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Mechanism of the inhibition of the γ-carboxylation of glutamic acid by *N*-methylthiotetrazole-containing antibiotics

(hypoprothrombinemia/vitamin K/moxalactam/glutathione/disulfiram)

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ABSTRACT **Antibiotics that contain a 1-*N*-methyl-5-thiotetrazole (MTT) side group have been associated with hypoprothrombinemia. In a detergent-treated rat liver microsomal system, MTT inhibited the carboxylation of the γ carbon of glutamic acid, a necessary reaction in the synthesis of four of the clotting factors. In the present work, the inhibition by MTT was found to be slow in onset, with a lag time of 15 min before significant inhibition occurred. A preincubation of MTT with the microsomes decreased the lag time and increased the extent of inhibition. Glutathione at 1 mM was found to markedly decrease the ability of MTT to inhibit this reaction. The disulfide dimer of MTT was a more potent inhibitor of the system than was MTT, with inhibition detected as low as 1 μM dimer. Disulfiram also inhibited the carboxylation system. These results indicate that the sulfhydryl group of MTT is important for the inhibitory effect of MTT and suggest that a slowly formed metabolite of MTT may be directly responsible for the observed inhibition. The inhibitory mechanism of MTT may be analogous to that of disulfiram, which would explain some pharmacologic effects in common with disulfiram. In addition, the *in vitro* observations presented here and a closer examination of the clinical evidence raise the possibility that MTT-containing antibiotic-induced hypoprothrombinemia may not be a vitamin K reversible phenomenon.**

An increased incidence of hypoprothrombinemia has been associated with the use of several new β-lactam antibiotics, including cefamandole, moxalactam, and cefoperazone (1–3). It was believed that the hypoprothrombinemia was corrected by the administration of vitamin K; therefore, two mechanisms that relate to vitamin K have been proposed to explain the hypoprothrombinemia. One is that these antibiotics, which are secreted into the bile, kill the intestinal bacteria that produce vitamin K (4). However, the nutritional role in humans of menaquinones, the bacterial forms of vitamin K, is uncertain (5), and many antibiotics are secreted into the bile but are not associated with an increased incidence of hypoprothrombinemia (6). The hypothesis that we and others (6, 7) have proposed is that a common structural feature of these antibiotics, a 1-*N*-methyl-5-thiotetrazole (MTT) side group, causes a more direct inhibition of the vitamin K-dependent step in clotting factor synthesis—i.e., the γ-carboxylation of glutamic acid. In support of this hypothesis, preliminary findings demonstrated that the MTT side group is capable of the *in vitro* inhibition of the γ-carboxylation of glutamic acid in a detergent-treated rat liver microsomal system (8). This system contains enzymes capable of the reduction of vitamin K and the carboxylation of glutamic acid. The MTT side group is attached by a methylene linkage to the 3-position of the six-membered ring of the antibiotic (Fig. 1*A*). The rationale for examining the activity of MTT was that it is in a "leaving group" position when the β-lactam bond of the antibiotic undergoes nucleophilic attack. This may occur when the antibiotic interacts with bacterial enzymes or nonenzymatically in alkaline solutions (9). Free MTT has been found in the plasma of subjects who had been given moxalactam (10). We present evidence in this paper that MTT is a potent inhibitor of the microsomal carboxylation system and that the mechanism of the inhibition may be related to the sulfhydryl portion of MTT.

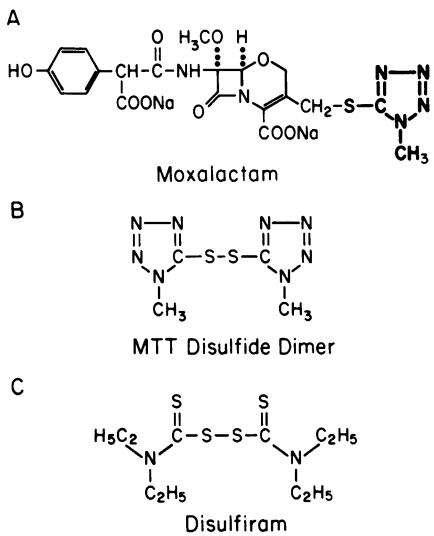

FIG. 1. (*A*) The structure of moxalactam. The MTT side group, shown in boldface, is attached to the carbon atom at the 3 position of the six-membered ring of moxalactam and is found also in cefamandole and cefoperazone. (*B*) The structure of the disulfide dimer of MTT. (*C*) The structure of disulfiram drawn to emphasize the structural similarity to the MTT disulfide dimer.

METHODS

Materials. The sodium salt of MTT and its disulfide dimer were a gift from Hoechst–Roussel Pharmaceuticals (Somerville, NJ). The pentapeptide L-phenylalanyl-L-leucyl-L-glutamyl-L-glutamylisoleucine, disulfiram, NADH, dithiothreitol, and *N*-ethylmaleimide were obtained from Sigma, and the $NaH^{14}CO_2$ was from New England Nuclear. Vitamin K1 was obtained as Aquamephyton (phylloquinone) from Merck Sharp & Dohme. Male Sprague–Dawley rats, 250–300 g, were obtained from Harland Sprague–Dawley (Walkersville, MD).

Microsome Preparation. Rat liver microsomes were prepared and the carboxylation of glutamic acid was determined

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Abbreviation: MTT, 1-*N*-methyl-5-thiotetrazole.

by a modification of the method of Houser *et al.* (11). Rats were fasted for 72 hr prior to decapitation and removal of the liver. Livers were homogenized, 33% (wt/vol), in 250 mM sucrose/0.08 M KCl/25 mM imidazole, pH 7.4, at 2°C. The homogenate was centrifuged at 10,000 × *g* for 10 min, and the resulting supernatant was centrifuged at 100,000 × *g* for 60 min. The pellet from this centrifugation was resuspended in the imidazole buffer to which had been added 1.5% (vol/vol) Triton X-100. The final volume of this solution was equal to the volume of the supernatant of the initial centrifugation. This suspension was centrifuged at 100,000 × *g* for 60 min, and the supernatant was taken as the microsomal preparation.

Carboxylation Assay. The carboxylation assay mixture, unless otherwise indicated, contained: the substrates, L-phenylalanyl-L-leucyl-L-glutamyl-L-glutamylisoleucine at 1.45 mM and 10 μCi (1 Ci = 37 GBq) of $NaH^{14}CO_2$ (43 mCi/mmol; final concentration, 0.4 mM); 500 μl of microsomal preparation (average = 3.5 mg of protein); and 2 mM NADH. Ten microliters of inhibitor was added to give a final volume of 580 μl. The pentapeptide, NADH, and inhibitor were added as solutions in imidazole buffer. After a 5-min preincubation at 22°C, reactions were initiated by the addition of 25 μg of vitamin K1 unless otherwise indicated in the text. The reaction mixture was incubated for 30 min at 22°C, the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid, and the mixture was centrifuged. The supernatant containing the radioactive peptide was removed and flushed with CO_2 gas for 15 min. An 0.8-ml aliquot was subjected to liquid scintillation counting to determine $^{14}CO_2$ incorporation into the peptide. Protein determinations were performed by the method of Lowry *et al.* (12).

RESULTS

The rate of γ-carboxylation by the microsomal system is shown in Fig. 2. After an initial lag period of about 2 min, this uninhibited rate was fairly linear over the 30-min incubation time. In the presence of 0.7 mM MTT, inhibition of activity was slow in onset, but significant inhibition began about 15 min after the start of the reaction. When MTT was preincubated with the microsomal system 15 min prior to the initiation of the reaction with vitamin K, the onset of the inhibition was more rapid and the degree of inhibition was greater.

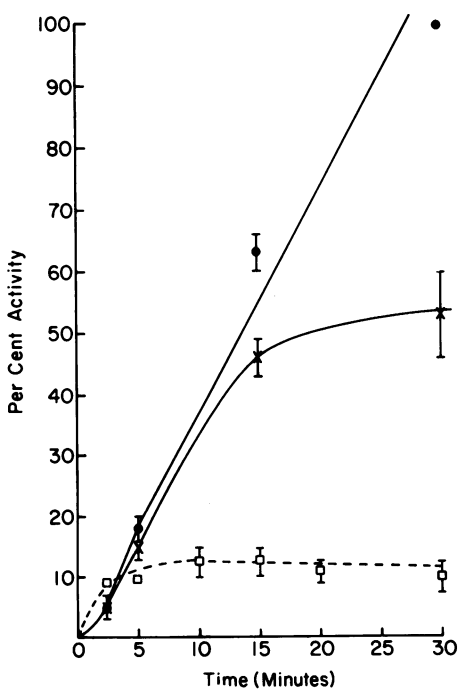

FIG. 2. Time course of the carboxylation reaction in the absence (●) and presence of 0.7 mM MTT with (□) and without (×) a 15-min preincubation of the microsomal system with MTT. The ordinate is the percentage of control carboxylation activity at 30 min. Points are the mean ± SEM of three to seven determinations.

Table 1. Effect on inhibition of an additional complement of reaction components and glutathione

Component	Amount added, μmol	% inhibition, mean ± SEM
Control	—	61 ± 3
NADH	1.16	64 ± 2
Pentapeptide	0.33	71 ± 3
$NaHCO_3$	0.23	76 ± 5
Vitamin K	0.03	66 ± 7
Glutathione	0.58	67 ± 4

To determine if this inhibition was due to depletion of one or more of the reaction components, additional reaction components were added individually to the microsomal system containing 1 mM MTT 15 min after beginning the reaction. None of the reaction components examined reversed the inhibition (Table 1).

Since greater inhibitory activity was observed with a 15-min preincubation of MTT with the microsomal system (Fig. 2), the effect of the duration of the preincubation on inhibition was examined. The inhibitory activity of 0.3 mM MTT increased in a linear manner with increasing time of preincubation (Fig. 3).

These results suggested that an interaction between MTT and the microsomes plays some part in the ultimate inhibition of the γ-carboxylation. The sulfhydryl group present on MTT might play a role in such an interaction. Therefore, the effect of glutathione on the ability of MTT to inhibit the γ-carboxylation system was examined. Glutathione was added to the reaction mixtures at the time of addition of MTT. In the presence of 1 mM glutathione, there was a considerable decrease in the ability of MTT to inhibit the reaction (Fig. 4). With glutathione present at 1 mM, it required 40 mM MTT to achieve 30% inhibition of the carboxylation system, whereas only 0.3 mM MTT was required in the absence of glutathione. Similar results were obtained with dithiothreitol. In the presence of 1 mM dithiothreitol, it required 20 mM MTT to produce a 30% inhibition.

The potential of glutathione to reverse existing inhibition was examined by adding glutathione 15 min after beginning the reaction. Glutathione was not able to reverse the inhibition (Table 1).

These findings are consistent with the hypothesis that the

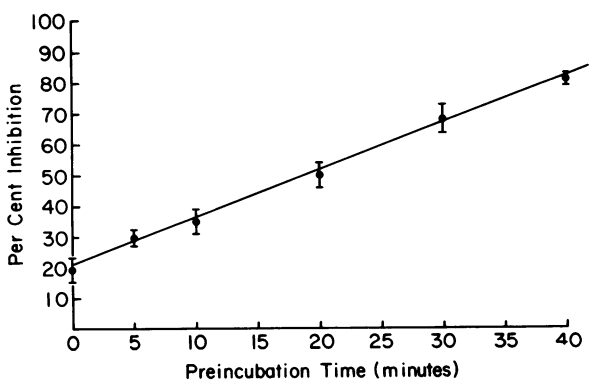

FIG. 3. The effect of the duration of preincubation of 0.3 mM MTT with microsomes on the extent of inhibition, measured at 30 min after the initiation of the reaction with vitamin K. Points are the mean ± SEM of three determinations.

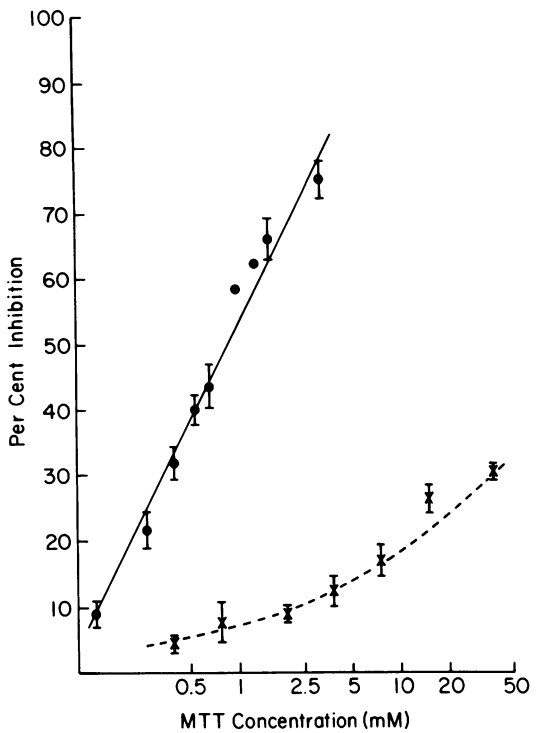

FIG. 4. The ability of MTT to inhibit the carboxylation system in the presence (×) and absence (●) of 1 mM glutathione. The concentration of the pentapeptide substrate was 2.9 mM, and the reaction was initiated by the addition of microsomes. Points are the mean ± SEM of at least three determinations. The points shown without the SEM are the average of two determinations.

interaction between MTT and the microsomal system may involve a sulfhydryl group or groups. To determine if the microsomal system itself contained such a sulfhydryl group, the effect of the sulfhydryl blocking agent *N*-ethylmaleimide on the microsomal system was examined. *N*-Ethylmaleimide inhibited the carboxylation system with a 50% inhibitory concentration of about 0.2 mM (Fig. 5).

If MTT were converted into a disulfide dimer (Fig. 1*B*) *in vivo*, it might interact with microsomes in this form. Fig. 6 shows a comparison of the degree of inhibition obtained with either MTT or the dimer. The disulfide dimer was a potent inhibitor of the carboxylation system, with inhibition detected at a concentration as low as 1 μM dimer, compared with MTT for which inhibition was not found below 100 μM.

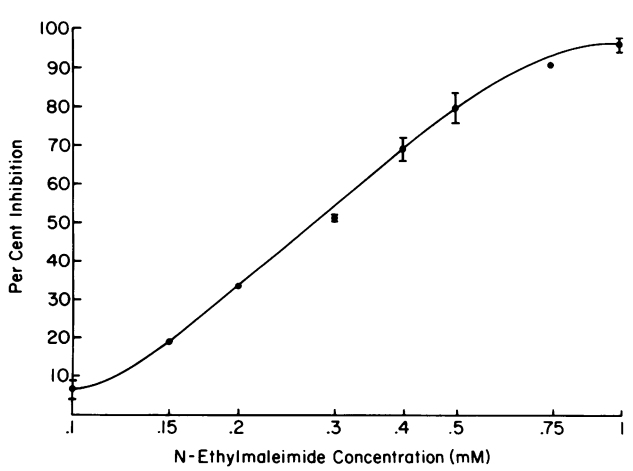

FIG. 5. The ability of *N*-ethylmaleimide to inhibit the carboxylation system. *N*-Ethylmaleimide was added to the reaction system prior to the initiation of the reaction with vitamin K. Points are the mean ± SEM of three determinations.

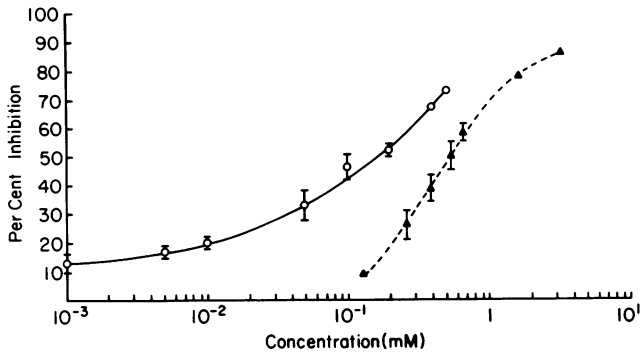

FIG. 6. The ability of MTT (▲) and its disulfide dimer (○) to inhibit the carboxylation system. Points are the mean ± SEM of at least three determinations.

The rationale for examining the disulfide dimer of MTT is that MTT as well as the parent antibiotics that contain MTT are capable of producing a disulfiram reaction *in vivo* (13). This reaction appears to be a consequence of the inhibition of aldehyde dehydrogenase and the subsequent toxic effects of the increased concentrations of acetaldehyde after ethanol ingestion (14). Disulfiram bears similar structural features to MTT and the disulfide dimer of MTT (Fig. 1*C*). To ascertain if there were further similarities in the pharmacologic effects of MTT and disulfiram, the ability of disulfiram to inhibit the carboxylation system was examined. Fig. 7 demonstrates that disulfiram inhibits the carboxylation system with inhibition detected in the micromolar range.

DISCUSSION

The data presented here support the hypothesis that MTT-containing antibiotics may produce hypoprothrombinemia by an effect of MTT on the γ-carboxylation system. It has been shown that the carboxylation system is relatively insensitive to inhibition by the parent antibiotics (8, 15). Thus, the inhibition of the γ-carboxylation system *in vivo* appears to be a consequence of the liberation of MTT from the parent antibiotic. Further support of this concept comes from the recent observation that MTT is able to produce hypoprothrom-

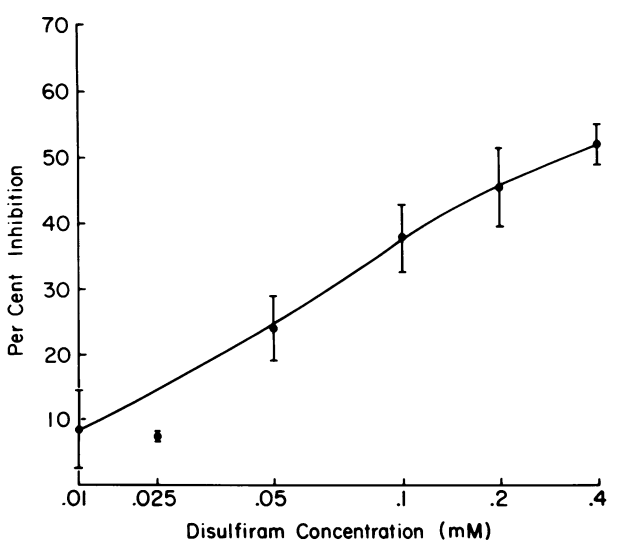

FIG. 7. The ability of disulfiram to inhibit the carboxylation system. Disulfiram was added in 10 μl of ethanol to the reaction mixture; 10 μl of ethanol was also added to controls. Points are the mean ± SEM of three determinations.

binemia when administered to rats maintained on vitamin K-deficient diets (16). The liberation of MTT *in vivo* may occur after secretion of the antibiotic into the bile and the breakdown of the antibiotic in the intestine. After liberation, MTT could be reabsorbed and circulated to the liver.

The finding that the disulfide dimer of MTT and disulfiram, another disulfide compound, are potent inhibitors of the carboxylation system suggests that a sulfhydryl group may be involved in the mechanism of the inhibition, as does the lower level of inhibition seen in the presence of glutathione or dithiothreitol. The stoichiometry of the preventive effect of glutathione on the inhibitory ability of MTT is inconsistent with a direct combination of glutathione and MTT. The failure of a 40-fold excess of MTT to completely overcome the effect of glutathione (Fig. 4) supports this conclusion.

Two possible explanations for the protective effect of glutathione are: first, that glutathione may maintain the reductive state of sulfhydryl groups in the microsomes, thus preventing their interaction with the drug; or second, that glutathione may combine with a metabolite of MTT that is responsible for the inhibition and directly blocks its further action. The requirement for MTT to form a metabolite also would be consistent with (*i*) the slow onset of inhibition by MTT (Fig. 2) and (*ii*) the enhancement of inhibitory activity of MTT by a preincubation (Fig. 3); thus, this may be a necessary step *in vivo*.

The failure of endogenous glutathione to prevent the inhibition from occurring *in vivo* may be due to localized production of the active metabolite of MTT. It is also possible that patients in whom the cellular levels of glutathione are low may be more susceptible to the development of hypoprothrombinemia from MTT-containing antibiotics. Therefore, the intracellular level of glutathione may be one of the factors responsible for the finding that only certain patients develop hypoprothrombinemia on these antibiotics.

The MTT dimer was a potent inhibitor of the carboxylation reaction. Dimer formation is consistent with the current understanding of the mechanism of action of the chemically and pharmacologically related compound disulfiram. In order to exert its effect *in vitro*, disulfiram must be in the intact disulfide form (17). *In vivo*, it is rapidly reduced to the less-active dithiodicarbonate form, but it may induce inhibition through disulfide re-formation or mixed disulfide formation. MTT may act in an analogous manner.

The observation that disulfiram inhibits the carboxylation system may provide a new explanation for the hypoprothrombinemia associated with this drug. Previously, it had been felt that the further prolongation of the prothrombin time caused by disulfiram in patients on warfarin was due to the inhibition by disulfiram of the metabolism of warfarin (18). The results presented here may indicate that disulfiram may have an additional and more direct effect on the carboxylation system.

Vitamin K added 15 min after the beginning of the reaction failed to reverse the inhibition (Table 1). This finding is consistent with clinical reports that it may take up to several days after the administration of vitamin K for recovery from MTT-containing antibiotic-induced hypoprothrombinemia to take place (1, 19). Although it has been commonly stated that the hypoprothrombinemia is vitamin K reversible, the delay in recovery seen clinically is not consistent with either vitamin K deficiency or a vitamin K reversible condition. In patients with true vitamin K deficiency, the correction of the hypoprothrombinemia by vitamin K administration takes place within hours (20).

The results presented in this paper support the theory that MTT-containing antibiotics produce hypoprothrombinemia through an effect of MTT on the γ-carboxylation system. The alternative hypothesis, that these antibiotics produce hypoprothrombinemia by killing intestinal bacteria that produce vitamin K, presupposes that the bacterial forms of vitamin K, menaquinones, produced in the colon, are a nutritional source of vitamin K for humans. Though this view has been held for many years (21), a careful review of the field indicates that there is little solid evidence for this view. Two studies in humans that have been quoted (22, 23) as evidence for this point involved a total of only 15 subjects, 12 on antibiotics and 3 not. In one study (22), neither the antibiotics nor their regimens were stated and, thus, it is impossible to assess the efficacy of the antibiotic regimens in killing intestinal bacteria. In the other study (23), neither neomycin nor tetracycline administration for 4 weeks was able to produce hypoprothrombinemia in four out of five subjects.

With animal studies, results are often confounded because of the failure to prevent coprophagy (24, 25), which allows for a dietary source of vitamin K. However, it is clear that hypoprothrombinemia can be induced in rats after 1 week by preventing coprophagia and administering a vitamin K-deficient diet without antibiotics (26). Intestinal bacteria were unable to prevent the development of hypoprothrombinemia under these conditions. We suggest that the commonly held belief that intestinal bacteria provide a nutritional source of vitamin K in humans be carefully reassessed.

Regardless of the outcome of this issue, the data in this paper indicate that MTT itself or a metabolite has a direct inhibitory effect on a necessary step in the synthesis of prothrombin. The complex microsomal carboxylation system assayed here is believed to be composed of three or more enzymes (27). It will be of interest to determine which part or parts of the system are inhibited by MTT and the molecular basis of that inhibition.

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