10-1-2016

Bioactivation of Trimethoprim to Protein-Reactive Metabolites in Human Liver Microsomes.

Jennifer Goldman
Children's Mercy Kansas City

Yakov M. Koen

Steven A. Rogers

Kelin Li

J Steven Leeder
Children's Mercy Hospital

See next page for additional authors

Recommended Citation

Goldman JL, Koen YM, Rogers SA, Li K, Leeder JS, Hanzlik RP. Bioactivation of Trimethoprim to Protein-Reactive Metabolites in Human Liver Microsomes. Drug Metab Dispos. 2016;44(10):1603-1607. doi:10.1124/dmd.116.072041

1521-009X/44/10/1603–1607$25.00
Drug Metabolism and Disposition

http://dx.doi.org/10.1124/dmd.116.072041
Drug Metab Dispos 44:1603–1607, October 2016

Short Communication

Bioactivation of Trimethoprim to Protein-Reactive Metabolites in Human Liver Microsomes

Received June 9, 2016; accepted July 22, 2016

ABSTRACT

The formation of drug-protein adducts via metabolic activation and covalent binding may stimulate an immune response or may result in direct cell toxicity. Protein covalent binding is a potentially pivotal step in the development of idiosyncratic adverse drug reactions (IADRs). Trimethoprim (TMP)-sulfamethoxazole (SMX) is a combination antibiotic that commonly causes IADRs. Recent data suggest that the contribution of the TMP component of TMP-SMX to IADRs may be underappreciated. We previously demonstrated that TMP is bioactivated to chemically reactive intermediates that can be trapped in vitro by *N*-acetyl cysteine (NAC), and we have detected TMP-NAC adducts (i.e., mercapturic acids) in the urine of patients taking TMP-SMX. However, the occurrence and extent of TMP covalent binding to proteins was unknown. To determine the ability of TMP to form protein adducts, we incubated [^{14}C]TMP with human liver microsomes in the presence and absence of NADPH. We observed protein covalent binding that was NADPH dependent and increased with incubation time and concentration of both protein and TMP. The estimated covalent binding was 0.8 nmol Eq TMP/mg protein, which is comparable to the level of covalent binding for several other drugs that have been associated with covalent binding–induced toxicity and/or IADRs. NAC and selective inhibitors of CYP2B6 and CYP3A4 significantly reduced TMP covalent binding. These results demonstrate for the first time that TMP bioactivation can lead directly to protein adduct formation, suggesting that TMP has been overlooked as a potential contributor of TMP-SMX IADRs.

Introduction

Trimethoprim (TMP)-sulfamethoxazole (SMX) is one of the most commonly prescribed antibiotics worldwide (Van Boeckel et al., 2014). Although TMP-SMX is considered safe and efficacious, it causes a higher rate of idiosyncratic adverse drug reactions (IADRs) compared with most other drugs (Boye and Gaustad, 1991; Macy and Poon, 2009; Björnsson, 2015). SMX is implicated in TMP-SMX–associated IADRs by evidence of SMX bioactivation, protein adduct formation, and immune stimulation (Cribb and Spielberg, 1992; Cribb et al., 1997; Naisbitt et al., 2002). However, SMX alone cannot explain all IADRs to the combination drug, because TMP (when prescribed alone) has also been associated with severe IADRs and the rates of IADRs are higher for TMP-SMX than for SMX alone (Harding et al., 1975; Bergan and Skjerven, 1979; Hawkins et al., 1993; Mortimer et al., 2005).

Although the mechanisms associated with the development of many IADRs have not been fully elucidated, bioactivation of drugs to reactive intermediates that covalently bind to proteins is a generally accepted pathway that can contribute to IADR development (Zhou et al., 2005; Nakayama et al., 2009; Park et al., 2011; Björnsson, 2015). In general, drugs found to be more commonly implicated in inducing IADRs tend to exhibit higher in vitro protein binding compared with those with a lower risk profile (Takakusa et al., 2008; Nakayama et al., 2009). Thus, a critical step for understanding the mechanism of TMP-induced IADRs is to determine its potential for covalent binding to proteins after bioactivation to reactive metabolites.

This research was supported in part by the National Institutes of Health National Center for Advancing Translational Sciences [Clinical and Translational Science Award KL2TR000119].
dx.doi.org/10.1124/dmd.116.072041.

Recent evidence suggests that TMP undergoes oxidative bioactivation, either by quinone imine formation or by *O*-demethylation followed by quinone methide formation (Lai et al., 1999; Damsten et al., 2008) (Fig. 1). These in vitro findings are consistent with our recent in vivo findings of TMP-derived mercapturic acids, such as *N*-acetyl cysteine (NAC) conjugates of TMP, in the urine of TMP-SMX–exposed children and demonstrate for the first time that TMP bioactivation and detoxification occurs in humans (van Haandel et al., 2014). Additional in vitro studies involving human liver microsomes (HLMs), recombinant cytochrome P450s (P450s), and specific P450 inhibitors demonstrated that CYP3A4 was the primary enzyme contributing to TMP bioactivation (Goldman et al., 2015). Although these data clearly demonstrated that TMP undergoes bioactivation in vitro and in human patients, the extent to which bioactivated TMP metabolites covalently bind to proteins remained unknown.

The main objective of this work was to characterize the extent of irreversible covalent binding of TMP to microsomal protein. [^{14}C]-TMP was synthesized for this purpose, because radiolabeled drugs have proven to be effective in the estimation of covalent binding to cellular macromolecules. Inhibition experiments targeting specific drug-metabolizing enzymes were used to define hepatic enzyme contribution to TMP protein covalent binding, and trapping experiments with NAC and *N*-acetyl lysine (NAL) were performed to demonstrate protection of proteins by surrogate nucleophiles.

Materials and Methods

Materials. EDTA, glucose-6-phosphate dehydrogenase, magnesium chloride, NAC, NAL, NADP, potassium phosphate dibasic, potassium phosphate monobasic, TMP, montelukast, quinidine, sulfaphenazole, and 1-benzylimidazole

ABBREVIATIONS: ADR, adverse drug reaction; DCM, dichloromethane; HLM, human liver microsome; IADR, idiosyncratic adverse drug reaction; NAC, *N*-acetyl cysteine; NAL, *N*-acetyl lysine; P450, cytochrome P450; SMX, sulfamethoxazole; TMP, trimethoprim.

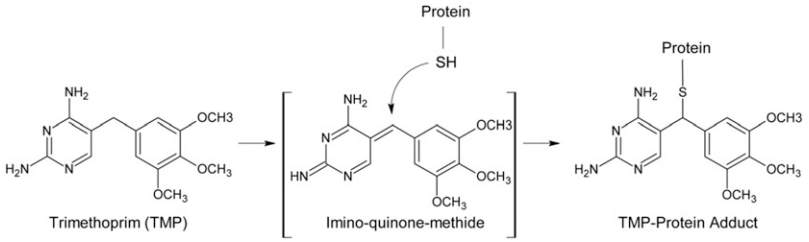

Fig. 1. Proposed pathway of TMP bioactivation and protein covalent binding.

were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Benzylnirvanol and thiotepa were purchased from Toronto Research Chemicals (Toronto, Canada). Ketoconazole was obtained from Research Biochemicals International (Natick, MA). Trichloroacetic acid was purchased from Fisher Scientific (Fairlawn, NJ). Pooled HLMs from 16 donors were purchased from XenoTech, LLC (Lenexa, KS). Microsomes were stored at −80°C and placed on ice prior to use, and the same lot of pooled HLMs was used for all experiments.

[^{14}C]-TMP Synthesis. The synthesis of 2-[^{14}C]-TMP was modeled after the procedure of Hoffer et al. (1971) (Fig. 2). The modification was first verified using nonradioactive materials to give 65%–75% yields of pure TMP (confirmed by thin-layer chromatography, high-performance liquid chromatography, and high-resolution mass spectrometry) on the same scale as planned for the radiosynthesis described below. [^{14}C]-Guanidine hydrochloride (500 μCi, approximately 50 Ci/mol, approximately 9 μmol; Moravek Biochemicals, Brea, CA) was transferred with 1 ml MeOH into a 13-mm × 100-mm culture tube and sodium methoxide in methanol (1.0 ml, 0.5 M) was added. The mixture was stirred at 70°C for 30 minutes. After the reaction mixture was cooled to room temperature, 3-anilino-2-(3,4,5-trimethoxybenzyl) acrylonitrile (48.8 mg, 150 μmol, mixture of *cis*/*trans* isomers; Toronto Research Chemicals) was added in one batch. The reaction tube was sealed with a screwcap and the mixture was stirred at 70°C for 16 hours. The reaction mixture was cooled to room temperature, and guanidine hydrochloride (9.55 mg, 100 μmol) in 1.0 ml methanol was added to the reaction mixture and stirred at 70°C for another 16 hours. The mixture was cooled to room temperature and transferred to a 50-ml round-bottom flask and evaporated to dryness. The residue was dissolved in 2.5% MeOH in dichloromethane (DCM) and loaded on a 4-g prepacked silica gel column. The column was washed with DCM (12 ml), 2.5% MeOH/DCM (30 ml), and 5% MeOH/DCM (30 ml) and the product was eluted with 10% MeOH/DCM, with monitoring by thin-layer chromatography. The combined fraction was evaporated to dryness under a vacuum (yield: 12.8 mg, 44.3 μmol, 41%), which was dissolved in MeOH (10 ml) and transferred to a scintillation vial for storage. The specific activity was 2.9 Ci/mol.

In Vitro Incubation Conditions. Incubations (150 μl) contained HLMs (500–1500 μg microsomal protein/ml), potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), and [^{14}C]-TMP (50–250 μM). The first step in setting up incubations involved transferring the required amount of [^{14}C]-TMP into the tubes using 50 μl methanol, which was then removed by evaporation under a gentle stream of nitrogen. After adding the other components, reactions were initiated by the addition of 15 μl of a NADPH-generating system consisting of NADP (10 mM), glucose 6-phosphate (50 mM), and glucose-6-phosphate dehydrogenase (10 U/ml). Reactions were incubated in a shaking water bath at 37 ± 1°C for 45–120 minutes. Incubations without the addition of NADPH were performed to control for background nonspecific covalent binding. All incubations were run in duplicate. For measurement of TMP metabolites covalently bound to protein, aliquots (100 μl) of the incubation mixture were applied to Whatman 3MM (Sigma-Aldrich) absorbent filter paper strips cut to fit into scintillation vials (11 × 75 mm). Strips were air-dried and then washed by immersion for 10−15 minutes in each of a series of five baths (130 ml each) composed of 10% trichloroacetic acid, 10% trichloroacetic acid again, 95% ethanol, 95% ethanol again, and finally ethyl acetate. After all washes, the strips were air-dried and submitted to liquid scintillation counting (Wallin et al., 1981; Dansette et al., 1991).

[^{14}C]-TMP Covalent Binding Inhibition Experiments. Incubations (1500 μg microsomal protein/ml, [^{14}C]-TMP 100 μM, 120 minutes) were performed in the presence and absence of NAC (5 mM) or NAL (5 mM) to determine the extent of covalent binding inhibition and implicate the putative covalent binding site as cysteine and/or lysine. Net covalent binding was also determined in the presence and absence of selective P450 inhibitors including thiotepa (CYP2B6, 50 μM), montelukast (CYP2C8, 5 μM), sulfaphenazole (CYP2C9, 10 μM), benzylnirvanol (CYP2C19, 10 μM), quinidine (CYP2D6, 5 μM), and ketoconazole (CYP3A4, 1 μM). 1-Benzylimidazole (1 mM) was used as a nonspecific P450 inhibitor. Inhibitor concentrations were approximately 30 times K_i. Inhibition experiments were performed in triplicate or quadruplicate.

Data Analysis. Correlation coefficients (r) between [^{14}C]-TMP protein covalent binding and incubation conditions (TMP concentration, protein concentration, and incubation time) were determined using least-squares regression analysis. Significance was determined by Pearson's regression analysis using a two-tailed t test with α equal to 0.05 by using GraphPad software (GraphPad Software Inc., La Jolla, CA). Effects of NAC, NAL, and the selective P450 inhibitors were determined by comparing the amount of covalent binding expressed as a percentage of the uninhibited control incubation.

Results and Discussion

In the presence of an NADPH-generating system, HLMs generated measurable [^{14}C]-TMP protein covalent binding up to 747 dpm/100 μg protein, which equates to approximately 0.8 nmol Eq bound TMP/mg protein. Covalent binding increased linearly with increasing TMP concentration, protein concentration, and incubation time (Fig. 3). For all incubations performed, nonspecific covalent binding observed in the absence of NADPH and/or HLMs was subtracted to determine net NADPH-dependent covalent binding (Fig. 4).

The presence of NAC (5 mM) decreased the amount of covalent binding by 73% ($P < 0.005$), whereas NAL had no effect on covalent binding of TMP (Fig. 3). Net TMP covalent binding was completely abolished with the addition of 1-benzylimidazole, a potent P450 inhibitor that may affect various P450 isoforms. In addition, several isoform-specific P450 inhibitors also significantly decreased the amount of covalent binding (Fig. 5). Thiotepa (a selective CYP2B6 inhibitor) and ketoconazole (a selective CYP3A4 inhibitor) inhibited TMP covalent binding by 64% and 51%, respectively ($P < 0.05$). Sulfaphenazole (a selective CYP2C9 inhibitor) inhibited TMP covalent binding by 34%, but this was not significant ($P = 0.10$).

The results presented here demonstrate several important findings. First, TMP metabolites definitely become covalently bound to proteins when TMP is incubated with HLMs, and the amount of covalent

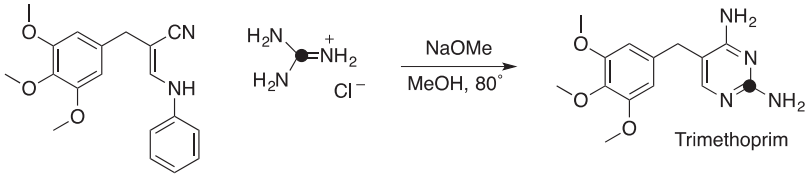

Fig. 2. Structure, synthesis, and labeling of [^{14}C]-TMP. The black dot indicates the position of the ^{14}C label.

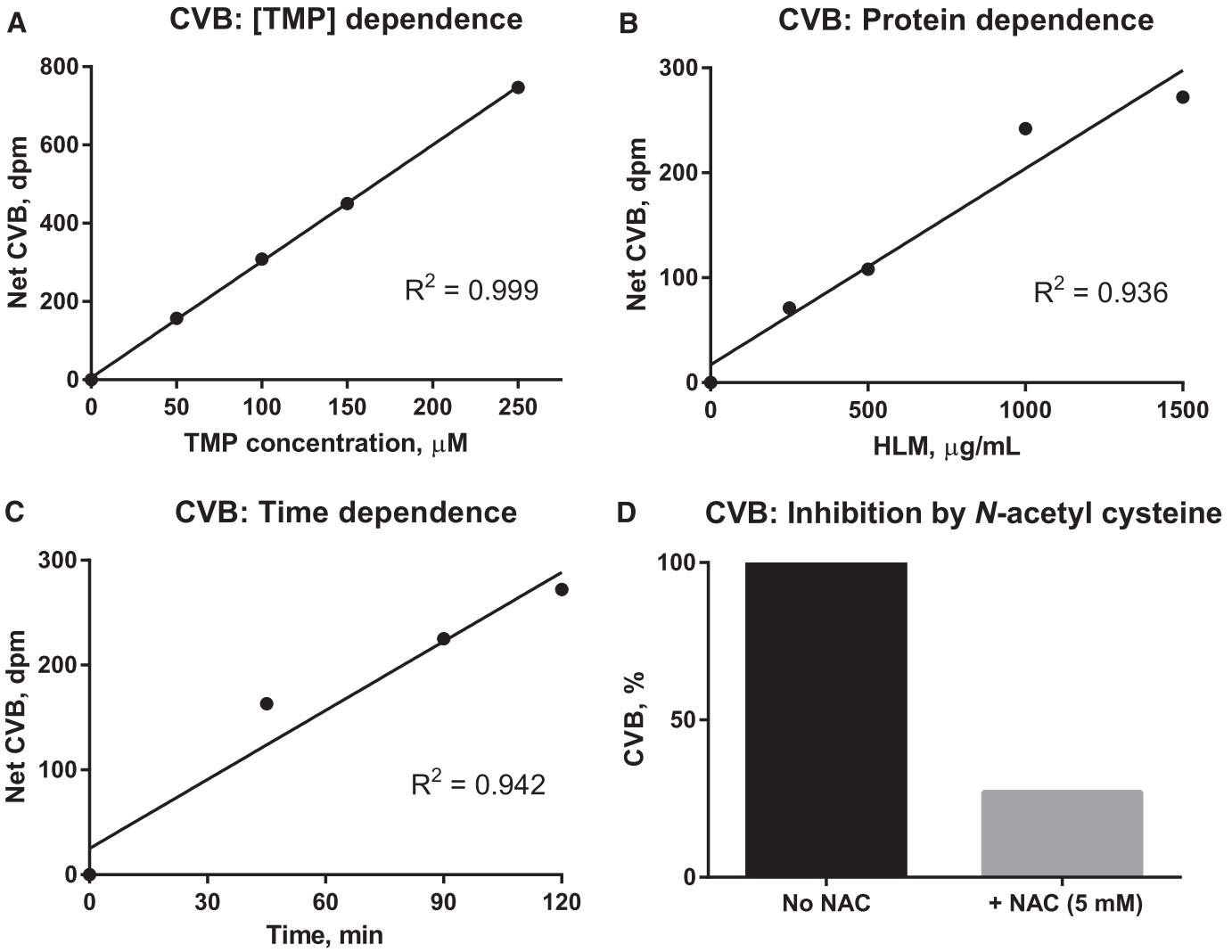

Fig. 3. Effects of TMP concentration, protein concentration, incubation time, and NAC on the covalent binding of [^{14}C]-TMP to HLMs. Pooled HLMs from 16 donors were incubated in the presence of NADPH at 37 ± 1°C. (A) TMP concentration ranged from 50 to 250 μM, whereas protein and time were constant at 1500 μg/ml protein and 120 minutes, respectively. (B) Protein concentrations ranged from 250 to 1500 μg/ml, whereas incubation time (120 minutes) and TMP (100 μM) were constant. (C) Incubation times ranged from 45 to 120 minutes, while protein (1500 μg/ml) and TMP (100 μM) concentrations were constant. (D) NAC (5 mM) was included in the incubation (100 μM TMP, 1500 μg protein/ml, 120 minutes). An aliquot (100 μl) of the incubation mixture was applied to absorbent filter paper strips and the strips were processed as outlined in the *Materials and Methods*. Radioactivity associated with the precipitated protein was estimated by liquid scintillation counting. CVB, covalent binding.

binding observed increases linearly with increases in incubation time, TMP concentration, and protein concentration. Second, the addition of 5 mM NAC significantly inhibited TMP protein covalent binding, whereas 5 mM NAL had no effect, thus implicating protein sulfhydryl groups as the likely target sites in proteins. Third, the selective inhibition of CYP2B6 or CYP3A4 significantly decreases the observed amount of TMP covalent binding.

The maximum covalent binding detected after incubations of [^{14}C]-TMP with HLMs was 0.8 nmol Eq/mg protein. This amount of covalent binding is comparable or greater than that reported for other drugs that are known to undergo bioactivation followed by protein covalent binding and subsequent adverse drug reactions (ADRs), including benzbromarone (0.39 nmol Eq/mg protein) (Takakusa et al., 2008), acetaminophen (0.09 nmol Eq/mg protein) (Nakayama et al., 2009), and flutamide (0.18 nmol Eq/mg protein) (Nakayama et al., 2009). It has been proposed that the covalent binding of chemically reactive metabolites to cellular proteins could result in direct organ toxicity as well as the formation of neoantigens that result in immune or even autoimmune reactivity (Koen et al., 2012). Interestingly, our results demonstrate that TMP has higher covalent binding capabilities in vitro with HLM incubations than previously observed with SMX (0.003 nmol Eq/mg protein) (Nakayama et al., 2009).

Until now, the ADRs observed with TMP-SMX have been attributed solely to the SMX component (or its metabolites), but our results show that TMP undergoes metabolic activation and covalent binding at least as efficiently as SMX and several other hepatotoxic drugs (Cheng et al., 2008). Our findings support the hypothesis that TMP has the potential to contribute to IADR development in these patients exposed to TMP-SMX via the pathway of bioactivation followed by protein adduct formation.

Modest concentrations of NAC (a soft nucleophile similar to glutathione) significantly inhibited TMP covalent binding, suggesting that protein covalent binding of TMP likely occurs on cysteine residues, whereas no inhibition was observed in the presence of NAL (a hard nucleophile). The observed effect of NAC on TMP covalent binding suggests that glutathione has the ability to decrease covalent binding of

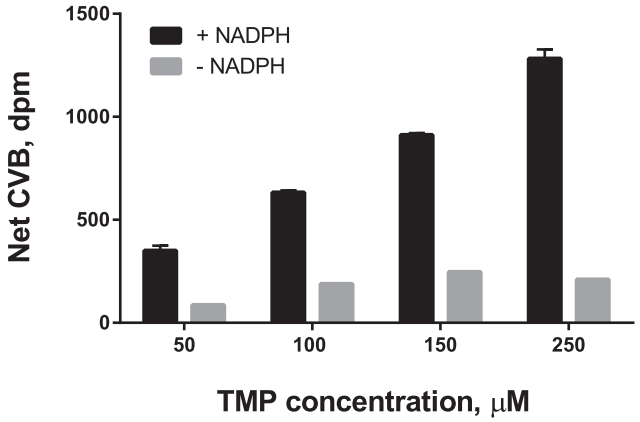

Fig. 4. Effect of NADPH on the covalent binding of [^{14}C]-TMP to HLMs. Pooled HLMs were incubated under standard conditions in the absence or presence of NADPH, with [^{14}C]-TMP (50–250 μM) at 37 ± 1°C for 120 minutes. An aliquot (100 μl) of the incubation mixture was applied to absorbent filter paper strips as outlined in the *Materials and Methods*. Radioactivity associated with the precipitated protein was estimated by liquid scintillation counting. A low level of apparent covalent binding was noted in those incubations without NADPH and this amount was considered background and subtracted. CVB, covalent binding.

TMP metabolites to protein. In vivo, the scavenging of reactive TMP metabolites by glutathione probably serves as a protective mechanism (Haouzi et al., 2000; Jaeschke et al., 2002) as well as a route to the formation of urinary mercapturic acids. Our previous findings revealed that all TMP-exposed individuals bioactivate TMP to some extent, as shown by their urinary excretion of TMP mercapturic acids (van Haandel et al., 2014). In addition, we found a 17-fold variation in TMP mercapturic acid formation among individual HLM preparations used in previous phenotyping experiments when NAC was added, suggesting that variability of bioactivation and protein covalent binding likely occurs in vivo (Goldman et al., 2015). However, the occurrence and extent of TMP protein adduct formation had not been determined until now.

With the inhibition of CYP2B6 and CYP3A4, a greater than 50% net decrease in TMP covalent binding was observed. These findings are in agreement with our previous work using HLMs, individual P450 isoforms, and isoform-selective P450 inhibitors, which revealed CYP3A4 as the isoform most strongly correlated with TMP-NAC generation (Goldman et al., 2015). We also found that the CYP2B6 inhibitor thiotepa inhibited TMP-NAC formation at the highest concentration used (150 μM), even though recombinant CYP2B6 did not appear to catalyze TMP-NAC formation (Goldman et al., 2015). This could be attributable to the fact that thiotepa is also known to be a mechanism-based inactivator of CYP3A enzymes. The high interindividual variation in TMP-mercapturate formation in HLMs supplemented with NAC may lead to differences in the extent of TMP covalent binding, and this may be directly or indirectly related to the idiosyncratic development of ADRs (Ozdemir et al., 2000; Lamba et al., 2010).

In summary, based on the extent of TMP protein adduct formation observed, further investigation of the role of protein covalent binding of TMP metabolites in the generation of IADRs is clearly warranted. A better understanding of the mechanisms associated with TMP covalent binding in vivo and potential downstream immune stimulation may provide predictive insight into which individuals are at greatest risk of developing an IADR. Currently, the extent of TMP protein adduct formation in vivo, and whether patients who develop an IADR have more extensive protein adduct formation, remains unknown.

Divisions of Clinical Pharmacology, Toxicology, and Therapeutic Innovation, Children's Mercy Hospital, University of Missouri, Kansas City, Missouri (J.L.G., J.S. L.); Department of Medicinal Chemistry, University of Kansas School of Pharmacy, Lawrence, Kansas (Y.M.K., R.P.H.); Baker & McKenzie LLP, Dallas, Texas (S.A. R.); and Center for Integrative Chemical Biology and Drug Discovery, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina (K.L.)

Jennifer L. Goldman
Yakov M. Koen
Steven A. Rogers
Kelin Li
James S. Leeder
Robert P. Hanzlik

Authorship Contributions

Participated in research design: Goldman, Koen, Rogers, Li, Hanzlik.
Conducted experiments: Goldman, Koen, Rogers, Li, Hanzlik.
Performed data analysis: Goldman, Koen, Hanzlik.
Wrote or contributed to the writing of the manuscript: Goldman, Koen, Leeder, Hanzlik.

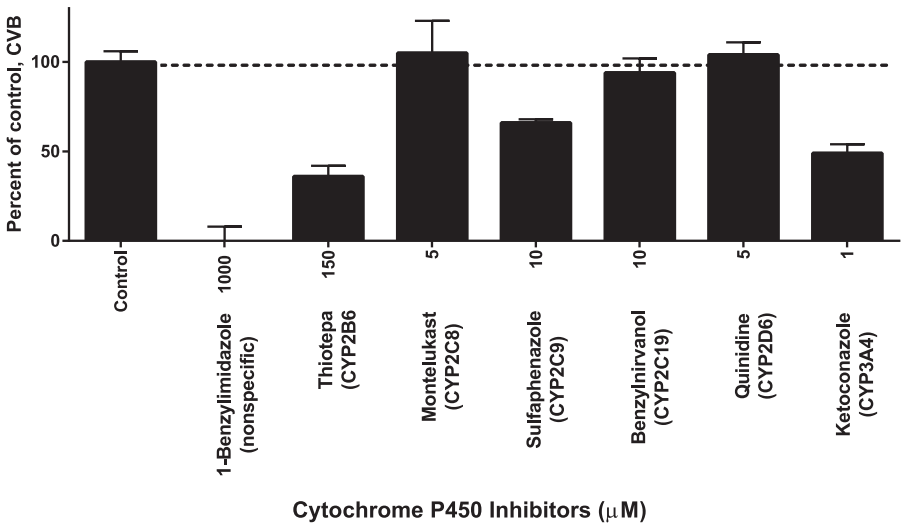

Fig. 5. Effects of various P450 isoform-selective inhibitors on the covalent binding of [^{14}C]-TMP to pooled HLMs were analyzed as described in the *Materials and Methods*. Each bar represents the mean and standard deviation. CVB, covalent binding.

References

Bergan T and Skjerven O (1979) Comparison of sulfamethoxazole alone and combined with trimethoprim in urinary tract infections. *Infection* **7**:14–16.

Björnsson ES (2015) Drug-induced liver injury: an overview over the most critical compounds. *Arch Toxicol* **89**:327–334.

Boye NP and Gaustad P (1991) Double-blind comparative study of ofloxacin (Hoe 280) and trimethoprim-sulfamethoxazole in the treatment of patients with acute exacerbations of chronic bronchitis and chronic obstructive lung disease. *Infection* **19** (Suppl 7):S388–S390.

Cheng L, Stewart BJ, You Q, Petersen DR, Ware JA, Piccotti JR, Kawabata TT, and Ju C (2008) Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. *Mol Pharmacol* **73**:1769–1775.

Cribb AE, Pohl LR, Spielberg SP, and Leeder JS (1997) Patients with delayed-onset sulfonamide hypersensitivity reactions have antibodies recognizing endoplasmic reticulum luminal proteins. *J Pharmacol Exp Ther* **282**:1064–1071.

Cribb AE and Spielberg SP (1992) Sulfamethoxazole is metabolized to the hydroxylamine in humans. *Clin Pharmacol Ther* **51**:522–526.

Damsten MC, de Vlieger JS, Niessen WM, Irth H, Vermeulen NP, and Commandeur JN (2008) Trimethoprim: novel reactive intermediates and bioactivation pathways by cytochrome p450s. *Chem Res Toxicol* **21**:2181–2187.

Dansette PM, Amar C, Valadon P, Pons C, Beaune PH, and Mansuy D (1991) Hydroxylation and formation of electrophilic metabolites of tienilic acid and its isomer by human liver microsomes. Catalysis by a cytochrome P450 IIC different from that responsible for mephenytoin hydroxylation. *Biochem Pharmacol* **41**:553–560.

Goldman JL, Leeder JS, Van Haandel L, and Pearce RE (2015) In vitro hepatic oxidative biotransformation of trimethoprim. *Drug Metab Dispos* **43**:1372–1380.

Haouzi D, Lekéhal M, Moreau A, Moulis C, Feldmann G, Robin MA, Lettéron P, Fau D, and Pessayre D (2000) Cytochrome P450-generated reactive metabolites cause mitochondrial permeability transition, caspase activation, and apoptosis in rat hepatocytes. *Hepatology* **32**:303–311.

Harding GK, Ronald AR, Boutros P, and Lank B (1975) A comparison of trimethorprim-sulfamethoxazole with sulfamethoxazole alone in infections localized to the kidneys. *Can Med Assoc J* **112**:9–12.

Hawkins T, Carter JM, Romeril KR, Jackson SR, and Green GJ (1993) Severe trimethoprim induced neutropenia and thrombocytopenia. *N Z Med J* **106**:251–252.

Hoffer M, Grunberg E, Mitrovic M, and Brossi A (1971) An improved synthesis of diaveridine, trimethoprim, and closely related 2,4-diaminopyrimidines. *J Med Chem* **14**:462–463.

Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, and Lemasters JJ (2002) Mechanisms of hepatotoxicity. *Toxicol Sci* **65**:166–176.

Koen YM, Sarma D, Williams TD, Galeva NA, Obach RS, and Hanzlik RP (2012) Identification of protein targets of reactive metabolites of tienilic acid in human hepatocytes. *Chem Res Toxicol* **25**:1145–1154.

Lai WG, Zahid N, and Uetrecht JP (1999) Metabolism of trimethoprim to a reactive iminoquinone methide by activated human neutrophils and hepatic microsomes. *J Pharmacol Exp Ther* **291**: 292–299.

Lamba V, Panetta JC, Strom S, and Schuetz EG (2010) Genetic predictors of interindividual variability in hepatic CYP3A4 expression. *J Pharmacol Exp Ther* **332**:1088–1099.

Macy E and Poon KYT (2009) Self-reported antibiotic allergy incidence and prevalence: age and sex effects. *Am J Med* **122**:778.e1–778.e7.

Mortimer NJ, Bermingham MR, Chapple SJ, and Sladden MJ (2005) Fatal adverse drug reaction to trimethoprim. *Aust Fam Physician* **34**:345–346.

Naisbitt DJ, Farrell J, Gordon SF, Maggs JL, Burkhart C, Pichler WJ, Pirmohamed M, and Park BK (2002) Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol* **62**: 628–637.

Nakayama S, Atsumi R, Takakusa H, Kobayashi Y, Kurihara A, Nagai Y, Nakai D, and Okazaki O (2009) A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab Dispos* **37**:1970–1977.

Ozdemir V, Kalow W, Tang BK, Paterson AD, Walker SE, Endrenyi L, and Kashuba AD (2000) Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method. *Pharmacogenetics* **10**:373–388.

Park BK, Laverty H, Srivastava A, Antoine DJ, Naisbitt D, and Williams DP (2011) Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity. *Chem Biol Interact* **192**:30–36.

Takakusa H, Masumoto H, Yukinaga H, Makino C, Nakayama S, Okazaki O, and Sudo K (2008) Covalent binding and tissue distribution/retention assessment of drugs associated with idiosyncratic drug toxicity. *Drug Metab Dispos* **36**:1770–1779.

Van Boeckel TP, Gandra S, Ashok A, Caudron Q, Grenfell BT, Levin SA, and Laxminarayan R (2014) Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infect Dis* **14**:742–750.

van Haandel L, Goldman JL, Pearce RE, and Leeder JS (2014) Urinary biomarkers of trimethoprim bioactivation in vivo following therapeutic dosing in children. *Chem Res Toxicol* **27**:211–218.

Wallin H, Schelin C, Tunek A, and Jergil B (1981) A rapid and sensitive method for determination of covalent binding of benzo[a]pyrene to proteins. *Chem Biol Interact* **38**:109–118.

Zhou S, Chan E, Duan W, Huang M, and Chen YZ (2005) Drug bioactivation, covalent binding to target proteins and toxicity relevance. *Drug Metab Rev* **37**:41–213.

Address correspondence to: Dr. Jennifer L. Goldman, Divisions of Pediatric Infectious Diseases and Clinical Pharmacology, Children's Mercy Hospitals and Clinics, 2401 Gillham Road, Kansas City, MO 64108. E-mail: jlgoldman@cmh.edu