Metabolism of Trimethoprim to a Reactive Iminoquinone Methide by Activated Human Neutrophils and Hepatic Microsomes

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ABSTRACT

The antibacterial agent, trimethoprim, is normally used synergistically with sulfonamides. Its use is associated with idiosyncratic reactions including liver toxicity and agranulocytosis. In this study, we demonstrated that trimethoprim was oxidized by activated human neutrophils, as well as a combination of myeloperoxidase/hydrogen peroxide/chloride or hypochlorous acid, to a reactive pyrimidine iminoquinone methide intermediate with a protonated molecular ion of m/z 289 as detected by mass spectrometry. In the presence of N-acetyl-L-cysteine (NAC), the pyrimidine iminoquinone methide could be trapped as three NAC adducts. The three NAC adducts were separable on HPLC, but showed the same protonated molecular ion of m/z 452. The proton NMR spectrum of the major adduct showed that the NAC group was at the 6 position of the pyrimidine ring. The mass spectra of the two minor NAC adducts indicated that they were the two diastereomers in which NAC was attached to the $\text{exo}$-cyclic prechiral carbon of the pyrimidine iminoquinone methide. Incubation of trimethoprim with isolated hepatic microsomes, both human and rat, in presence of NAC gave the same set of trimethoprim-NAC adducts. We propose that the formation of this pyrimidine iminoquinone methide by both hepatic microsomes and neutrophils may be responsible for trimethoprim-induced idiosyncratic hepatotoxicity and agranulocytosis.

Trimethoprim is widely used in combination with sulfonamides as an effective antibacterial agent against many bacterial species. However, the use of this combination has been associated with various adverse reactions, such as skin rashes, liver toxicity, blood dyscrasias, and generalized hypersensitivity reactions (Frisch, 1973; Haaverstad and Kaneloning, 1984; Myers and Jick, 1997). Skin disorders that are related with trimethoprim/sulfonamide therapy range from mild drug rashes and urticaria to toxic epidermal necrolysis (Roujeau and Stern, 1994; Roujeau et al., 1995). Although the incidence of trimethoprim/sulfonamide-associated blood dyscrasias, including agranulocytosis, thrombocytopenia, leukopenia, and aplastic anemia is low (1 case/18,000 prescriptions), they are potentially fatal (Williamson and Grote, 1972; Anonymous, 1989; Keisu et al., 1990, 1992). The incidence of idiosyncratic reactions seems to be increased dramatically by some viral infections. For example, when patients with AIDS were treated with trimethoprim/sulfamethoxazole for pneumocystis carinii pneumonia, the incidence of adverse reactions increased to almost 50% (Medina et al., 1990). Evidence suggests that most adverse reactions associated with the combination of trimethoprim/sulfamethoxazole are caused by the sulfamethoxazole component (Rieder et al., 1988; Leeder et al., 1991; Cribb et al., 1996). The use of trimethoprim as a single agent is increasing because of the high incidence of adverse reactions associated with trimethoprim/sulfamethoxazole. However, when trimethoprim was used alone, several idiosyncratic reactions, including skin rashes (e.g., toxic epidermal necrolysis) and neutropenia, were also reported (Nwokolo et al., 1988; Das et al., 1988; Hawkins et al., 1993). Furthermore, using trimethoprim to rechallenge AIDS patients with a history of hypersensitivity to trimethoprim/sulfamethoxazole, Carr et al. (1993) were able to demonstrate that about 20% of the hypersensitivity cases were due to trimethoprim. The mechanism of trimethoprim-induced adverse drug reactions is still unknown. However, it has been proposed that the hemat...
logic, hepatic, and cutaneous reactions associated with trimethoprim are based on an immunologic rather than a directly toxic mechanism and the characteristics of these reactions are consistent with this hypothesis (Frisch, 1973).

Although idiosyncratic reactions are a serious clinical problem associated with many drugs, there is no preclinical model to predict such reactions in humans. It has been demonstrated that many marketed medicines associated with idiosyncratic agranulocytosis can be bioactivated by neutrophils to Michael acceptors that bind covalently to nucleophilic proteins (Uetrecht, 1992; Uetrecht et al., 1994). Many other drugs, such as amodiaquine and acetaminophen, that cause chemically induced hepatic toxicity were found to be oxidized to quinone imines by liver microsomes and bind irreversibly to liver proteins (Maggis et al., 1988; Parkinson, 1996). In the following study, we used an in vitro approach to investigate the oxidation of trimethoprim to reactive intermediates by both human neutrophils and hepatic microsomes.

**Experimental Procedures**

**Materials.** Trimethoprim, N-acetyl-L-cysteine (NAC), and phorbol 12-myristate-13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium hypochlorite (NaOCl) was purchased from Aldrich Chemical Company (Milwaukee, WI). Hydrogen peroxide (H$_2$O$_2$) was obtained from Mallinckrodt Canada Inc. (Pointe-Claire, Quebec, Canada). The concentration of NaOCl was determined by a spectrophotometric method (Hussarin et al., 1970). All solvents used for HPLC and liquid chromatography interfaced with mass spectrometry (LC/MS) analyses were HPLC grade. Myeloperoxidase (MPO) was obtained from Cortex Biochemical (San Leandro, CA). One unit of MPO activity was defined as the amount of enzyme that decomposed 1 mmol of H$_2$O$_2$/min at 25°C and pH 6. The neutrophils were isolated from venous blood collected from normal subjects.

**Analytical Methods.** The HPLC analyses were carried out on a Shimadzu HPLC system containing a LC-600 pump, a SPD-6A UV detector set at 254 nm and a C-R6A integrator (Shimadzu Corporation, Kyoto, Japan). The chromatography columns, packed with 5 μm Ultracarb ODS 30, were supplied by Phenomenex (Torrance, CA). The column used for all analytical work was 2 × 100 mm with a 2 × 30 mm guard column. The column used for isolation of the NAC adduct was 10 × 150 mm with a 10 × 60 mm guard column. A mobile phase of water/acetonitrile/acetic acid (90:10:1, v/v) with 2 mM ammonium acetate was used, unless otherwise specified.

LC/MS and LC interfaced with fragmentation MS were performed on a Sciex API III mass spectrometer (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) with an IonSpray interface. Analyses were carried out with an ionizing voltage of 5 kV and an orifice voltage of 65 V. $^1$H NMR spectra were recorded at 500 MHz with a Varian Unity Plus 500 spectrometer with $^1$H$_2$O as the solvent.

**Neutrophil Isolation.** Blood (60 ml), collected from normal subjects, was mixed with 3% dextran (molecular mass, 500 kDa; Sigma) in 0.9% NaCl at a ratio of 4:1 (v/v). The erythrocytes were allowed to settle for 30 min. The supernatant was carefully drawn off and underlaid with Picoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) at a 5:2 (v/v) ratio. After centrifugation at 500g for 25 min, neutrophils were collected as a pellet and the supernatant was discarded. The supernatant was centrifuged at 350g for 5 min, and the cell pellet was washed twice with Hank’s balanced salt solution (HBSS) without phenol red (Media Services, University of Toronto) before finally suspending the cells in HBSS (10 ml). The neutrophils were stained with 0.1% trypan blue and counted with a hemocytometer. Trypan blue exclusion showed the cell viability to be more than 95% in all isolations.

**Metabolism of Trimethoprim by Activated Neutrophils in the Presence of NAC.** To 1.7 × 10$^7$ neutrophils suspended in HBSS (3 ml) was added an ethanolic solution of trimethoprim (1.5 μM, final concentration 5 μM), an aqueous solution of NAC (75 μl, final concentration 5 mM), and PMA [120 ng in 1.2 μl of dimethyl sulfoxide (DMSO)]. The samples were incubated at 37°C for 60 min. After incubation, the suspension was centrifuged at 500g for 10 min, the supernatant was collected, and the solvent was removed with a stream of nitrogen at 25°C. The samples were redissolved in water and analyzed by LC/MS using selective ion monitoring (SIM) at m/z 291 (trimethoprim) and m/z 452 (trimethoprin-NAC). In the control experiments, DMSO replaced the DMSO solution of PMA.

**Metabolism of Trimethoprim by Rat and Human Liver Microsomes in the Presence of NAC.** Rat-liver microsomes were prepared from male Sprague-Dawley rats (average weight 300 g). The animals were sacrificed by cervical dislocation, and their livers were removed and minced in ice-cold sucrose buffer (0.25 M sucrose, 15 mM hydrochloric acid-modified tris(hydroxymethyl)aminomethane, 0.1 mM EDTA, pH 6.8). The liver homogenates were prepared using a homogenizer. The liver homogenates were filtered through a piece of cheesecloth and centrifuged at 1000g for 11 min at 4°C. The pellets were resuspended in fresh sucrose buffer and recentrifuged. The combined supernatants were centrifuged at 10,000g for 30 min at 4°C, and the pellets were resuspended in fresh sucrose buffer and recentrifuged. The combined 10,000g supernatants were further centrifuged at 100,000g for 90 min at 4°C. The microsome pellets were finally resuspended in a storage buffer (100 mM potassium phosphate, 10% sucrose, pH 7.5) and stored at −80°C before use. Human liver microsomes were a gift from Prof. Tadanobu Inaba (Department of Pharmacology, University of Toronto) and were obtained from the livers of accident victims.

Microsomal cytochrome P-450 content was qualitatively demonstrated by the reduced carbon monoxide difference spectrum method of Omura and Sato (1964). To phosphate buffer (0.1 M, pH 6.8, 747.4 μl with 5 mM MgCl$_2$) was added a microsome suspension (82.6 μl, final concentration 2 mg protein/ml), an ethanolic solution of NAC (50 μl, final concentration 5 mM), and a NADPH−phosphoglucoisomerase (EC 5.3.1.9) generating system (100 μl, final concentration 0.5 mM NADP+, 5 mM glucose 6-phosphate and 0.25 U glucose 6-phosphate dehydrogenase). The suspension was incubated at 37°C for 3 h, after which an equal volume of acetonitrile was added to precipitate the proteins in the mixture. After centrifugation at 500g for 10 min, the supernatant was isolated and dried with a stream of nitrogen. The residue was redissolved in water and analyzed by LC/MS using SIM at m/z 291 (trimethoprin) and m/z 452 (trimethoprim-NAC). In the control experiments, the NADPH−generating system was omitted.

**Oxidation of Trimethoprim by HOCl.** An ethanolic solution of trimethoprim (10 μl, 10 mM) and an aqueous solution of NaOCl (2 μl, 100 mM) were added to an aqueous solution of 60% (v/v) ethanol with 0.2% (v/v) acetic acid (88 μl). The reaction mixture (5 μl) was immediately injected into the mass spectrometer although a HPLC injector. The solvent (methanol) flow rate was set at 200 μl/min and decreased to 20 μl/min with a splitter.

The oxidation of trimethoprim by HOCl was also monitored by a Hewlett Packard diode-array spectrophotometer (HP8452A; Hewlett Packard Company, Palo Alto, CA). An ethanolic solution of trimethoprin (100 μl, 10 mM) and an aqueous solution of NaOCl (20 μl, 100 mM) were added to phosphate buffer (0.1 M, pH 6, 1880 μl) in a quartz cuvette with rapid stirring by a micromagnetic stirring bar. The reaction mixture was immediately scanned by the spectrophotometer at 5-s intervals for 180 s over a wavelength range of 200 to 600 nm.
Trapping HOCl Oxidation Product of Trimethoprim with NAC. Ethanolic solutions of trimethoprim (10 mM, 10 μl) were added to phosphate buffers (0.1 M, 83 μl) with pHs of 5.8, 6.0, 6.4, 6.8, 7.0, 7.2, and 7.6, respectively. The oxidation was initiated by adding aqueous solution of NaOCl (100 mM, 2 μl). Immediately after addition of oxidant, a phosphate-buffered (0.5 M, pH 8.5) NAC solution (200 mM, 5 μl) was added to each reaction mixture. The resulting mixtures were analyzed by LC/MS using SIM at m/z 452 for trimethoprim-NAC adducts.

Preparation of the Major NAC Adduct of the Reactive Intermediate of Trimethoprim for NMR Study. Trimethoprim (100 mg, 0.34 mmol), acetonitrile (15 ml), and phosphate buffer (30 ml, 0.1 M, pH 6) were added to a 150-ml Erlenmeyer flask. An aqueous solution of NaOCl (0.971 ml, 700 mM, 0.68 mmol) was rapidly added to the mixture with vigorous stirring. The solution immediately became bright yellow. After 5 to 10 s, NAC (222 mg, 1.36 mmol) in phosphate buffer (13.6 ml, 0.5 M, pH 8) was rapidly added to the mixture. The reaction mixture was stirred at room temperature for 1 h before it was extracted three times with an equal volume of chloroform. The aqueous phase was separated and then washed with a small amount of pentane to remove any chloroform residue. Methanol was then added to the aqueous phase, and the solution was concentrated to about 1 ml using a rotavap. Methanol was then added to the methanol solution, and the mixture was vacuum-dried. The crude product obtained was further purified by HPLC using a mobile phase of water/acetonitrile/acetic acid (90:10:1, v/v) at a flow rate of 5 ml/min, and the fraction with a retention time of 23 min was collected. HPLC analysis showed that the purity of the final product was more than 95%. The typical yield for the preparation was about 10%. Separation of the other two isomers was not successful.

Oxidation of Trimethoprim by MPO Enzyme System. An ethanolic solution of trimethoprim (10 μM, 10 mM) and MPO (1 μl, 1 U/μl) was added to PBS (81.5 μl, 0.1 M, pH 7), and the reaction was initiated by addition of H2O2 (2.5 μl, 80 mM). After 30 s at 25°C, an aqueous solution of NAC (5 μl, 200 mM) was added. The reaction mixture was then incubated at 25°C for 1 h and analyzed by LC/MS using SIM at m/z 291 (trimethoprim) and m/z 452 (trimethoprim-NAC). In the control experiments, H2O2 was replaced by water.

**Results**

Metabolism of Trimethoprim by Neutrophils and Microsomes in the Presence of NAC. Trimethoprim was metabolized by PMA-activated human neutrophils at pH 7.4. In the presence of NAC, three trimethoprim-NAC adducts with protonated molecular ions at m/z 452 were detected using LC/MS in the SIM mode. The major trimethoprim-NAC adduct had a HPLC retention time of 23 min, whereas the two minor adducts had retention times of 7.5 and 8.5 min, respectively (Fig. 1). No significant metabolism was detected in the control experiment in which the cells were not activated. The approximate ratio of the three trimethoprim-NAC adducts was 17:1:1.

Upon incubation of trimethoprim with human-liver microsomes in the presence of NAC and a NADPH-generating system at pH 6.8, three trimethoprim-NAC adducts with protonated molecular ions of m/z 452 were again observed using LC/MS in the SIM mode (Fig. 2a). The three trimethoprim-NAC adducts showed the same HPLC retention times as those obtained using isolated neutrophils. Similar results were obtained when rat-liver microsomes were used (Fig. 2b). Although the peak with a retention time of 23 min
remained dominant, the approximate ratio of the three trimethoprim-NAC adducts generated in human liver microsomal system was 5:1:1, whereas the ratio in the rat liver microsome system was 4:1:1.

With collision-activated dissociation (CAD) MS, the two minor trimethoprim-NAC adducts with equal intensities gave identical patterns of molecular ion fragmentation (Fig. 3a). The fragment ions were at m/z 258 (15%; MH\(^+\) - C\(_9\)H\(_8\)O\(_3\)N - 2CH\(_3\)), 289 (100%; MH\(^+\) - C\(_9\)H\(_8\)O\(_3\)NS - H), 308 (15%; MH\(^+\) - C\(_9\)H\(_8\)O\(_3\)N - CH\(_3\) + H) and the parent ion at m/z 452 (20%; MH\(^+\)). Under similar CAD conditions, the major trimethoprim-NAC adduct with a retention time of 23 min showed fragmentation ions at m/z 155 (80%; MH\(^+\) - C\(_7\)H\(_6\)O\(_3\)N - C\(_6\)H\(_13\)O\(_3\)), m/z 181 (25%; MH\(^+\) - C\(_7\)H\(_6\)O\(_3\)NS - C\(_6\)H\(_14\)N\(_4\) - H), m/z 290 (20%; MH\(^+\) - C\(_9\)H\(_8\)O\(_3\)NS), m/z 323 (100%; MH\(^+\) - C\(_9\)H\(_8\)O\(_3\)N + H), and the parent ion at m/z 452 (50%; MH\(^+\); Fig. 3b). The major fragment ions of the two minor trimethoprim-NAC adducts (m/z 289) were generated from losing the whole NAC moiety, whereas the major fragment ion of the major trimethoprim-NAC adduct (m/z 323) was generated from losing the NAC moiety with sulfur still attached to trimethoprim. This suggested that in the major trimethoprim-NAC adduct, the NAC was bound to the pyrimidine ring, and the minor adducts were diastereoisomers of trimethoprim-NAC with NAC attached to the methylene carbon bridging the pyrimidine and phenyl rings.

Oxidation of Trimethoprim by HOCI. Trimethoprim was readily oxidized by HOCI. The data from diode array spectrophotometry showed an increased absorption at 320 nm immediately after addition of hypochlorite. The absorption increased to a maximum 40 s after adding hypochlorite and then decreased with a half-life of ~1.5 min (Fig. 4). When the reaction mixture of trimethoprim and hypochlorite was directly analyzed by mass spectrometry, a small but significant iminoquinone methide ion peak (MH\(^+\) - 289) was observed (Fig. 5). When the reaction mixture was analyzed by LC/MS with a mobile phase of water/acetonitrile/acetic acid (90:10:1, v/v), stable products were found with protonated molecular ions of m/z 307 (trimethoprim + OH), MH\(^+\) - 325 (trimethoprim + Cl), MH\(^+\) - 343 (trimethoprim + OH + Cl), and MH\(^+\) - 377 (trimethoprim + OH + 2Cl), which were presumably caused by reactions of the pyrimidine iminoquinone methide with chloride ion and/or water (Fig. 6). In the presence of NAC, the reactive iminoquinone methide could be trapped as the same set of three NAC adducts of m/z 452, and the ratio was pH dependent. At pH 7.0, the ratio of the NAC adducts with retention times of 23, 8.5, and 7.5 min was approximately 1:1:1 (Fig. 7a). At low pH, the major NAC adducts were those with retention times of 8.5 and 7.5 min, whereas at high pH, the NAC adduct with a retention time of 23 min became dominant (Fig. 8).

\(^1\)H NMR of the Major Trimethoprim-NAC Adduct. The major trimethoprim-NAC adduct with a HPLC retention time of 23 min was purified as described above. The proton NMR spectrum of this major NAC adduct consisted of peaks with \(\delta\) 1.81 ppm (3H, s), \(\delta\) 3.34 ppm (1H, dd, \(J = 14.4\) Hz, \(J' = 9.1\) Hz), \(\delta\) 3.70 ppm (1H, dd, \(J = 14.4\) Hz, \(J' = 3.9\) Hz), \(\delta\) 3.73 ppm (3H, s), \(\delta\) 3.79 ppm (6H, s), \(\delta\) 3.83 ppm (1H, s), \(\delta\) 3.85 ppm (1H, s), \(\delta\) 4.38 ppm (1H, dd, \(J' = 9.1\) Hz, \(J'' = 3.9\) Hz), \(\delta\) 6.53 ppm (2H, s) (Fig. 9). The three doublet of doublet peaks were attributed to the two diastereotopic protons and the chiral proton of NAC. Compared with the spectrum of trimethoprim, the proton on the heterocyclic ring of the major NAC adduct is missing. In addition, the two previously equivalent protons of the exo-cyclic methylene group in trimethoprim became diastereotopic due to the presence of chiral NAC, and they gave two close, but separated peaks at \(\delta\) 3.83 and \(\delta\) 3.85 ppm, respectively. This supported the assignment of the major NAC adduct’s structure in which the NAC was attached to the pyrimidine ring of trimethoprim shown as adduct C in Fig. 10.

![Fig. 3.](image-url) a, CAD spectra of trimethoprim-NAC adduct A, with a HPLC retention time of 7.5 min. b, CAD spectrum of trimethoprim-NAC adduct C with HPLC retention time of 23 min.

![Fig. 4.](image-url) Repetitive absorption spectra from the reaction of trimethoprim with HOCI at pH 6. The dotted curve is the UV spectrum of trimethoprim, and the dashed curve is the first UV spectrum of the reaction. Integration time and total run time were 0.5 and 180 s, respectively. The spectra were plotted with a 20-s interval. The concentration of trimethoprim was 0.5 mM and that of HOCI was 1 mM.
Oxidation of Trimethoprim by MPO Enzyme System.

Trimethoprim was oxidized by MPO/H₂O₂/Cl₂ in a similar manner as by HOCl. In the presence of NAC, the same three NAC adducts were also observed by LC/MS in the SIM mode. The ratio of the NAC adducts was about 0.4:1:1 (Fig. 7b). In the absence of NAC, one of the stable chlorinated metabolites with a MH⁺ ion of m/z 325 (trimethoprim + Cl) and a reten-

**Fig. 5.** Mass spectra of the reactive pyrimidine iminoquinone methide intermediate produced by the reaction of trimethoprim with HOCl obtained in the IonSpray mode. a, mass spectrum of the iminoquinone methide obtained with computer extraction of the ion at m/z 289. b, mass spectrum of the reaction mixture containing both the iminoquinone methide (m/z 289) and unreacted trimethoprim (m/z 291).

**Fig. 6.** LC/MS spectra of the stable products of trimethoprim oxidation by HOCl when the reaction mixture was allowed to stand at 25°C without addition of trapping agents. The spectra were obtained with computer extraction of the ions at m/z 307, m/z 325, m/z 343, and m/z 377. The reaction was carried out in 0.1 M pH 6 phosphate buffer at 25°C, and the concentrations of trimethoprim and HOCl were 1 and 2 mM, respectively. The HPLC mobile phase was water/acetonitrile/acetic acid (90:10:1) with 2 mM ammonium acetate. The number in the upper right hand corner of each trace is the total ion current for that ion.

**Fig. 7.** Selective ion monitoring of trimethoprim-NAC adducts generated by HOCl in 0.1 M, pH 7.0, phosphate buffer at 25°C (a) and MPO in presence of H₂O₂ in 0.1 M, pH 7.0, PBS (b). The concentrations for substrates were 1 mM for trimethoprim, 2 mM for HOCl, 1 U for MPO, 2 mM for H₂O₂, and 10 mM for NAC. The mass spectra were obtained in the IonSpray mode using LC/MS at a flow rate of 200 μl/min.

**Fig. 8.** LC/MS-SIM ion currents of trimethoprim-NAC adducts when trimethoprim was oxidized by HOCl in presence of NAC at different pHs. The concentration for reactants were 1 mM for trimethoprim, 2 mM for NaOCl, and 10 mM for NAC.
tion time of 26 min was also observed as the major product by LC/MS using a mobile phase of water/acetonitrile/acetic acid (90:10:1, v/v) (data not shown). The NAC adducts were not detected in the control experiments when H₂O₂ was replaced by water.

**Discussion**

Trimethoprim was metabolized to a reactive metabolite by both activated human neutrophils and hepatic microsomes. The intermediate proved to be electrophilic and reacted with sulfhydryl-containing nucleophiles, such as NAC, to give three adducts with protonated molecular ions of m/z 452. The CAD mass spectra of the NAC adducts and the NMR spectrum of the major adduct provide strong evidence of the assigned structures shown in Fig. 10.

The two trimethoprim-NAC adducts (NAC adducts A and B) with shorter retention times on HPLC (7.5 min and 8.5 min) show an identical CAD molecular ion fragmentation spectrum (Fig. 3a). The major fragment is at m/z 289, which corresponds to the cleavage of the exo-cyclic methylene carbon-sulfur bond and the loss of the attached NAC group along with its sulfur atom. The LC/MS peaks of these two trimethoprim-NAC adducts are of equal intensity. Together, these data strongly support the assertion that the two trimethoprim-NAC adducts with shorter HPLC retention times are diastereomers generated by the attack of chiral NAC on the prechiral exo-cyclic carbon of an iminoquinone methide intermediate.

The major trimethoprim-NAC adduct formed in both the neutrophil and microsome systems with a longer retention time on HPLC (23 min, NAC adduct C) gives very different CAD molecular ion fragments (Fig. 3b). The major MS/MS fragment is at m/z of 323, which is generated by losing the NAC moiety except for the sulfur atom. This major fragment is able to further lose the trimethoxymethyl moiety to give the second most abundant fragment at m/z 155. The proton NMR spectrum of the trimethoprim-NAC adduct C (Fig. 9) confirms the proposed structure. Compared with the proton NMR spectrum of trimethoprim itself (data not shown), the two protons of exo-cyclic methylene group become diastereotopic due to the presence of chiral NAC, and they now give two peaks with slightly different chemical shifts (δ 3.83 and δ 3.85 ppm, respectively), and the proton on the heterocyclic ring is missing.

The formation of this major NAC adduct (NAC adduct C) produced by the neutrophil and liver microsome oxidation systems was also attributed to the iminoquinone methide intermediate. Unlike quinone methides generated from phenolic compounds, which preferentially react with nucleophiles at the exo-cyclic methylene carbon (Bolton et al., 1997), the major site of reaction for the iminoquinone methide of trimethoprim was on the ring. However, the ratio of cyclic to exo-cyclic products was pH dependent. At low pH, the protonated iminoquinone methide may exist partially as the exo-cyclic carbocation leading to more exo-cyclic adduct. At high pH, the iminoquinone methide of trimethoprim predominantly reacts with nucleophiles (i.e., NAC) on the pyrimidine ring. This phenomenon is consistent with the reactivities of heterocyclic aromatic compounds in which nucleophilic additions often occur preferentially in the 2 and 4 positions relative to the hetero atom because of its electron-withdrawing effect. Figure 10 summarizes the proposed pathway for the bioactivation of trimethoprim and the formation of NAC adducts.

Trimethoprim is also oxidized by HOCl and MPO/H₂O₂/Cl⁻ in a similar manner as by neutrophils and liver microsomes. The production of the same set of NAC adducts implies that the same iminoquinone methide intermediate is involved. The pyrimidine iminoquinone methide is presumably formed from one of the possible chloramines. On addition of HOCl, trimethoprim gives a new UV absorption peak with a λmax at approximately 320 nm. This new peak increases to maximum intensity in 40 s, which presumably represents the formation of the iminoquinone methide intermediate. The half-life of the iminoquinone methide, as determined by UV spectrometry, is about 1.5 min, which is considerably shorter than many quinone methides generated from phenolic compounds; thus, it may be much more reactive than most quinone methides in vivo. In the absence of nucleophilic trapping agents, the pyrimidine iminoquinone methide reacts with water and/or chloride to produce several relatively stable products including α-hydroxytrimethoprim (m/z 307) (Fig. 6). This α-hydroxytrimethoprim has been identified as one of the major metabolites of trimethoprim both in vivo (Meshi and Sato, 1972) and in vitro (van’t Klooster et al., 1992). It could be formed by the direct oxidation of trimethoprim by cytochrome P-450, or it could come from the reaction of the quinone methide metabolite with water.

The short-lived pyrimidine iminoquinone methide intermediate can also be detected by MS in the flow system (Fig. 5). The combination of evidence from the UV spectrum, mass spectrum, and the structures of the NAC adducts formed by trapping the reactive intermediate strongly support the formation of a pyrimidine iminoquinone methide from trimethoprim as proposed.

It has been demonstrated that cytochrome P-450 enzymes or peroxidases can oxidize phenolic compounds with appropriate alkyl substituents in the para- position to quinone methides (Peter, 1989; Thompson et al., 1993). The formation of quinone methides has been linked to many adverse reactions, including hepatotoxicity, pulmonary toxicity and carcinogenicity (Gyton et al., 1993; Takahashi, 1988; Mizutani et al., 1983; Thompson et al., 1998; Mayalarp et al., 1996). We have demonstrated the formation of a pyrimidine iminoquinone methide from tri-
methoprim, a commonly used antibacterial agent, which is associated with infrequent, but sometimes serious, idiosyncratic agranulocytosis and hepatotoxicity. Although the mechanism responsible for drug-induced agranulocytosis is not well known, it has been shown that most drugs associated with a high incidence of agranulocytosis are also oxidized to reactive intermediates by activated neutrophils (Uetrecht, 1992; Uetrecht et al., 1994). The formation of the trimethoprim iminoquinone methide by activated neutrophils is consistent with this pattern. Therefore, we propose that the iminoquinone methide formed by neutrophils, or neutrophil precursors in the bone marrow that contain myeloperoxidase, is responsible for trimethoprim-induced agranulocytosis. Likewise, the formation of the pyrimidine iminoquinone methide by hepatic cytochrome P-450 is likely responsible for trimethoprim-induced hepatotoxicity. Although, the pyrimidine quinone methide may also be responsible for other trimethoprim-induced idiosyncratic drug reactions, the relevant site of formation for these reactions is more speculative.

References


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