. Time Course of the Vitamin K-Dependent Carboxylation Stimulated with 1 and/or MK-4 in Human Liver Microsome Preparation at 25 °C](image)

![Fig. 5. Lineweaver-Burk Plots for the Vitamin K-Dependent Carboxylation Stimulated with Prodrugs](image)

### Table 3: Apparent Kinetic Parameters for the Vitamin K-Dependent Carboxylation Stimulated with the Prodrugs in Human Liver Microsome Preparation at pH 7.2 and 25 °C

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ ($\times 10^{-4}$ mol·mg$^{-1}$)</th>
<th>$V_{\text{max}}$ (dpm·mg$^{-1}$·min$^{-1}$)</th>
<th>$V_{\text{max}}/K_m$ ($\times 10^{-12}$ dpm·min$^{-1}$·mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.80</td>
<td>1091</td>
<td>1.39</td>
</tr>
<tr>
<td>2</td>
<td>1.30</td>
<td>20</td>
<td>0.145</td>
</tr>
</tbody>
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Note: Carboxylation rate is presented as the quantity of product formed per min per mg of microsomal protein.
susceptibility to oxidation. The prodrugs were concluded to be hydrolyzed to MKH, which was subsequently oxidized to MK-4 quantitatively. The kinetics of regeneration of MKH from the prodrugs can be described by the Michaelis-Menten model. The kinetic data from the studies were analyzed using the Lineweaver-Burk equation (Eq. 5), where $v$ is the initial rate of the hydrolysis, $S_0$ is the initial concentration of the ester, $K_m$ is the Michaelis constant, and $V_{max}$ is the maximal hydrolytic rate for saturation for a given enzyme concentration.

The kinetic parameters of $V_{max}$ and $K_m$ generated from the initial rate data and a linear regression analysis of Eq. 5 are listed in Table 4 along with $V_{max}/K_m$. The values of $V_{max}/K_m$ are the most meaningful kinetic parameters for comparing different substrates. A good substrate will have a large $V_{max}$ and a low $K_m$ (tight binding) and hence a large $V_{max}/K_m$ value. Compound 1 was more rapidly hydrolyzed than 2, indicating that the enzymatic reactivity of the prodrugs appears to predominantly depend on the esterified position. The liver microsomal enzymatic reconversion characteristics of the prodrugs were in the same order as the carboxylation activities of the prodrugs (mentioned above), indicating the reductive activation-independent carboxylation stimulated with the prodrugs was attributed to the MKH that was regenerated from the prodrugs. This result clearly indicates that prodrug 1 is the most useful candidate for the prodrug of MKH. Based on these findings, further studies were carried out using only prodrug 1.

**Effects of an Esterase Inhibitor on the MKH Regeneration and the Carboxylation** The effect of eserine, an esterase inhibitor, on the hydrolysis of 1 and on the carboxylation stimulated with 1, was studied in the human liver microsome system. The human liver microsome catalytic hydrolysis of 1 was strongly inhibited by the addition of eserine (Fig. 6). It has been postulated that the carboxylesterase is present in human liver microsome and that eserine is an inhibitor for carboxylesterase. Thus, the results suggested that the reversion of the prodrug was mainly catalyzed by the liver microsomal carboxylesterase. Eserine inactivated the carboxylation activity stimulated with 1 in the same manner as the inactivation of the microsomal enzymatic hydrolysis of 1 (Fig. 6). These results indicated that the carboxylation stimulated with the prodrugs required the hydrolytic process of the prodrugs to MKH. The DTT independent activity and the requirement of the cleavage to MKH of the prodrug in the carboxylation clearly indicate that MKH hydrolytically regenerated from the prodrug can act as a cofactor for the carboxylase itself without the reductive activation pathway of MKH.

**Carboxylation Activity of the Prodrug in the Warfarin-Poisoned Human Liver Microsome** To assess the usefulness of the prodrug in the treatment of warfarin-poisoning conditions, the warfarin effect on the carboxylation stimulated with 1 was determined and is shown in Fig. 7. In the presence of warfarin, the carboxylation stimulated with 1 exhibited significant activity, whereas the carboxylation stimulated with MK-4 in the presence of DTT was strongly inhibited. The inhibition effect of warfarin on the carboxylation stimulated with MK-4 in the presence of DTT indicates that the function of the coumarin-sensitive reductive activation pathway is provided in this human liver microsome test system. Thus, the significant carboxylation activity of 1 under the warfarin-poisoned conditions, where the vitamin K cycle was strongly inhibited, was clearly a result of the reductive activation-independent delivery of MKH.

The most important condition for the successful therapeutic application of prodrugs is the selective reconversion of

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**Table 4. Kinetic Parameters for the Regeneration MKH from the Prodrugs in the Human Liver Microsome Preparation at pH 7.2 and 25 °C**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ $(\times 10^{-4} \text{ mol} \cdot \text{mg}^{-1})$</th>
<th>$V_{max}$ $(\times 10^{-10} \text{ mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1})$</th>
<th>$V_{max}/K_m$ $(\times 10^{-9} \text{ min}^{-1})$</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>7.10</td>
<td>4.75</td>
<td>6.69</td>
</tr>
<tr>
<td>2</td>
<td>4.61</td>
<td>0.484</td>
<td>1.05</td>
</tr>
</tbody>
</table>

*a* The rate is presented as the quantity of MK-4 formed per min per mg of microsomal protein.
the prodrug in the human liver (site of action of MKH) relative to its reconversion at other sites and the activity of regenerated MKH as a cofactor for the carboxylase without the bioreductive activation process. In this study, we confirmed that esterification of MKH with N,N-dimethylglycine may be a potentially useful approach to obtain a prodrug for a systemic bioreductive activation-independent delivery system for MKH. As has been demonstrated, it is feasible to have the l-monoester I display a high aqueous solubility, high susceptibility to the human liver enzymatic hydrolysis, and activity as a cofactor for the carboxylase without the bioreductive activation process. It has also been confirmed that the bioreductive activation-independent carboxylation characteristic of I can produce significant carboxylation activity in the warfarin-poisoned human liver. Therefore, the bioreductive activation-independent delivery of MKH via a prodrug might lead to an efficient and reliable method for the treatment of hypoprophthrombinaemia induced in patients undergoing coumarin and cephalosporin therapies, in whom the bioreductive activation pathway of quinone vitamin K was inhibited.

REFERENCES