In Vitro Effect of Beta-Lactam Antibiotics and N-Methyl-Tetrazolethiol on Microsomal Vitamin K Epoxide Reductase in Rats

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Abstract—Liver microsomal vitamin K epoxide reductase activity was determined by measuring the formation of menaquinone-4 from the substrate menaquinone-4 2,3-epoxide. The enzyme was active when dithiothreitol (DTT) was used as a reducing agent, and the activity increased gradually with increasing concentrations of DTT. Glutathione and cysteine also functioned as reductants, but these physiological reductants showed less than 15% of the activity detected with 0.5 mM DTT. Addition of various beta-lactam antibiotics to the assay mixture for vitamin K epoxide reductase caused a slight inhibition of the activity. N-Methyltetrazolethiol (NMTT) and other heterocyclic thiol compounds also inhibited the enzyme activity in vitro depending on their concentrations. Most of these antibiotics and heterocyclic thiol compounds inhibited the enzyme activity only 10–25% in the in vitro assay system even when higher concentrations were added (5–10 mM). Among the compounds tested, methyl-thiadiazolethiol was the only compound that caused 50% inhibition of the enzyme activity. NMTT-induced inhibition was diminished gradually by increasing DTT concentrations. Kinetic analysis of the inhibitory action of heterocyclic thiol compounds showed competitive inhibition against the reductant DTT and non-competitive inhibition against the substrate. On the other hand, warfarin, a typical anticoagulant, showed different patterns in the inhibitory action: non-competitive inhibition against DTT and mixed-type inhibition against the substrate.

Recent clinical work has shown that several beta-lactam antibiotics, having an N-methyltetrazolylthiomethyl group at the 3-position of the cephem nucleus, cause vitamin K reversible hypoprothrombinemia in some cases (1–3). However, these antibiotics did not produce hypoprothrombinemia in vitamin K-sufficient volunteers (4). When vitamin K-deficient rats, but not K-sufficient rats, were given antibiotics, hypoprothrombinemic alterations in blood coagulation parameters such as prolongation of prothrombin time, decrease in prothrombin level and increase in descarboxyprothrombin were detected even in animals (5), which was consistent with the clinical data. Subsequently, Bechtold et al. demonstrated the transient increase in plasma concentration of vitamin K epoxide when subjects who had been pretreated with antibiotics were given vitamin K (6). Administrations of NMTT or NMTT-containing antibiotics to vitamin K-deficient rats caused them to develop hypo--

Abbreviations: NMTT, N-methyltetrazolethiol (1-methyl-1H-tetrazole-5-thiol); HTT, 1-(2-hydroxyethyl)-1H-tetrazole-5-thiol; DATT, 1-(2-dimethylamino)ethyl-1H-tetrazole-5-thiol; TDT, 1,3,4-thiadiazole-2-thiol; MTDT, 2-methyl-1,3,4-thiadiazole-5-thiol; LMOX, latamoxef; CMD, cefamandole; CPZ, cefoperazone; FMOX, flomoxef; CTM, cefotiam; CEZ, cefazolin; DTT, dithiothreitol; HPLC, high performance liquid chromatography; MK-4, menaquinone-4; MK-4-0, menaquinone-4 2,3-epoxide, MK-4-H2, dihydromenaquinone-4.
prothrombinemia concomitant with a depression of liver microsomal vitamin K epoxide reductase activity, although gamma-carboxylation and vitamin K reductase activities were not inhibited (5, 7–8). These data suggested that the antibiotics-induced hypoprothrombinemia developed under vitamin K-deficient conditions results from inactivation of the liver vitamin K epoxide reductase by antibiotics.

Vitamin K epoxide reductase is known to be highly sensitive to inhibition by coumarin anticoagulant drugs and is believed to be the target for their anticoagulant effect (10, 11). We thus compared the in vitro effect of beta-lactam antibiotics and related compounds with that of warfarin. The findings presented here indicate that NMTT and other heterocyclic thiol compounds display an in vitro inhibitory action on liver microsomal vitamin K-epoxide reductase, although only when very high concentrations are used. This in vitro inhibitory action of the heterocyclic thiol compounds could be completely reversed by thiol compounds, such as glutathione and DTT.

Materials and Methods

Animals: Jcl:Sprague Dawley strain male rats (7–8 weeks old) were used. Animals were kept in an air-conditioned room (25±1°C, 50–60% humidity) lighted 12 hr a day (8:00–20:00) and maintained on commercial rat chow (CA-1, Clea Japan, Inc., Tokyo) and water ad libitum. All animals were allowed at least 7 days to become acclimatized to the housing conditions prior to use in experiments.

Preparation of enzyme samples: Liver microsomes used for the activity determination were obtained from overnight-fasted rats as follows: fasted animals were killed by decapitation and their livers were quickly removed. Liver samples were homogenized in ice-cold 0.25 M sucrose containing 50 mM Tris-HCl buffer (pH 7.4), and the microsomal fraction was obtained by differential centrifugation as described previously (12). Microsomal pellets were stored for several days at −70°C under a nitrogen atmosphere, without any loss of the enzyme activity. The protein concentration of the sample was determined by the method of Lowry et al. (13) using bovine serum albumin as a standard.

Determination of enzyme activity: Microsomal vitamin K epoxide reductase activity was determined essentially as described previously (14) with slight modifications. One milliliter of reaction mixture contained microsomes (about 1.5 mg protein), 20 μM substrate (MK-4-0), 0.5 or 0.02 mM DTT, 0.15 M KCl and 0.2 M Tris-HCl buffer (pH 7.4). The substrate solution was prepared by mixing MK-4-0 in ether with an equimolar of 0.02% Emulgen 913 (Kao Atlas, Tokyo), followed by evaporation of ether and then mixing with 0.2 M Tris-HCl buffer (pH 7.4) containing 0.15 M KCl. The reaction was initiated by adding DTT. After 10-min incubation at 25°C with moderate shaking, the reaction was terminated by adding 2 ml of a mixture of isopropanol and hexane (3:2, v/v) and mixing on a Thermomics mixer (Thermomics Co., Tokyo) for about 10–15 sec. After centrifugation, 0.5 ml of the hexane layer was removed, evaporated under a nitrogen atmosphere and redissolved in 0.2 ml of isopropanol. All these procedures were carried out under dark conditions under an orange light. A portion of this extract (0.1 ml) was analyzed by HPLC using an instrument equipped with a Shimadzu LC-3A pump, a
Shimadzu SPD-2A detector (set at 254 nm) and a Shimadzu C-R2A chromatopac. The column was Cosmosil 5C18 (4.6 mm i.d.×150 mm) with a MPLC guard column (4 mm i.d.×30 mm, Brownlee Lab.). The mobile phase used was a mixture of methanol and water (99:1, v/v) at the flow rate of 1.0 ml/min. Under these analytical conditions, derivatives of vitamin K were separated and detected as shown in Fig. 1, and the impurities derived from microsomal components did not interfere with the signals of vitamin K derivatives. The epoxide reductase activity was determined by calculating the amount of vitamin K formed. After the addition of 3.1-5.5 μg of MK-4 and MK-4-0 to the microsomal suspension, the mixture was treated as above, with about 86 and 91% recoveries of MK-4 and MK-4-0, respectively.

**Antibiotics and chemicals:** LMOX, CMD and FMOX were obtained from Shionogi & Co. (Osaka), CPZ from Toyama Chemical Co. (Tokyo), CTM from Takeda Chemical Industries (Osaka), and CEZ from Fujisawa Pharmaceutical Co. (Osaka). NMTT, DATT, HTT, TDT and MTDT were prepared in the Shionogi Research Laboratories. Chemical structures of antibiotics and heterocyclic thiol compounds employed for the experiments are shown in Fig. 2. MK-4-H2 and MK-4-0 were also synthesized in our laboratories, and these derivatives were purified chromatographically prior to use. Other chemicals of the purest grade available were obtained commercially and used for the experiments without further purification.

**Results**

Microsomal vitamin K epoxide reductase: Under the assay conditions described in "Methods", enzymatic conversion of MK-4-0 to MK-4 was detected by HPLC techniques. Several assay conditions were compared to estimate the optimum assay system. Enzyme activity increased slightly with increasing pH, but the pH-activity curve showed no optimum pH between pH 6–9. Thus, the pH of the reaction system was set at 7.4. Since the solubilization of microsomal enzymes with detergent caused only slight activation (about 10%) of the activity, intact microsomes were employed as the enzyme source. The require-
ment of reductants for the action of vitamin K epoxide reductase is generally accepted, although its physiological reductant(s) is still unknown (10, 14). When the epoxide reductase activity was measured using various reductants, the activity increased markedly with increasing DTT concentrations. On the other hand, lower enzyme activity was detected with the addition of physiological reductants such as glutathione or cysteine (Table 1). The activity detected by the addition of physiological reductants was less than 15% of that obtained with 0.5 mM DTT. When the cytosol fraction was added to the reaction mixture as the source of physiological reductants, lower activity was also detected. We thus decided to use DTT as the reductant, although it is not a physiological component.

Under the assay conditions employed, the reaction proceeded linearly for about 10 min, and a close correlation between the formation of MK-4 and microsomal concentration was obtained when the reaction mixture (1 ml) contained 1–3 mg of microsomal proteins. Using our assay system, the enzyme activity in liver microsomes obtained from intact and overnight-fasted rats were 51.6±10.6 and 68.5±6.4 pmol/min/mg protein (n=5), respectively, in the presence of 0.5 mM DTT. Thereafter, fasted animals were used for the experiments. Based on these results, the microsomal vitamin K epoxide reductase activity was determined under the conditions described in "Methods".

In vitro effects of beta-lactam antibiotics and 3'-position substituents on vitamin K epoxide reductase: The effects of various beta-lactam antibiotics on the epoxide reductase activity were determined in the reaction mixture containing 0.02 mM DTT as the reductant. As shown in Table 2 (Exptl groups 1 and 2), addition of a high concentration (10 mM) of various beta-lactam antibiotics caused a slight inhibition of the enzyme activity; 10–25% decrease in the activity was detected with the NMTT-containing antibiotics (LMOX and CPZ) or the HTT-containing antibiotic (FMOX). Although no inhibition was detected with CEZ, slight inhibition was observed with CTM, which has DATT and not NMTT as the 3'-position substituent of the cephem nucleus (Fig. 2). Addition of various heterocyclic thiol compounds, which are 3'-position substituents of beta-lactam antibiotics, caused no or slight inhibition of the enzyme activity. Among the thiol compounds tested, MTDT showed a marked inhibitory effect on the enzyme activity (Table 2, Exptl group 2).

Using NMTT as a model of heterocyclic thiol compounds, the effect of DTT on the inhibitory action of NMTT was determined. As shown in Fig. 3A, microsomal epoxide reductase activity detected in the presence of 5 mM DTT was clearly suppressed by adding 5 mM NMTT, but the inhibitory action of NMTT was diminished gradually by increasing concentrations of DTT. It was also suppressed by addition of a high concentration of glutathione instead of DTT (data not shown). The activity detected in the presence of 0.02 mM DTT was depressed gradually with increasing NMTT concentrations (Fig. 3B). The results indicated that higher DTT concentrations would not be suitable for analyzing the in vitro inhibitory

Table 1. Effects of various reductants on microsomal vitamin K epoxide reductase

| Reductant added | Concentration (mM) | Activity (pmol/min/mg protein) |
|-----------------|--------------------|-------------------------------|
| DTT             | 0.5                | 65.37±7.29 (100)              |
| DTT             | 0.1                | 25.23 (38.6)                  |
| DTT             | 0.05               | 16.66 (25.5)                  |
| Glutathione     | 5.0                | 3.14 (5.4)                    |
| Cysteine        | 5.0                | 9.41 (14.4)                   |

Microsomal enzyme activity was determined as described in "Methods", except that various concentrations of reductants were added to the reaction mixture. The enzyme activity obtained in the presence of 0.5 mM DTT represents the mean±S.E. of 3 different determinations. Relative enzyme activity is shown in parenthesis.
action of heterocyclic thiol compounds, although higher activities of vitamin K epoxide reductase were detected in the presence of higher concentrations of DTT.

Mechanism of the in vitro inhibitory action of NMTT: As described above, microsomal vitamin K epoxide reductase was inhibited in vitro by antibiotics and heterocyclic thiol compounds in the presence of lower concentrations of reductant. Preincubation of enzyme samples with NMTT did not enhance the inhibitory action of NMTT (data not shown). Figure 4 (inset) indicates the relationship between the epoxide reductase activity and the inhibitory effect of 5 mM MNTT with various DTT concentrations. A Lineweaver-Burk plot showed competitive inhibition by NMTT of the epoxide reductase activity.

Table 2. Effects of various beta-lactam antibiotics and their 3'-position substituents on microsomal vitamin K epoxide reductase

| Expt group | Concentration (mM) | Activity (pmol/min/mg protein) | Relative activity |
|------------|-------------------|--------------------------------|------------------|
| 1          |                   |                                |                  |
| Control    | 5                 | 10.9±0.1**                    | 89.3             |
| LMOX      | 10                | 10.3±0.1**                    | 84.4             |
| CMD       | 10                | 11.3±0.2*                     | 92.6             |
| CPZ       | 10                | 11.3±0.2*                     | 92.6             |
| CTM       | 10                | 11.3±0.2*                     | 92.6             |
| FMOX      | 10                | 10.3±0.1**                    | 84.4             |
| NMTT      | 5                 | 12.2±0.5                      | 100              |
| 2          |                   |                                |                  |
| Control    | 5                 | 12.9±0.3                      | 105.7            |
| FMKX      | 10                | 12.7±0.3                      | 104.1            |
| CEZ       | 10                | 11.2±0.2                      | 91.8             |
| NMTT      | 5                 | 12.7±0.3                      | 104.1            |
| HTT       | 5                 | 11.6±0.3                      | 95.1             |
| TDT       | 5                 | 6.1±0.4**                     | 60.0             |
| MTTD      | 5                 | 12.9±0.3                      | 105.7            |
| DATT      | 5                 | 12.9±0.3                      | 105.7            |

Antibiotics and their 3'-position substituents were added to the reaction mixture at the concentration shown in the table. The reaction was carried out in the presence of 0.02 mM DTT. The values in the table represent the mean±S.E. of 3 different determinations. *, **: statistically different (P<0.05 and P<0.01, respectively) from the control.
indicating that NMTT and DTT compete for the same binding site on the enzyme molecule (Fig. 4). The inhibitor constant ($K_i$) of NMTT against DTT was calculated to be 22.2 mM. The relationship between the epoxide reductase activity and the inhibitory effect of 5 mM NMTT with various concentrations of substrate, MK-4-0, is shown in Fig. 5 (inset). A Lineweaver-Burk plot of the epoxide reductase activity showed a non-competitive inhibitory pattern against the substrate, and the inhibitor constant of NMTT against the substrate was 24.4 mM. The results indicated that there were different binding sites for NMTT and the substrate MK-4-0 (Fig. 5). Since MTDT showed the strongest inhibitory action on vitamin K epoxide reductase among the heterocyclic thiol compounds tested (Table 2), its inhibitory pattern was compared with that of NMTT. The inhibitory action of MTDT was suppressed markedly by 0.5 or 1 mM DTT, as found for NMTT. Kinetic studies on the in vitro action of MTDT on the epoxide reductase demonstrated that the competitive and non-competitive inhibitions by MTDT of the enzyme activity against DTT and substrate,
respectively, were similar to those for NMTT (data not shown).

In vitro effect of warfarin: Warfarin is known to inhibit vitamin K-epoxide reductase in both in vivo and in vitro systems (11, 14). This in vitro effect on microsomal vitamin K-epoxide reductase was then determined to compare it with that of NMTT. As shown in Fig. 6, the epoxide reductase activity, detected in the presence of various concentrations of DTT and MK-4-0, was inhibited markedly by adding warfarin. A Lineweaver-Burk plot indicating the inhibitory effect of warfarin at various DTT concentrations showed a non-competitive inhibitory pattern. On the other hand, the plot indicating the inhibitory effect of warfarin at various MK-4-0 concentration showed a mixed inhibitory pattern. The results indicated that the inhibitory pattern of warfarin differed from that of heterocyclic thiol compounds.

In the reaction system containing 0.02
mM DTT, the in vitro effect of various concentrations of warfarin on the epoxide reductase activity was determined and compared with those of heterocyclic thiol compounds. As shown in Fig. 7, the activity could be inhibited by adding 1 μM or less concentrations of warfarin. The epoxide reductase activity was also inhibited concentration-dependently by heterocyclic thiol compounds, MTDT and NMTT, but millimolar concentrations were required to inhibit the activity.

Discussion

Warfarin and many other 4-hydroxycoumarin drugs are potent inhibitors of vitamin K-dependent protein formation and have consequently found extensive use in clinical medicine. Warfarin is known to inhibit both DTT-dependent vitamin K reductase and vitamin K 2,3-epoxide reductase activities in rat liver microsomes in vitro (15–17). Administration of warfarin causes the depression of liver microsomal vitamin K 2,3-epoxide reductase concomitant with the accumulation of endogenous substrates for vitamin K-dependent carboxylase in plasma and various organs (18). The in vitro inhibitory action of warfarin was also detected in our experimental system as shown in Figs. 6 and 7.

Recently, NMTT-containing antibiotics have been reported to produce hypoprothrombinemia in vitamin K-deficient patients and rats (1–3, 19, 20). Uchida et al. (7, 21) checked the effects of antibiotics on the blood coagulation system in vitamin K-deficient conventional and germ-free rats, and they concluded that menaquinones synthesized by intestinal bacteria do not play a major role in synthesis of clotting factors. Their data suggested that the inhibition site of antibiotics is the biosynthetic process of clotting factors in mammalian liver, but not the intestinal bacteria synthesizing menaquinones. In the antibiotics-induced hypoprothrombinemic rats, the accumulation of endogenous substrates for gamma-glutamylcarboxylase was detected (22). However, microsomal carboxylation activity in the hypoprothrombinemic rats was not altered when the activity was determined by the use of either MK-4-H2 or MK-4 plus NADH as a cosubstrate (5, 7, 23). The results suggested that the antibiotics did not modify the activities of both gamma-glutamylcarboxylase, an essential enzyme for the synthesis of clotting factor, and vitamin K reductase. On the other hand, administration of NMTT-containing antibiotics and NMTT itself caused a decrease in vitamin K-epoxide reductase (8), similar to treatment with warfarin. Liver microsomal vitamin K epoxide reductase was inhibited by various antibiotics and their 3’-position substituents even in the in vitro reaction system (Table 2). However, the concentrations of antibiotics and heterocyclic thiol compounds required to inhibit the enzyme activity were very high compared with that of warfarin (Fig. 7), indicating a weak inhibitory action of antibiotics and their substituents.

Most of the NMTT-containing antibiotics and NMTT itself showed the inhibitory activity in the reaction system employed here. Inhibition of enzyme activity was also detected with FMOX and its 3’-position substituent HTT (Fig. 2). On the other hand, CEZ did not affect the enzyme activity, although its 3’-position substituent MTDT showed the strongest inhibition of the activity among the heterocyclic thiol compounds examined. These results suggest no structural correlation in the in vitro inhibitory action between antibiotics and their 3’-position substituents. The inhibitory activity of these thiol compounds was thought to be due to the interaction of the thiol group of the compounds with the enzyme protein, because the inhibition was diminished by DTT concentration-dependently and competitively (Figs. 3 and 4).

In the in vitro system, the inhibitor constant of NMTT was 22–24 mM. When LMOX was administered to rats intravenously at 1,000 mg/kg, the highest LMOX levels in plasma and liver were 3.1 mg/ml and 480 μg/g, respectively. The peak plasma and liver levels of NMTT released in the body were 21.7 μg/ml (0.19 mM) and 11.2 μg/g (97 nmoles/g), respectively. Furthermore, LMOX and NMTT disappeared rapidly from plasma with t1/2 of 15.4 and 20.9 min, respectively, and the liver clearance (t1/2) of LMOX and NMTT was 38.0 and 32.9 min, respectively (24). These results indicate that the concen-
tration of NMTT required to inhibit microsomal vitamin K epoxide reductase in vitro is very high compared with the plasma and liver levels of NMTT even after the higher doses of LMOX. It suggests that the inhibition mechanism of hepatic vitamin K epoxide reductase in vivo differs from that of the mechanism in vitro. Creedon and Suttie suggested that NMTT is required to convert more active metabolite(s) for the reaction of inhibitory action. We cannot presently identify the active metabolite(s), and thus further studies are required to clarify the effects of antibiotics and heterocyclic thiol compounds on the liver vitamin K metabolizing system.

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