EFFECT OF BILIARY DIVERSION ON THE ABILITY OF CEFAMANDOLE TO INHIBIT VITAMIN K METABOLISM

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SUMMARY

The effect of biliary diversion on the ability of cefamandole, a methyltetrazole-thiol (MTT) containing antibiotic, to alter hoth hepatic vitamin K metabolism and the gamma-carboxylation of glutamic acid were examined in the rat, in order to understand the hypoprothrombinemia associated with MTT-containing antibiotics. At a dose of 3gm/kg, cefamandole decreased the activity of hepatic vitamin K epoxide reductase at 24 but not at 4 hours after its administration. This inhibition occurred with or without diversion of the bile duct from the intestine. When vitamin K was used as the cofactor in the enzymatic reaction, carboxylation of glutamic acid was also found to be reduced in both biliary diverted as well as in biliary intact rats. Carboxylation of glutamic acid was not reduced when vitamin K hydroquinone was used. These results suggest that part of the mechanism underlying the

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hypoprothrombinemia associated with MTT containing antibiotics is linked to the ability of the MTT group to inhibit vitamin K metabolism. Furthermore, biliary secretion of the intact antibiotic may not be required for the effect upon vitamin K metabolism to be observed.

I. INTRODUCTION

Antibiotics containing the methyltetrazole-thiol (MTT) leaving group have been associated with hypoprothrombinemia /1/. Since this effect is a result of decreased activity of vitamin K dependent clotting factors /2, 3/, studies of the mechanism underlying the hypoprothrombinemia have focused on vitamin K metabolism and the synthesis of vitamin K dependent clotting factors.

The mechanism by which MTT-containing antibiotics produce hypoprothrombinemia is the subject of controversy. Originally, it was thought that these antibiotics caused hypoprothrombinemia by depleting the intestinal flora, thereby causing a decrease in menaquinones, the forms of vitamin K which are produced by intestinal bacteria /4/. That theory has been challenged, since many antibiotics, including those without the MTT group, cause a decrease in the intestinal flora, but not all of these antibiotics are associated with an increased incidence of hypoprothrombinemia /5/. A theory which involves the MTT group has been put forth to explain the hypoprothrombinemia /5, 6/. MTT is a heterocyclic sulfhydryl group that is liberated when the beta-lactam bond of the antibiotic is cleaved /7/. MTT has been shown to inhibit *in vitro* the vitamin K dependent step in clotting factor synthesis, the gamma-carboxylation of glutamic acid /8, 9/. It has also been shown that MTT, *in vivo,* can inhibit the activity of vitamin K-epoxide reductase, the enzyme which reduces vitamin K-epoxide to the quinone form. /10/. Inhibition of this step may ultimately effect carboxylation in that this enzyme is involved in the metabolic cycle (Figure 1) of vitamin K, related to the carboxylation reaction. Vitamin K is ingested in the quinone form (K). The carboxylation reaction requires vitamin K to be in the hydroquinone (KH_2) form which is generated by the reduction of the quinone. The active hydroquinone is converted to an epoxide (KO) concurrent with the carboxylation of glutamic acid. This epoxide is reduced back to the quinone form by the epoxide reductase

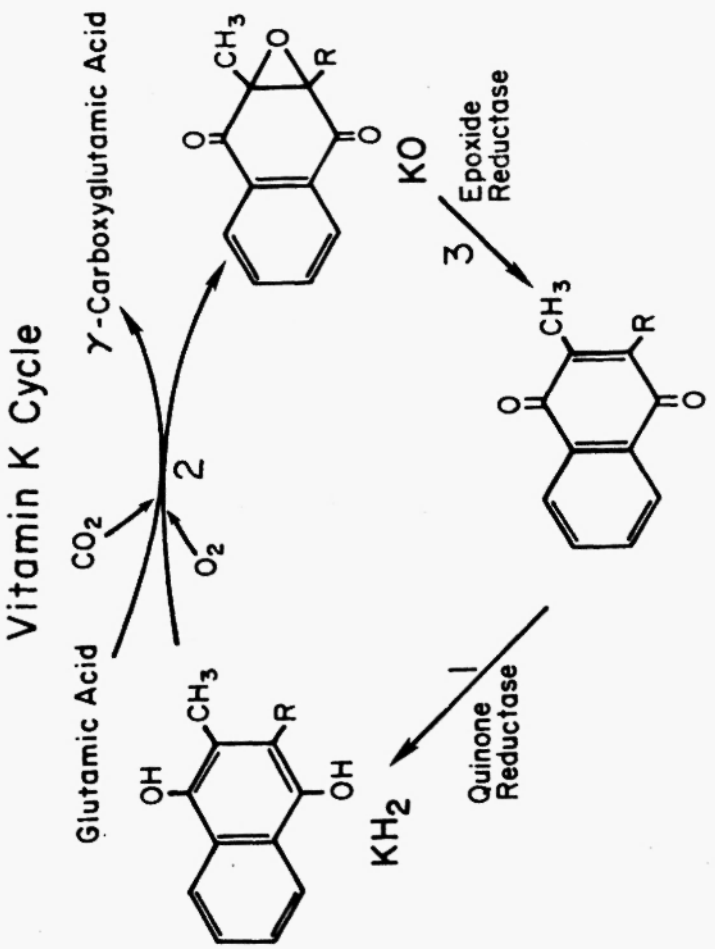

Fig. 1: Diagram of the vitamin K cycle.
The abbreviations used are K: vitamin K quinone, KH_2: vitamin K hydroquinone, and KO: vitamin K epoxide.

enzyme. As the body stores of vitamin K are low, this recycling must occur in order for the carboxylation to proceed /11/.

Since it has been shown that MTT *in vivo* inhibits vitamin K epoxide reductase, in these studies we have examined the ability of the intact, MTT-containing antibiotic, cefamandole, to inhibit this enzyme as well as the gamma-carboxylation of glutamic acid when administered *in vivo* to the rat. Preliminary experiments have suggested that in order to exert its effects, MTT must be liberated from the parent antibiotic. Because one possible site of liberation of MTT is the intestine, where, after biliary secretion of the antibiotic, the beta-lactam bond would be susceptible to either beta-lactamases of intestinal bacteria or the higher pH of the intestine, we have also examined the effect of biliary diversion on the potential of cefamandole to inhibit vitamin K epoxide reductase and the gamma-carboxylation of glutamic acid.

II. METHODS

2.1 Liver Microsome Preparation

Male Sprague Dawley rats, 250-300g , were obtained from Harlan Sprague Dawley and were fed vitamin K deficient diets (ICN Biochemicals Inc., Cincinnati, Ohio) for 48 hours and then fasted for 24 hrs prior to decapitation. Rats were housed in cages designed to prevent coprophagy. Carbon dioxide anesthesia was used prior to decapitation. The liver was immediately removed, minced, and immersed in 2ml/gm liver of a buffer containing 25mM imidazole, pH7.4, 250mM sucrose, 0.08M KCl. The liver was then homogenized for approximately 30 seconds with a Polytron homogenizer. The resulting suspension was then centrifuged at 10000g for 10 minutes, the supernatant was collected and centrifuged at 100000g for 60 min. The resulting pellet was homogenized in the buffer which contained 8.0mM CHAPS and was diluted with this buffer to a final concentration of 10mg protein/ml. This microsomal preparation was frozen at -70°C until use.

Measurement of vitamin K-epoxide reductase activity.

The activity of vitamin K epoxide reductase was assayed by a modification of the method of Wallin and Martin /12/ in the

microsomal preparation which contained 5.0mg of microsomal protein and 40uM vitamin K epoxide in ethanol which had been prepared by the method of Tischler, et al. /13/. The reaction was initiated by the addition of dithiothreitol (DTT) (Sigma Chemical Company, St Louis Mo), to give a final concentration of 0.5mM. Incubations were carried out at 21°C for 30 minutes. The reaction was terminated by the addition of 2ml of a 1:1 isopropanol:hexane mixture. Tubes were then vortexed for 20 seconds and centrifuged for 5min at 1300g. One ml of the resulting upper layer was removed and evaporated under a nitrogen atmosphere. The resulting solid was redissolved in 0.1ml of methanol and was stored at −70°C until analysis could be performed.

Vitamin K-epoxide reductase activity, defined as the degree of conversion of vitamin K epoxide to vitamin K quinone, was measured with a C-18 HPLC column with methanol as the mobile phase.

Measurement of the gamma-carboxylation of glutamic acid

The gamma-carboxylation of glutamic acid was assayed by the incorporation of ^{14}C labeled carbon dioxide into a pentapeptide containing glutamic acid as described /9/. In brief, reactions were run using the microsomal preparation described above. The components were L- phenylalanyl- L- leucyl- L- glutamyl- L- glutamylisoleucine, 1.45mM, and 10uCi of $NaH^{14}CO_2$ (43mCi/mM; final concentration 0.4mM); 500ul of microsomal preparation, and either .5mM DTT or 2mM NADH as described in the text. The pentapeptide and DTT were added as solutions in the imidazole buffer. Reactions were initiated by the addition of 25ugm of vitamin Kl, phylloquinone (used as Aquamephyton, Merck Sharp and Dohme), or vitamin K hydroquinone prepared by the reduction of vitamin K quinone /14/ and terminated 30 minutes later by the addition of trichloroacetic acid. Following centrifugation, the supernatant solution was flushed with unlabeled CO_2 and the incorporation of radioactive carbon dioxide into the pentapeptide was determined by liquid scintillation counting of an aliquot of the supernatant solution.

Antibiotic treatment

Rats were treated with 3g/kg intraperitoneally of cefamandole (Eli Lilly and Co., Indianapolis) 24 hours prior to sacrifice or with a

comparable volume of 0.9% saline unless otherwise indicated in the text.

2.2 **Surgical treatment**

Rats undergoing cannulization were anesthetized with 60mg/kg sodium pentobarbital. Following ventral midline laparotomy, canalization of the common bile duct was accomplished with PE-10 tubing which was diverted outside of the body.

Statistical evaluation

Data were initially analyzed by analysis of variance. Where differences were found, individual groups were compared using the t test which was corrected for multiple comparisons by the method of Bonferroni /15/.

III. RESULTS

In a preliminary experiment, the activity of hepatic epoxide reductase was measured four hours after the administration of 3gm/kg cefamandole to intact animals. This time interval was chosen because a previous study by others demonstrated that epoxide reductase activity was reduced at four hours after the administration of 100mg/kg of MTT /10/. The results in Table 1 indicate that epoxide reductase

TABLE 1

Activity of hepatic epoxide reductase 4 hours after the administration of cefamandole

Treatment	Epoxide Reductase Activity*
Control (n = 2)	20.1
Cefamandole (n = 2)	19.7

*pmoles vitamin K formed/mg microsomal protein/min

activity was the same in both controls and treated animals. Therefore the activity of epoxide reductase was examined at a later time, 24 hours after the administration of cefamandole. The results in Table 2 indicate that in the animals receiving cefamandole without biliary diversion there was a decrease in activity of epoxide reductase by approximately 43 per cent, as compared to animals which received saline. Animals which underwent biliary diversion also had a similar reduction, 42 per cent, in epoxide reductase activity. Therefore diversion of the bile from the intestine did not alter the ability of cefamandole to effect epoxide reductase. Nor did surgical manipulation of the bile duct alone effect enzyme activity, as controls of both non-diverted and diverted animals had similar activities. The results in Table 2 also indicate that the route of administration, whether intraperitoneal or subcutaneous, had no additional effect. Since the enzyme which reduces the epoxide to the quinone form of vitamin K may be the same enzyme which reduces the quinone (K) to the hydroquinone (KH_2) (steps 1 and 3 in Figure 1) this latter activity was also measured. However, the product of the enzyme, the hydroquinone form of vitamin K, is very unstable in the microsomal system. As the quinone must be reduced to the hydroquinone

TABLE 2

Effect of cefamandole on hepatic epoxide reductase activity at 24 hours

	Epoxide Reductase Activity* (mean ± SD) Treatment		
Biliary Manipulation	Control	Cefamandole	
		IP	SQ
None	19.3 ± 1.8 (n=8)	10.2 ± 1.6 (n=5)	11.3 (n=2)
Diverted	19.8 ± 2.7 (n=5)	11.6 ± 2.4+ (n=7)	12.8 (n=2)

* pmoles vitamin k formed/mg microsomal protein/min

\+ p 0.001 compared to control

in order for carboxylation to occur, the quinone reductase activity was assayed indirectly by measuring the carboxylation reaction starting with vitamin K in the quinone form (steps 1 and 2, Figure 1). The results in Table 3 indicate that there was an inhibition of carboxylation activity in the microsomes from rats treated with cefamandole as compared to controls and that biliary diversion did not alter this effect. This decrease in carboxylation activity could have been due to either an inhibition of the reduction of vitamin K quinone to the hydroquinone (step 1, Figure 1) or to a decrease in the carboxylation (step 2, Figure 2). In order to determine which was the case, the activity of the carboxylation reaction was assessed following the addition of the hydroquinone form of the vitamin. The results in Table 3 indicate that the carboxylation activity was the same in both the cefamandole treated and the control animals. Therefore, the decreased activity of the carboxylation reaction observed when the quinone was used to support the reaction was likely to be due to the inhibition of the reduction of the quinone.

IV. DISCUSSION

The results presented demonstrate that the activities of both hepatic vitamin K epoxide reductase and a DTT dependent quinone reductase are inhibited after the administration of cefamandole. However, there was no significant difference in the effect of cefamandole on rats that were treated and not bile duct cannulized and those that were treated and cannulized. Since *in vitro* studies /8/ suggest that free MTT, rather than the intact antibiotic, is needed for the inhibition of these enzymes then this result suggests that MTT release is not dependent on the secretion of cefamandole in the bile and its breakdown in the intestine. Liberation of MTT could occur elsewhere in the body, e.g. the liver. Mizojiri et al. /16/ found that in rats with the biliary system intact, approximately 11 per cent of labeled MTT in moxalactam was liberated from the parent compound. However, if the biliary system was diverted from the small intestine, there was still an approximate 4 per cent release of MTT. Therefore, in the case of another MTT containing antibiotic, moxalactam, MTT release can occur in the absence of excretion into the small intestine. These results may explain our observation that similar inhibition was found whether the biliary system was intact or diverted.

TABLE 3

Effect of cefamandole on the gamma-carboxylation of glutamic acid

	Carboxylation Activity* (mean ± SD) Condition			
	Quinone + DTT		Hydroquinone	
Biliary Manipulation	Control	Cefamandole	Control	Cefamandole
None	6893 ± 2851 (n=5)	2073 ± 860+ (n=5)	1366 ± 533 (n=5)	1254 ± 565 (n=5)
Diverted	9671 ± 4694 (n=5)	3264 ± 816+ (n=5)		

* CPM ^{14}C incorporated into glutamic acid.
+ $p < .03$ compared to control

The observation that the activities of both vitamin K epoxide reductase and the DTT dependent quinone reductase were reduced to approximately the same degree may indicate that the two activities may be a function of the same enzyme, a concept which has been proposed by others /11/. This inhibition is a function of the free MTT group rather than the intact antibiotic as previous studies by others have shown that MTT itself, when administered *in vivo* inhibits hepatic epoxide reductase activity /10/. In those studies, the administration of 100mg/kg of MTT resulted in enzyme inhibition that was maximal after three hours. Although the dose of cefamandole used in the present study contains in the intact molecule approximately seven and one half times the amount of MTT used in the previous study, inhibition of epoxide reductase was not detected at four hours, but was significant within 24 hours. The difference in timing of the inhibitory effect between intact antibiotic and free MTT may reflect the additional time needed for the release of MTT and the subsequent inhibition of the enzyme.

The dose of cefamandole, used in the present study, 3gm/kg, could release approximately 750mg/kg of MTT, if all the cefamandole were degraded; however, this is unlikely as it is known that 75 per cent of the administered cefamandole in the rat remains intact /17/. Although it is not known how much cefamandole releases MTT, it is known as mentioned above, that for the MTT-containing drug moxalactam, about 11 per cent of the administered dose releases free MTT in the bile duct intact rat. Therefore, if the extent of metabolism of cefamandole is similar to that of moxalactam, a dose of 3gm/kg of cefamandole may result in the release of approximately 75mg/kg of MTT. In biliary diverted rats, about 4 per cent of moxalactam releases MTT, and if cefamandole behaves in a similar fashion, then a 3gm/kg dose would result in the release of about 30mg/kg of MTT.

Although less MTT may have been released in the rats that had their bile ducts diverted, the degree of inhibition, 43 per cent, was similar to that in rats with bile ducts intact (47 per cent). This reduction in activity is similar to the fifty per cent reported by Creedon and Suttie /10/ when 100mg/kg of free MTT were administered. This latter study did not examine the effect of lower doses of MTT. The fact that similar extent of inhibition was found in both experiments may indicate that fifty per cent inhibition is the maximal extent of inhibition caused by

MTT. Further studies with both intact MTT containing antibiotics as well as with free MTT may clarify this issue.

The finding that the vitamin K-epoxide reductase activity was decreased 24 hours after administration of cefamandole suggests that the inhibition of vitamin K-epoxide reductase is irreversible. The half-life of cefamandole in the rat is 42 minutes /17/ and that of MTT is 21.5 minutes /16/. Therefore, at 24 hours virtually all of the cefamandole and free MTT should have been eliminated. Furthermore the method used to prepare the microsomal fraction would dilute any MTT or cefamandole remaining in the microsomes. If the inhibition were reversible, then a restoration of activity might be expected.

V. CONCLUSIONS

In conclusion, it was found that intraperitoneal administration of cefamandole succeeded in inhibiting the vitamin K-epoxide reductase activity as well as a quinone reductase activity in rat livers. Also, there was no significant difference between the effects of cefamandole in rats which received cefamandole intraperitoneally with or without bile duct cannulization, suggesting that intestinal degradation of cefamandole is not necessary for the inhibition of vitamin K-epoxide and quinone reductase activity.

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VII. REFERENCES

1. Lipsky, J.J. Antibiotic-associated hypoprothrombinemia. *J. Antimicrob. Chemother.,* 1988; **21**:281-300.
2. Mackie, I.J., Walshe, K., Cohen, H., et al. Effects of N-methyl-thiotetrazole cephalosporin on haemostasis in patients with reduced serum vitamin K^1 concentrations. *J. Clin. Pathol.,* 1986; **39**:1245-1249
3. MacLennan, F.M., Ah-See, A.K., Wong, A.E., Anderson J A., and Bennett B. Severe depletion of vitamin-K-dependent clotting factors during post-operative latamoxef therapy. *Lancet.* 1983; i:1215.
4. Hockman, R., Clark, J., Rolla, A., Thomas, S., Kaldany, A., and D'Elia J.A. Bleeding in patients with infections. *Arch. Intern. Med.,* 1982; **142**:1440-1442.
5. Neu, H.C. Adverse effects of new cephalosporins. *Ann. Intern. Med.,* 1983; **98**:415-416.
6. Smith, C.R., and Lipsky, J.J. Hypoprothrombinemia and platelet dysfunction caused by cephalosporin and oxalactam antibiotics. *J. Antimicrob. Chemother.,* 1983; **11**:496-499.
7. Boyd, D.B. and Lunn, W.H.W. Electronic structures of cephalosporins and penicillins. 9. Departure of a leaving group in cephalosporins. *J. Med. Chem.,* 1979; **22**:778-784.
8. Lipsky, J.J. N-methyl-thio-tetrazole inhibition of the gamma-carboxylation of glutamic acid: possible mechanism for antibiotic-associated hypoprothrombinemia. *Lancet,* 1983; ii:192-193.
9. Lipsky, J.J. Mechanism of the inhibition of the gamma-carboxylation of glutamic acid by N-methylthiotetrazole-containing antibiotics *Proc. Natl. Acad. Sci. USA.* 1984; **81**:2893-2897.
10. Creedon, K.A. and Suttie, J.W. Effect of N-methyl-thiotetrazole on vitamin K epoxide reductase. *Thrombosis Res.,* 1986; **44**:147-153.
11. Suttie, J.W. Recent advances in hepatic vitamin K metabolism and function *Hepatology.* 1987; **7**:367-376.
12. Wallin, R. and Martin, L.F. Warfarin poisoning and vitamin K antagonism in the rat and human liver. *Biochem. J.,* 1987; **241**:389-396.
13. Tishler, M., Fieser, L.F., and Wendler, N.L. Hydro, oxido and other derivatives of vitamin K_1 and related compounds. *J. Amer. Chem. Soc.,* 1940; **62**:2866-2871.
14. Sadowski, J.A., Esmon, C.T., and Suttie, J.W. Vitamin K-dependent carboxylase. Requirements of the rat liver microsomal enzyme system. *J. Biol. Chem.,* 1976; **251**:2770-2776.
15. Godfrey, K. Statistics in Practice. Comparing the means of several groups. *New Eng. J. Med.,* 1985; **313**:1450-1456.
16. Mizojiri, K., Norikura, R., Takashima, A., et al., Disposition of moxalactam and N-methyltetrazolethiol in rats and monkeys. *Antimicrob. Agents Chemother.,* 1987; **31**:1169-1176.
17. Sullivan, H.R., Due, S.L., Dau, D.L.K., Quay, J.F., and Miller, W M. Metabolic fate of [^{14}C] cefamandole, a parenteral cephalosporin antibiotic in rats and dogs. *Antimicrob. Agents Chemother.,* 1977; **12**:73-79.