CEFAZOLIN ADMINISTRATION AND 2-METHYL-1,3,4-THIADIAZOLE-5-THIOL IN HUMAN TISSUE: POSSIBLE RELATIONSHIP TO HYPOPROTHROMBINEMIA

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ABSTRACT:

Cephalosporin antibiotics with structures that include the heterocyclic leaving group 1-methyltetrazole-5-thiol (MTT) can cause hypoprothrombinemia and hemorrhage as a result of MTT-dependent inhibition of the \( \gamma \)-carboxylation of glutamate. The structure of cefazolin also includes a heterocyclic thiol, 2-methyl-1,3,4-thiaziazole-5-thiol (MTD), and this compound can also inhibit the \( \gamma \)-carboxylation of glutamate. However, unlike MTT, which is known to be present in vivo after the administration of drugs that include this structure, there have been no reports that MTD is present in vivo after cefazolin administration. We set out to determine whether MTD might be present in the tissues of patients treated with cefazolin prior to surgery. To do that, we took advantage of the fact that heterocyclic thiols can undergo S-methylation catalyzed by the genetically polymorphic drug-metabolizing enzyme thiopurine S-methyltransferase (TPMT). Initially, we tested recombinant human TPMT as a “reagent” to S-methylate MTD.

MTD was a substrate for TPMT-catalyzed S-methylation, with an apparent \( K_m \) value of 63 \( \mu \)M. Recombinant TPMT, with [\(^{14}\)C-methyl]S-AdoMet as a cosubstrate, was then used to radioactively label a methyl acceptor substrate present in liver and kidney cytosol preparations from patients who had been treated preoperatively with cefazolin. Pooled renal cytosol from 10 of those patients was used to purify and isolate the methylated product. That methylated compound coeluted with S-methyl MTD. When the methylated product was subjected to tandem mass spectrometry, it was identified as S-methyl MTD. Therefore, MTD is present in the tissues of patients treated with cefazolin. These observations also raise the possibility that the TPMT genetic polymorphism may represent a risk factor for cefazolin-induced hypoprothrombinemia since subjects who genetically lack TPMT would be unable to catalyze this MTD biotransformation pathway.

Cephalosporin antibiotics such as moxalactam, cefamandole, and cefoperazone can cause life-threatening hypoprothrombinemia and hemorrhage (Reddy and Bailey, 1980; Weitkamp and Aber, 1983; Dupuis et al., 1984; Lipsky, 1988). That adverse reaction is thought to be due to the in vivo release of a heterocyclic leaving group, 1-methyltetrazole-5-thiol (MTT), which is present in the structures of these drugs (Black et al., 1983; Lipsky, 1983, 1984, Lipsky et al., 1984). MTT inhibits the \( \gamma \)-carboxylation of glutamic acid, a vitamin K-dependent reaction required for the formation of active clotting factors (Sutie, 1978). Hypoprothrombinemia has also been reported to occur, but much less frequently, after the administration of another cephalosporin, cefazolin (Lerner and Lubin, 1974; Clark et al., 1983; Dupuis et al., 1984). The structure of cefazolin also includes a heterocyclic sulfhydryl moiety, 2-methyl-1,3,4-thiaziazole-5-thiol (MTD) (Fig. 1A). MTD, like MTT, is a potent in vitro inhibitor of the \( \gamma \)-carboxylation of glutamic acid (Kerremans et al., 1985; Lipsky et al., 1986). However, unlike MTT, MTD has not been reported to be present in vivo after the clinical administration of cefazolin.

We set out to determine whether MTD, like MTT, might be present in human tissue after cefazolin administration. Cefazolin is often used to treat patients prior to surgery, thus making it possible to obtain surgical “waste” tissue after the administration of known doses of this antibiotic. Both MTD and MTT have been reported to be substrates for S-methylation catalyzed by the AdoMet-dependent phase II drug-metabolizing enzyme TPMT (Kerremans et al., 1985). As a result, MTD can be radioactively labeled by TPMT in the presence of [\(^{14}\)C-methyl]AdoMet (Kerremans et al., 1985) (Fig. 1B). TPMT is genetically polymorphic, with approximately 89% of Caucasian subjects homozygous for the trait of high enzyme activity, approximately 11% heterozygous with intermediate activity, and 1 of every 300 subjects homozygous for the allele for very low or undetectable TPMT activity (Weinshilboum and Sladek, 1980; Weinshilboum et al., 1999). This pharmacogenetic trait could be of clinical significance as a risk factor for cefazolin-induced hypoprothrombinemia if MTD is present in human tissues after the administration of this cephalosporin antibiotic, as we demonstrate in the present study.

These studies were supported in part by National Institutes of Health Grants R01 GM28157, R01 GM35720, and U01 GM61388. 1 Present address: Beyond Genomics, 40 Bear Hill Road, Waltham, MA 02451 2 Abbreviations used are: MTT, 1-methyltetrazole-5-thiol; MTD, 2-methyl-1,3,4-thiaziazole-5-thiol; AdoMet, S-adenosyl-L-methionine; TPMT, thiopurine S-methyltransferase; HSS, high speed supernatant; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MS/MS, tandem mass spectrometry.

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Materials and Methods

Materials. MTD was purchased from Aldrich Chemical Company (Milwaukee, WI). [¹⁴C-Methyl]AdoMet, 60 mCi/mmol, was purchased from PerkinElmer Life Sciences (Boston, MA). Nonradioactive AdoMet-HCl was purchased from Sigma-Aldrich (St. Louis, MO).

Tissue Acquisition and Preparation. Renal tissue was obtained from patients undergoing clinically indicated nephrectomy, and hepatic tissue was obtained from patients undergoing partial hepatectomy for the removal of tumor. All tissue was obtained under guidelines reviewed and approved by the Mayo Clinic Institutional Review Board. Grossly normal surgically resected tissue was immediately placed on dry ice and was stored at −80°C. All patients had received 1 g of intravenous cefazolin 1 h prior to surgery. The surgery was homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 9 volumes of 5 mM potassium phosphate buffer, pH 7.4, followed by centrifugation at 100,000 × g for 1 hr at 4°C to obtain high speed supernatant (HSS) cytosol preparations.

Recombinant Human TPMT. Recombinant human TPMT was prepared by transfecting COS-1 cells with the eukaryotic expression vector p91023(b) that contained the human TPMT cDNA (Honchel et al., 1993). The transfection procedure as well as characteristics of the recombinant human TPMT have been described previously (Honchel et al., 1993; Szumlanski et al., 1996). Preparations from COS-1 cells transfected with “empty vector” were used as a control.

TPMT-Catalyzed MTD Methylation. The ability of TPMT to catalyze the S-methylation of MTD was determined by using a modification of the TPMT assay described by Weinshilboum et al. (1978) with recombinant human TPMT as the enzyme source. During the reaction, MTD was converted to S-methyl MTD with [¹⁴C-methyl]AdoMet (24 μCi/μmol) as the methyl donor. Nonradioactive AdoMet was the methyl donor for reactions in which mass spectrometry was to be performed. The methylated reaction product was extracted into toluene (Kerremans et al., 1985) rather than the 20% isooamy alcohol in toluene used when the assay is performed with 6-mercaptopurine as a substrate (Weinshilboum et al., 1978). All assays were performed in triplicate, and values reported are averages of those three determinations. Reactions for substrate kinetic experiments used MTD concentrations that ranged from 6.25 to 100 μM. Reactions performed to determine relative methyl acceptor substrate concentrations in individual cytosol preparations used individual human kidney and liver cytosol as a source of “substrate” and recombinant human TPMT as the enzyme source. Specifically, approximately 1000 units (1 unit equals 1 nmol of 6-methylmercaptopurine formed per hour of incubation) of recombinant TPMT were added to each sample analyzed. In addition, a pooled renal cytosol preparation that contained equal volumes of cytosol from 10 individual renal tissue samples was used as a substrate source for some experiments. Blanks for all assays were identical samples that did not contain methyl acceptor substrate (i.e., samples that contained neither MTD, renal cytosol, nor hepatic cytosol).

HPLC Isolation of Methylated Reaction Product. To prepare samples for the HPLC isolation of methylated products, methylation reactions were performed, and the reaction products were partitioned into 2.5 ml of toluene. Toluene (1.5 ml) was pooled from each of 10 identical reactions, and 120 μl of 30% acetonitrile in 7.0 mM ammonium acetate was added. The tolune was then evaporated under a stream of nitrogen, and 100 μl of the acetonitrile-ammonium acetate solution that remained after evaporation was subjected to HPLC. Initial separation was performed with a 4.6 × 250-mm Phenomenex C₅-reverse phase column, 5 μ particle size (Phenomenex, Torrance, CA) using a HP1090 liquid chromatograph (Hewlett Packard Analytical Di rect, Wilmington, DE). The mobile phase consisted of components “A” and “B”. Mobile phase component A was 10 mM ammonium acetate in water, and component B was acetonitrile. The flow rate was 800 μl/min with the following mobile phase compositions: 10% B and 90% A for 2 min, followed by a linear increase from 10 to 50% B over 30 min, with a subsequent linear increase to 95% B over 5 min, followed by 5 min of 95% B prior to returning to the starting condition (10% B and 90% A) for 8 min to re-equilibrate the column. Fractions that varied from 10 to 30 s (133 to 400 μl) were collected to characterize the elution profile of the compound of interest. Radioactively labeled methylation product eluted reproducibly in a 30-s (400 μl) fraction with a retention time of between 17.0 and 17.5 min.

A second HPLC separation was then performed with a 1 × 150-mm Michrom BioResources Inc. (Auburn, CA) MagicMS C₁₈ reverse phase column, 5 μ particle size, 10 Å pore size, with a Michrom BioResources UMA micro-scale liquid chromatograph at a flow rate of 50 μl/min. In this case, mobile phase component A was 1% 10 mM ammonium acetate in acetonitrile, and mobile phase component B was 10% 10 mM ammonium acetate in acetonitrile. A linear multistep gradient from 0 to 60% B over 30 min was used.
to elute the column, followed by a 2 min “ramp” to 95% B. The column was then eluted with 95% B for 5 min before re-equilibration with 0% B for 10 min. A 1-mm column was selected for the second HPLC separation to enhance mass spectrometric detection by taking advantage of the 16-fold concentration that occurred by eluting compounds at 50 μl/min versus the 800 μl/min flow rate that was used during the initial HPLC separation. To achieve this reduction in flow rate, it was necessary to use a Brownlee 3 × 15 mm-C_{18} preconcentration cartridge as the sample injection loop for the second HPLC separation. We also found that it was necessary to reduce the acetonitrile content in the eluant from the initial HPLC columns by a 10-fold dilution of the 400-μl fraction with 10 mM ammonium acetate. The diluted fraction was then preconcentrated onto the cartridge with the injector in the sample load position. The sample injector was then switched to place the cartridge in-line with the 1 × 150-mm C_{18} column.

Mass Spectrometric Product Identification. Liquid chromatography/mass spectrometry (LC/MS) and LC/tandem mass spectrometry (LC/MS/MS) analyses were performed with a Micromass Q-Tof II mass spectrometer (Micromass Inc., Manchester, UK) using the Z-spray electrospray ionization interface with the source block at 80°C, the desolvation gas at 125°C, and an electrospray ionization spray voltage of 3100 V. LC/MS data were collected over an m/z range from 50 to 800. LC/MS mass data were acquired at a mass resolution of 9000 full width at half maximum by adding a mass reference solution of 0.5 ng/μl tripropylamine after the column at a flow rate of 10 μl/min. The protonated ion of tripropylamine, m/z 144.1752, was used as an internal standard to calibrate the LC/MS data for purposes of elemental composition calculations. LC/MS/MS experiments were performed by passing only m/z 147 through the mass-analyzing quadrupole (Q1). The m/z 147 precursor ions were then fragmented by collision with argon (collision-induced dissociation) within the hexapole collision cell. The fragment ions that resulted were analyzed by the time-of-flight mass analyzer to identify all fragment ions formed within the mass-analyzing quadrupole (Q1). The m/z 147 precursor ions were then fragmented by collision with argon (collision-induced dissociation) within the hexapole collision cell. The fragment ions that resulted were analyzed by the time-of-flight mass analyzer to identify all fragment ions formed within the mass-analyzing quadrupole (Q1).

Results

TPMT-Catalyzed MTD Methylation. As an initial experiment, we used recombinant TPMT as an enzyme source in an attempt to confirm that it could catalyze MTD methylation. The relationship between enzyme activity and six different concentrations of MTD that ranged from 6.25 to 100 μM is shown in Fig. 2A. A double inverse plot of those same data is shown in Fig. 2B. The apparent K_{m} value of 63 μM calculated from these data was very similar to a previous report of 68 μM for human TPMT with MTD as a substrate (Kerreman et al., 1985). These observations confirmed that TPMT could be used to radioactively label MTD to determine whether this inhibitor of the γ-carboxylation of glutamate (Kerreman et al., 1985) might be present in tissue preparations from subjects exposed to ceftazolin in a clinical setting.

Methyl Acceptor Substrate in Human Renal and Hepatic Cyto- sol. The initial experiments in this series of studies were performed with pooled human kidney cytosol from patients treated with ceftazolin as a potential source of methyl acceptor substrate. That is, in these studies renal cytosol was used as a substrate, a source of methyl acceptor, in the presence of TPMT and [14C-methyl]AdoMet. We observed a linear relationship between quantity of cytosol protein and methylated product extracted over a range from 3.75 to 30 μl of cytosol, equivalent to 23.4 to 187 μg/ml of cytosol protein (data not shown). Subsequent analyses performed with individual human kidney and liver cytosol preparations revealed the presence of methyl acceptor substrate in all samples tested from patients exposed to ceftazolin (Table 1). The data listed in the table were obtained using 30 μl of cytosol from each sample, with a range of protein concentrations in individual samples from 123 to 209 μg/ml for kidney and 364 to 589 μg/ml for liver. The table also shows values for concentration of the methylated metabolite calculated from the specific activity of the [14C-methyl]AdoMet. To determine whether this methylated product was S-methyl MTD, we used HPLC to purify the methyl-labeled product, followed by the use of mass spectrometry for definitive identification.

HPLC Isolation of Methylated Product. Pooled renal cytosol was used as a source of methyl acceptor substrate for these experiments. Kidney was selected because those preparations generally gave higher counts per minute per microliter of cytosol and, thus, a better signal-to-noise ratio than did hepatic preparations (Table 1). We initially subjected the radioactively labeled methylated product isolated from the pooled renal preparation to HPLC performed with a C_{18} reverse phase column. The methylated product eluted reproducibly within a 30-s interval between 17.0 to 17.5 min. Peak heights were 2860 cpm for the endogenous substrate and 36,457 cpm for MTD as a substrate, with “background” fractions having 100 cpm or less. Authentic methylated MTD (i.e., the product of a reaction performed with 1 mM MTD as substrate) eluted from the column at the same time as did the methylated substrate from tissue. Fractions from this initial HPLC separation were collected and diluted. Those fractions, after “dilution” as described under Materials and Methods, were then subjected to a second round of HPLC performed with a C_{18} reverse phase column. Once again, the methyl acceptor substrate eluted with authentic S-methyl MTD. In this case, the peak height for the tissue substrate was 1356 cpm, whereas the peak height for the product of a reaction

Figure 2. TPMT-catalyzed methylation of MTD.

A: the relationship between MTD concentration and the formation of methylated product is shown. B: a double inverse plot of the data in (A) is shown.
Therefore, the same pooled renal cytosol sample was heated at 95°C for use in mass spectrometric analysis. Heating the tissue preparations might result in the liberation of greater quantities of this leaving group for use during mass spectrometry. However, we reasoned that if the column were so low that it was difficult to collect adequate material the HPLC peak for the tissue substrate that eluted from this second column represents values after subtraction of the blank. The final concentrations of methylated product were calculated on the basis of the specific activity of the [14C]-methyl[AdoMet].

The “blank”, recombinant TPMT without the addition of cytosol, was 108 cpm. The “net cpm” column represents values after subtraction of the blank. The final concentrations of methylated product were calculated on the basis of the specific activity of the [14C]-methyl[AdoMet].

**Discussion**

The cephalosporins are important antibiotics. However, some third generation cephalosporins (e.g., moxalactam) caused life-threatening hypoprothrombinemia and hemorrhage (Reddy and Bailey, 1980; Weitkamp and Aber, 1983; Dupuis et al., 1984; Lipsky, 1988). That adverse reaction was shown to be due to inhibition of the γ-carboxy-

**TABLE 1**

Methylated product formed with 10 individual kidney and 10 individual liver biopsy cytosol samples as substrate

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Net cpm</th>
<th>cpm/μg Protein</th>
<th>Mols Methylated Product/mg Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>706</td>
<td>103</td>
<td>105</td>
</tr>
<tr>
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<td>530</td>
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</tr>
<tr>
<td>K10</td>
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<td>157</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>L1</td>
<td>601</td>
<td>33</td>
<td>89</td>
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<tr>
<td>L2</td>
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<td>L10</td>
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<td>29</td>
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performed with MTD as substrate was 23,700. Counts per minute in the HPLC peak for the tissue substrate that eluted from this second column were so low that it was difficult to collect adequate material for use during mass spectrometry. However, we reasoned that if the methyl acceptor substrate in tissue was MTD released from cefazolin, heating the tissue preparations might result in the liberation of greater quantities of this leaving group for use during mass spectrometry.

**Accurate Mass Spectrometric Identification of Methylated Product.** All mass spectrometric analyses were carried out at high resolution to determine the elemental composition of both the intact molecular ion as well as product (fragment) ions accruing from MS/MS experiments. Authentic nonradioactive S-methyl MTD was purified and isolated by use of the two HPLC steps, with the second column connected to the mass spectrometer. Under those conditions, the HPLC peak displayed an m/z value of 147 (Fig. 4A). Accurate mass measurements of the HPLC peak at m/z 147, using tripropylamine as an internal mass calibrant, gave a mass value of 147.0054 versus the calculated theoretical mass of 147.0051 for authentic S-methyl MTD, affording a molecular ion formula of C₇H₇N₂S₂. The same peak with the same theoretical mass was observed with methylated product isolated from heated renal cytosol (Fig. 4B, trace i). As a control, the separation was also performed using fractions from a blank sample [i.e., the reaction was performed without a source of methyl acceptor substrate (Fig. 4B, trace ii)]. In addition, mock-transfected COS-1 cell preparations, preparations lacking recombinant TPMT, were used as an enzyme source with renal cytosol as the substrate source (Fig. 4B, trace iii). Both of these controls had profiles that lacked the m/z peak at 147. Subsequently, accurate tandem mass spectrometric analysis was carried out with both authentic S-methylated MTD (Fig. 5A) and the methylated product obtained with heated renal cytosol as a substrate for the reaction (Fig. 5B). In both cases, the precursor ion at m/z 147.0051 afforded prominent product ions near m/z 99.0017 (+ 0.0050) and m/z 78.9676 (+ 0.0050), the anticipated values. These ions corresponded to fragments containing elemental compositions of C₆H₆N₂S and CH₃S₂, respectively. Proposed fragment pathways and resulting product ions for S-methyl MTD are shown in Fig. 6. Within experimental error, the product ion spectrum of authentic S-methyl MTD (Fig. 5A) was identical to that of the methylated product obtained with heated renal cytosol as a substrate (Fig. 5B), unequivocally identifying the presence of MTD in the patient samples.

**HPLC of Methylated Heated Renal Cytosol Substrate and Methylated MTD**

![Fig. 3. HPLC of the methylated product generated with heated renal cytosol and with MTD as substrates after chromatography on (A) a C₈ reverse phase and (B) a C₁₈ reverse phase column.](Image)

HSS is a high speed supernatant preparation.
ylation of glutamate residues in clotting factors by MTT, a sulfhydryl leaving group present in the structures of the cephalosporin antibiotics most often associated with hypoprothrombinemia (Black et al., 1983; Lipsky, 1983, 1984; Lipsky et al., 1984). Although the structure of cefazolin also includes a potential sulfhydryl leaving group, MTD, and although MTD is also a potent in vitro inhibitor of the $\gamma$-carboxylation of glutamate (Kerremans et al., 1985), hypoprothrombinemia is an unusual complication of cefazolin therapy (Lerner and Lubin, 1974; Clark et al., 1983; Dupuis et al., 1984). In addition, even though MTT can be easily detected in the tissues of subjects treated with cephalosporins which contain that structure (Black et al., 1983), MTD has not been reported to be present in human tissues after cefazolin exposure. The purpose of the present experiments was to determine whether MTD was present in the tissues of patients treated with cefazolin. To test that hypothesis, we took advantage of the routine prophylactic treatment of surgical patients with cefazolin and the fact that MTD has been reported to be a substrate for the genetically polymorphic drug-metabolizing enzyme TPMT (Kerremans et al., 1985), making it possible to radioactively label MTD in tissue preparations by using recombinant human TPMT as a reagent.

The present experiments confirmed that MTD was a substrate for S-methylation catalyzed by TPMT (Kerremans et al., 1985). We were then able to use recombinant human TPMT to test the hypothesis that MTD might be present in renal or hepatic tissue resected from patients treated preoperatively with 1 g of intravenous cefazolin. All of the tissue samples that we studied contained a substrate capable of acting as a methyl acceptor in the presence of TPMT (Table 1), and we demonstrated that that compound was MTD. These observations raise several questions. If MTD, like MTT, is a leaving group that is released in human tissues, and if MTD is, as previously reported, more potent than MTT as an inhibitor of the $\gamma$-carboxylation of glutamate (Kerremans et al., 1985), why does hypoprothrombinemia occur so much less frequently after exposure to cefazolin than after exposure to cephalosporins that include MTT in their structures. It should be noted that cefazolin administration has been associated with hypoprothrombinemia, not only in patients (Lerner and Lubin, 1974; Clark et al., 1983; Dupuis et al., 1984) but also in vitamin K depleted rats (Lipsky

Fig. 4. LC/MS ion chromatograms of m/z 147.
A, chromatogram obtained from material purified by HPLC when recombinant human TPMT was used to methylate MTD; B, material purified by HPLC when trace i, recombinant human TPMT, was incubated with heated renal cytosol; trace ii, recombinant human TPMT, was incubated without heated renal cytosol; trace iii, a mock-transfected COS-1 cell preparation, the source of the recombinant TPMT, was incubated with heated renal cytosol.

Fig. 5. Accurate mass LC/MS/MS spectra showing mass product ions of m/z 147 from (A) material purified by HPLC after recombinant human TPMT was incubated with MTD, and (B) material purified by HPLC after recombinant human TPMT was incubated with heated renal cytosol.

The (+) indicates unfragmented precursor ion that was used as a “lock mass” (m/z 147.0051) to calibrate the MS/MS spectra for purposes of elemental composition calculations.

Fig. 6. Proposed MS/MS fragmentation pathways for S-methyl MTD based on elemental composition assignments of the MS/MS accurate mass product ions in the spectra shown in Fig. 6.

The values reported for each proposed fragment of methylated MTD are the calculated theoretical mass values.
et al., 1986). One possible explanation for the low incidence of hypoprothrombinemia after exposure to cefazolin is that MTD is a poorer leaving group than is MTT. Another possibility is that MTD undergoes rapid S-methylation in vivo. In support of that possibility is the fact that the apparent $K_m$ value of human kidney TPMT for MTD was approximately 4-fold greater than that for MTT and that the $V_{max}/K_m$ ratio for MTD was over an order of magnitude greater than that for MTT (Kerremans et al., 1985). In addition, S-methyl MTD is at least 2 orders of magnitude less potent as an inhibitor of the γ-carboxylation of glutamate than is the parent free sulfhydryl (Kerremans et al., 1985). As these observations seem to indicate, if S-methylation of MTD is “protective”, then the 1 in 300 white subjects homozygous for low or absent TPMT might be at increased risk for cefazolin-induced hypoprothrombinemia and hemorrhage because those subjects would be unable to perform this biotransformation. Obviously, that hypothesis will have to be tested systematically in the course of future clinical studies. If the hypothesis that the TPMT genetic polymorphism represents a risk factor for cefazolin-induced hemorrhage is confirmed, our studies may help make it possible to predict and prevent an adverse reaction to this frequently used cephalosporin antibiotic.

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References

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