In Vitro Effects of Various Heterocyclic Thiol Compounds and \( \beta \)-Lactam Antibiotics on Vitamin K-Dependent \( \gamma \)-Glutamylcarboxylation Activity in Liver Microsomes

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Abstract—The in vitro effect of N-methyltetrazolethiol (NMTT), one of the common substituents at the 3'-position of the cephem in various \( \beta \)-lactam antibiotics, on liver microsomal \( \gamma \)-glutamylcarboxylation (\( \gamma \)-carboxylation) activity was examined using solubilized rat liver enzyme. The enzyme activity was inhibited by coexisting with NMTT and NADH, and this inhibitory activity could be suppressed by the addition of a sulfhydryl compound such as dithiothreitol (DTT), glutathione or cysteine. Various five-membered heterocyclic thiol compounds exhibited concentration-dependent inhibition of microsomal \( \gamma \)-carboxylation activity. These inhibitory actions diminished markedly in the presence of 1 mM DTT. In vitro \( \gamma \)-carboxylation activity also decreases upon addition of various \( \beta \)-lactam antibiotics at 1 or 10 mM, depending upon the concentration of the drug. Among the heterocyclic thiol compounds, there is a correlation between their inhibitory activities and hydrophobicities. Thus, the in vitro inhibitory activity of heterocyclic thiol compounds and \( \beta \)-lactam antibiotics on microsomal \( \gamma \)-carboxylation activity is not correlated with their molecular structures, but rather depends on their hydrophobicities and with the concentrations in the reaction mixture.

The vitamin K-dependent \( \gamma \)-glutamylcarboxylase (\( \gamma \)-carboxylase) system in liver microsomes catalyzes the post-translational modification of prothrombin precursor to form biologically active prothrombin and other vitamin K-dependent plasma clotting factors: VII, IX, and X (1-3). The modification involves the carboxylation of specific glutamyl residues to form \( \gamma \)-carboxyglutamyl residues.

Recently, repeated administration of some \( \beta \)-lactam antibiotics, having the N-methyltetrazolylthiomethyl group as the 3'-position substituent of the cephem nucleus, have been reported to produce hypoprothrombinemia (4-7). As a possible mechanism of this increased incidence, Lipsky pointed out that the common side chain structure, N-methyltetrazolethiol (1-methyl-1\( H \)-tetrazole-5-thiol, NMTT), could inhibit the liver microsomal \( \gamma \)-glutamylcarboxylation (\( \gamma \)-carboxylation) reaction in vitro, and he proposed that it may cause hypoprothrombinemia in vivo (8-10). Subsequently, several investigators examined the in vitro effects of \( \beta \)-lactam antibiotics and NMTT on microsomal enzymes (11-14). NMTT is known to inhibit the enzyme activity only in the presence of NADH (14, 15).

\( \beta \)-Lactam antibiotics contain various heterocyclic thiol compounds as the 3'-position substituent of the cephem nucleus. We tried to find whether these heterocyclic thiol compounds inhibit the vitamin K-dependent \( \gamma \)-carboxylation activity, like NMTT does. This paper reports that all the heterocyclic thiol compounds tested show the inhibition under the experimental conditions employed.
Materials and Methods

Animals: Sprague-Dawley male rats (7–9 weeks old) were obtained from Clea Japan, Inc. (Ishibe, Shiga-ken) and were kept in an air conditioned room (24–26°C, 50–60% relative humidity), lighted 12 hr a day (8.00–20.00), and fed an ordinary rat diet (CA-1, Clea Japan, Inc., Tokyo) ad libitum. Rats were starved for one night before they were killed, to induce a higher activity of liver microsomal γ-carboxylation (16). During this starvation, the rats were kept on suspended wire nets to prevent vitamin K intake by coprophagy. Tap water was supplied ad libitum throughout all the experiments.

Chemicals: Heterocyclic thiol compounds employed for this study were 1,3,4-thiadiazole-5-thiol (TDT), 2-methyl-1,3,4-thiadiazole-5-thiol (MTDT), 1-methyl-1H-1,3,4-triazole-5-thiol (4-MTT), 1H-1,2,3-triazole-5-thiol (2-TT), NMTT, 1-(2-dimethylamino)ethyl-1H-tetrazole-5-thiol (DATT), 1-hydroxyethyl-1H-tetrazole-5-thiol (HTT), 1-carboxymethyl-1H-tetrazole-5-thiol (CMTT) and 1-phenyl-1H-tetrazole-5-thiol (PTT), and their chemical structures are shown in Fig. 1. All these heterocyclic compounds and the pentapeptide substrate (Phe-Leu-Glu-Glu-Leu) were prepared in our laboratories. Latamoxef (or moxalactam, LMOX) and cefamandole (CMMD) were obtained from Shionogi & Co. (Osaka), cefotiam (CTM) from Takeda Chemical Industries (Osaka), cefoperazone (CPZ) from Toyama Chemical Co. (Tokyo), cefazoline (CEZ) from Fujisawa Pharmaceutical Co. (Osaka) and cefoxitin (CFX) from Daiichi Pharmaceutical Co. (Tokyo). Other reagents of the purest grade available were obtained commercially and used without further purification.

Determination of γ-carboxylation activity: Vitamin K-dependent γ-carboxylation activity was determined using liver microsomes prepared from overnight-fasted rate (16). Microsomal pellets were suspended in SIK buffer (0.25 M sucrose, 50 mM imidazole and 0.5 M KCl at pH 7.2) containing 0.6% (v/v) Triton X-100, and they were used for the assay as an enzyme source (Ms-suspension) in some experiments. The resulting microsomal suspension was centrifuged at 105,000×g for 60 min, and the supernatant

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Chemical structure</th>
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<tr>
<td>TDT</td>
<td><img src="image" alt="TDT structure" /></td>
</tr>
<tr>
<td>MTDT</td>
<td><img src="image" alt="MTDT structure" /></td>
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<tr>
<td>2-TT</td>
<td><img src="image" alt="2-TT structure" /></td>
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<tr>
<td>CMTT</td>
<td><img src="image" alt="CMTT structure" /></td>
</tr>
<tr>
<td>PTT</td>
<td><img src="image" alt="PTT structure" /></td>
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Fig. 1. Chemical structure of five-membered heterocyclic thiols.
fraction was used as the source of enzyme (Ms-supernatant) in most of the experiments. Except where specified otherwise, the reaction mixture (0.5 ml SIK buffer at final volume) contained 0.2 ml microsomal preparation (0.9–1.2 mg protein), 0.24% (v/v) Triton X-100, 1 mM pentapeptide substrate, 2 mM NADH and the vitamin K cofactor (0.1 mM phylloquinone, VK₁). In some cases, 0.8 mM pyridoxal-5’-phosphate (PAL-P) was added to the mixture.

The reaction was initiated by adding VK₁ in 10 μl ethanol, test compound in SIK buffer, and sodium [¹⁴C]-bicarbonate solution (10 μCi, final concentration of 0.35 mM). Heterocyclic thiol compounds were dissolved in SIK buffer, and the resulting solution was adjusted to pH 7.2. The reaction was carried out at 17 °C for 60 min in the dark and then stopped by adding 2 ml of 10% (w/v) trichloroacetic acid. After centrifugation, the supernatant fraction was gassed with carbon dioxide (1 liter/min) for 5 min, and the radioactivity, fixed in a 0.5 ml aliquot, was determined by liquid scintillation spectrometry.

The microsomal total protein content was determined by the Bio-Rad Protein Assay Kit (17) using bovine serum albumin as a standard.

Determination of hydrophobic parameter: The parameter (log Pc) showing the hydrophobicity of various test compounds was calculated by the CLOGP3 program (version 3.33, Pomona College, Calif., U.S.A.) using the partition coefficient between water and n-octanol (18, 19). An experimental hydrophobic parameter of the compounds (Rₘ value) was also obtained by a reversed phase t.l.c. method (20). A high performance thin layer plate (RP-2 F₂₅₄; Merck, Germany) was used for the determination. The mobile phase employed was veronal buffer (pH 7.35) or sodium acetate/HCl buffer (pH 6.11). Acetone (30%, v/v) was added to both cases when used.

Results

Effects of sulfhydryl reductant: As reported previously (16), the microsomal γ-carboxylation activity is stimulated by some sulfhydryl reagents such as DTT, but inhibited in the presence of NADH by NMTT (14, 15), which contains the thiol moiety within the molecule. Because these differing effects of thiol com-

![Fig. 2. Effects of various concentrations of thiol compounds on the inhibitory action of NMTT on microsomal γ-carboxylation in vitro. Enzyme activity was determined using the reaction mixture described in Methods, except that the mixture contained various concentrations of sulfhydryl compounds such as DTT (A; circles), GSH (B; triangles), and cysteine hydrochloride (C; boxes). In addition, PAL-P (0.8 mM) was added to the mixture and Ms-supernatant (0.2 ml, 1.2 mg protein) was used for the assay. The reaction was carried out without preincubation, and the relative activity detected against the drug-free control (specific activity: 2880±117 dpm/mg protein/hr) was plotted as a function of the thiol compound concentration in the reaction mixture. Solid and dotted lines represent the relative activity in the presence and absence of 1 mM NMTT, respectively. Each point in the figure represents the mean±S.E. of three different determinations. * and **: statistically significant (P<0.05 and P<0.01, respectively) against the NMTT-free control.](image)
pounds on the microsomal γ-carboxylation reaction have been observed, we examined the effect of several thiol compounds on the inhibitory action of NMTT. As the concentration of DTT or glutathione (GSH) was increased, the γ-carboxylation activity increased (Fig. 2, A and B). Cysteine showed dual effects on microsomal γ-carboxylation; the activity was stimulated by adding 0.1–0.3 mM cysteine, but decreased concentration-dependently by adding higher concentrations of cysteine (Fig. 2C). Addition of 1 mM NMTT to the sulfhydryl reductant-free reaction mixture caused a decrease, but this inhibitory action could be suppressed by increasing concentrations of sulfhydryl reductant. Based on the graphical data, the concentration of DTT, GSH and cysteine required to diminish by 50% the inhibitory action of 1 mM NMTT was 0.03, 0.05 and 0.1 mM, respectively (Fig. 2). DTT- or GSH-induced stimulation and NMTT-dependent inhibition of the γ-carboxylation activity were observed similarly in both enzyme systems, Ms-suspension and Ms-supernatant. When microsomal enzyme samples were preincubated in the presence of both 1 mM NMTT and NADH, a marked decrease in the γ-carboxylation activity was detected as described previously (10, 13, 14). A sulfhydryl reductant such as DTT also caused attenuation of the inhibitory action of NMTT even in the preincubation system (data not shown).

Effects of five-membered heterocyclic thiol compounds: The effects of various heterocyclic thiol compounds that occur as a 3'-substituent of β-lactam antibiotics or analogues of such compounds on microsomal γ-carboxylation activity were determined using the in vitro reaction system (Fig. 3).

![Fig. 3](image_url)  
**Fig. 3.** Inhibitory effect of heterocyclic thiol compounds on liver microsomal γ-carboxylation reaction in vitro. The reaction mixture contained Ms-supernatant (0.9–1.1 mg protein), pentapeptide, PAL-P, NADH, VK, and 0.24% (v/v) Triton X-100 in SIK buffer (pH 7.2) as described in Methods. The specific activity of the drug-free control in the absence and presence of 1 mM DTT were 10504±1079 and 16708±651 dpm/mg protein/hr, respectively, and the relative enzyme activities in the presence of 1 mM (dotted columns) or 10 mM (heavily dotted) of test compound against the drug-free control are shown in the figure. Each value represents the mean±S.E. of three or four determinations. * and **: statistically significant (P<0.05 and P<0.01, respectively) against the control.
Addition of test compounds at 1 or 10 mM to the DTT-free reaction mixture caused decreases in the $\gamma$-carboxylase activity depending upon the concentration of the compounds. Except for a few compounds (MTDT and CMTT) that showed only weak inhibition at 1 mM, almost all exhibited statistically significant inhibition at the same concentration. PTT showed the strongest inhibition among the compounds tested. The inhibitory activity was not correlated with the structure of the ring moiety, i.e., whether it was thiadiazolyl (TDT and MTDT), triazolyl (4-MTT and 2-TT) or a tetrazolyl derivative (NMTT, DATT, HTT and PTT). The inhibition was also not correlated with the side chain structure at the 1-position nitrogen of the tetrazolyl rings. As in the case of NMTT (Fig. 2), the inhibitory action of heterocyclic thiol compounds was reduced remarkably by adding 1 mM DTT in most cases. DTT canceled the inhibition caused by nearly all of the test compounds at 1 mM, and by many of them at 10 mM, although 10 mM MTDT and CMTT still showed the inhibitory action even in the presence of 1 mM DTT. The PTT-induced inhibition of the $\gamma$-carboxylation activity was not recovered by 1 mM DTT.

Effect of $\beta$-lactam antibiotics: Figure 4 compares the effect of some $\beta$-lactam antibiotics on the microsomal $\gamma$-carboxylation reaction in vitro. When using 0.1 mM antibiotics, all drugs exhibited no inhibition (data not shown); and at 1 mM, they tended to inhibit the reaction to different degrees, but none to a statistically significant level. When 10 mM drug was used, all of the antibiotics showed stronger and statistically significant inhibition. The magnitude of inhibition of each antibiotic was not correlated with the ring structure of the substituent at the 3'-position of the cepham nucleus, such as the tetrazolyl (CMD, LMOX, CTM and CPZ) or thiadiazolyl (CEZ) group, nor with the side chain structure at the 1-position nitrogen of the tetrazolyl ring. CFX, having a noncyclic substituent (carbamoyloxymethyl group) at the 3-position, exhibited nearly the same inhibitory activity on the carboxylation reaction in vitro as observed using NMTT-containing antibiotics. Preincubation of microsomal enzymes together with antibiotic and NADH caused much stronger inhibition, as in the case of NMTT (data not shown).

Since the $\gamma$-carboxylase is known to be a microsomal membrane bound enzyme (1, 3, 18), the hydrophobic characteristics of heterocyclic thiol compounds were supposed to be an important factor for the interaction with the microsomal enzyme. Because of this, the "log $P_e$ value" of several heterocyclic thiol compounds that showed the concentration-dependent inhibitory action were calculated. The "$R_m$ value" of each compound was obtained by a reversed phase t.l.c. method at pH

![Fig. 4. Effects of some $\beta$-lactam antibiotics on microsomal $\gamma$-carboxylation activity in vitro. The reaction mixture contained Ms-suspension (1.2 mg protein), pentapeptide, NADH, VK, and 0.24% (v/v) Triton X-100 in SIK buffer (pH 7.2). The reaction was initiated by adding NaH$^{14}$CO$_3$ and test compound. The dotted columns (containing 1 mM or 10 mM (heavily dotted) antibiotic) represent the relative activity against the control (open column and broken line, 933±101 dpm/mg protein/hr). Each column represents the mean±S.E. of three different determinations. * and **: statistically significant (P<0.05 and P<0.01, respectively) against the drug-free control.]
7.35 or pH 6.11. The log P<sub>c</sub> values correlated quite well (correlation coefficient, r=0.979) with those of R<sub>m</sub>(pH 7.35) rather than with those of R<sub>m</sub>(pH 6.11) indicating that the log P<sub>c</sub> values reflect the hydrophobic characters of the compounds at neutral pH. The residual r-carboxylation activity (RA<sub>10</sub>) obtained in the presence of 10 mM heterocyclic thiol compounds (Table 1) correlated with the log P<sub>c</sub> value (r=-0.792), as shown in Fig. 5. Almost the same result (r=-0.731) was obtained in the relationship between the RA<sub>10</sub> and the R<sub>m</sub>(pH 7.35). These results indicated that hydrophobicity of the heterocyclic thiol compounds at neutral pH correlates well with their inhibitory activity on microsomal r-carboxylase.

### Table 1. Residual activities of the microsomal r-glutamylcarboxylation reaction and the hydrophobic parameters of heterocyclic thiol compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>log P&lt;sub&gt;c&lt;/sub&gt;</th>
<th>pH 7.35</th>
<th>pH 6.11</th>
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<tr>
<td>PTT</td>
<td>23.2%</td>
<td>1.485</td>
<td>0.768</td>
<td>-0.006</td>
</tr>
<tr>
<td>MTDT</td>
<td>88.2%</td>
<td>0.695</td>
<td>0.180</td>
<td>0.159</td>
</tr>
<tr>
<td>2-TT</td>
<td>60.6%</td>
<td>0.667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMTT</td>
<td>77.0%</td>
<td>-0.304</td>
<td>0.233</td>
<td>-0.225</td>
</tr>
<tr>
<td>CMTT</td>
<td>103.1%</td>
<td>-0.586</td>
<td>0.738</td>
<td>0.549</td>
</tr>
<tr>
<td>HTT</td>
<td>59.3%</td>
<td>-0.941</td>
<td>0.683</td>
<td>-0.651</td>
</tr>
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</table>

The residual activities were determined in the presence of 1 mM or 10 mM of test compound (see Fig. 3). The values of log P<sub>c</sub> were calculated according to the CLOGP3 program, using the partition coefficient between water and n-octanol. Experimental hydrophobic parameters of compounds (R<sub>m</sub> values) were obtained by reversed phase t.l.c. methods (details are described in the text).

### Discussion

Microsomal r-carboxylation activity was inhibited in vitro by various five-membered heterocyclic thiol compounds depending upon the concentration added (Fig. 3). Most of these compounds are 3'-position substituents of β-lactam antibiotics and are analogues of NMTT (Fig. 1). Interestingly, almost the same level of inhibition was observed with these compounds, although PTT exhibited the strongest inhibition. The inhibitory activity of these side chain-analogues was not correlated with the ring structures. Addition of 1 mM DTT suppressed the inhibitory action of these heterocyclic compounds, as in the case of NMTT, though inhibition of the enzyme activity by 10 mM PTT or MTDT was observed even in the presence of 1 mM DTT (Fig. 3). This may be considered as a protective action of DTT to the oxidative denaturation of the active sulfhydryl group in the enzyme. PTT and MTDT were supposed to have a high affinity to the enzyme molecule, which overcomes the protective action of DTT. The inhibitory activity of these heterocyclic thiol compounds was correlated with their hydrophobicity at neutral pH (Fig. 5), but not with the polarity of the thiol group in each compound (data not shown).

All of the β-lactam antibiotics examined here similarly inhibited microsomal r-carboxylation activity as NMTT did in vitro (Fig. 4), especially when they were used at higher concentrations (10 mM). The antibiotics...
contain various heterocyclic thiol groups at the 3'-position of the cephem nucleus; CMD, LMOX, CTM or CPZ have NMTT, and CEZ has the thiadiazolylthio group. Interestingly, although CFX has the carbamyloxymethyl group which is not a heterocyclic thiol compound, as the 3-position substituent, it exhibited inhibitory activity similar to the other β-lactam antibiotics and NMTT (Fig. 4). These results strongly suggested that the in vitro inhibitory activity of these antibiotics is correlated simply both with their concentrations in the reaction mixture and with their hydrophobicity. Nearly the same results were obtained in another assay system using vitamin K hydroquinone (12). As found among these in vitro experiments, the inhibition required higher levels of β-lactam antibiotics (3–10 mM), but not necessarily the heterocyclic ring in the 3-position side chain.

In conclusion, the liver microsomal γ-carboxylation reaction to exogenous peptide substrate was inhibited by various β-lactam antibiotics or by their fragments (heterocyclic thiol compounds) in vitro. The inhibition did not occur due to the presence of NMTT but depended on the drug concentration. Relatively high drug concentrations (millimolar or more) were required for the inhibitory action and far less amount of DTT or GSH could suppress these inhibitory activities in vitro. The maximum plasma levels of NMTT have been reported to be 0.03 mM in human volunteers (21) who had been given LMOX (3 g × 4 doses, at 8-hr intervals) and 0.16 mM in rat at 5 min after the administration (i.v.) of 1 g/kg LMOX, but the levels decreased rapidly (22, 23). These levels are too low to cause the inhibition in vitro. Therefore, if in vivo inhibition proceeds similarly as Lipsky assumed (8–10), this inhibition of the liver microsomal γ-carboxylation reaction should not occur in vivo. Moreover, animal livers usually contain several millimolar GSH (24, 25), so this inhibition would be less likely to occur in vivo.

Acknowledgment: We sincerely thank Drs. M. Narisada and M. Yoshioka for their preparation of various heterocyclic thiol compounds and Dr. K. Inouye for the synthesis of the pentapeptide substrate.

References


