METABOLISM OF TICLOPIDINE BY ACTIVATED NEUTROPHILS: IMPLICATIONS FOR TICLOPIDINE-INDUCED AGRANULOCYTOSIS

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ABSTRACT:

Ticlopidine is associated with a relatively high incidence of agranulocytosis and aplastic anemia. We have shown that other drugs associated with agranulocytosis are metabolized to reactive metabolites by activated human neutrophils or by HOCl, which is the major oxidant produced by activated neutrophils. We set out to test the hypothesis that ticlopidine also fits this pattern and is oxidized to a reactive intermediate by activated neutrophils and HOCl. As much as 8% ticlopidine was metabolized by activated human neutrophils to a dehydro-ticlopidine; however, this product did not account for all of the decrease in ticlopidine concentration. The oxidation products of ticlopidine by the combination of myeloperoxidase and hydrogen peroxide were the same as those by HOCl: dehydrogenated ticlopidine and 2-chloroticlopidine. A neutrophil-derived reactive metabolite of ticlopidine was trapped with GSH and the same ticlopidine-GSH conjugate was found in both the myeloperoxidase and HOCl systems. Evidence for the identity of the reactive metabolite was obtained by reaction of ticlopidine with HOCl in a flow reaction system coupled to a mass spectrometer. The mass spectra suggested that the reactive metabolite was a thiophene-S-chloride. We conclude that ticlopidine follows the same pattern of reactive metabolite formation by activated neutrophils as other drugs associated with a high incidence of agranulocytosis, and the putative thiophene-S-chloride formed by activated neutrophils may be responsible for ticlopidine-induced agranulocytosis.

The development of ticlopidine was the result of a search for a nonacidic nonsteroidal anti-inflammatory drug. Ticlopidine was shown to have no anti-inflammatory effects, but possessed platelet antiaggregating properties (Saltiel and Ward, 1987). Although the risk versus benefit has been shown to be positive for patients at increased risk for stroke, the use of ticlopidine is limited by a low but significant incidence (1–2%) of agranulocytosis (Hass et al., 1989; Ono et al., 1991), and there are also several reports of aplastic anemia (Tsatalas et al., 1995).

The mechanism by which ticlopidine causes agranulocytosis and aplastic anemia remain unclear at present. It has been suggested that ticlopidine-induced agranulocytosis is due to either direct cytotoxicity or an immune-mediated reaction (Ono et al., 1991). Ticlopidine contains a thiophene ring; thiophene rings have been associated with a significant incidence of adverse reactions in other drugs, such as ticrynafen (tienilic acid) (Lopez-Garcia et al., 1994). Mansuy and coworkers have provided evidence for the formation of a thiophene S-oxide; this reactive intermediate appears responsible for immune-mediated tienilic acid-induced hepatitis (Valadon et al., 1996).

We have demonstrated that HOCl, the principal oxidant generated by activated human neutrophils and monocytes (Weiss, 1989), can oxidize many drugs to reactive intermediates, which may be responsible for the drug-induced adverse reactions such as agranulocytosis (Uetrecht, 1992). We set out to determine whether HOCl oxidizes ticlopidine to reactive intermediates, to identify those reactive intermediates that are formed, and to characterize the reaction pathway. Our working hypothesis was that ticlopidine-induced agranulocytosis is due to a reactive intermediate formed by oxidation of the thiophene ring by activated neutrophils or neutrophil precursors in the bone marrow.

Experimental Procedures

Materials. Ticlopidine hydrochloride, phorbol 12-myristate-13-acetate (PMA)2, N-acetylcysteine, and N-acetylllysine were obtained from Sigma Chemical Company (St. Louis, MO). Sodium hypochlorite and GSH were obtained from Aldrich Chemical Company (Milwaukee, WI). Hydrogen peroxide was purchased from Mallinkrodt Canada Inc. (Pointe-Claire, Quebec). All solvents used for HPLC analysis were HPLC grade. The concentration of sodium hypochlorite was determined spectrophotometrically (Hussain et al., 1970). Human myeloperoxidase (MPO) was obtained from Cortex Biochem Inc. (San Leandro, CA). One unit of MPO activity was defined as the amount of enzyme that would decompose 1 μM hydrogen peroxide/min at 25°C and pH 6.

Analytical. Most of the HPLC was performed using a Shimadzu system (LC-600 pump, SPD-6A UV detector set at 254 nm, and C-R6A integrator; Shimadzu Corporation, Kyoto, Japan). The HPLC columns were packed with 5 μm of Ultracarb ODS 30 (Phenomenex, Torrance, CA). The dimensions of the columns used for analytical work were 2 × 100 mm with a 2–× 30-mm
guard column. A mobile phase of 1 mM ammonium acetate/acetoniitrile/acetic acid (85:15:1, v/v/v) was used with a flow rate of 0.2 ml/min unless otherwise specified. Under these conditions, the retention time of ticlopidine was 10 min and that of the major metabolite (a dehydrogenated ticlopidine) was 12 min. With the mobile phase consisting of 1 mM ammonium acetate/acetoniitrile/ acetic acid (70:30:1, v/v/v), the retention time of ticlopidine was 3 min, and that of 2-chloroticlopidine was 11 min. The standard curves for ticlopidine with these two mobile phases were linear from 0.5 to 100 μM with correlation coefficients of 0.999 for both. We did not have sufficient quantities of the metabolites to determine standard curves for them, so we estimated the concentrations of 2-chloroticlopidine and the dehydro-ticlopidine metabolite by using the approximation that the extinction coefficients of these two metabolites were the same as that of ticlopidine. Although this is unlikely to be an accurate estimate, it seemed preferable to reporting absorbance units with no indication of approximate concentrations.

Mass spectrometry and combined liquid chromatography-tandem mass spectrometry were carried out on a Sciex API III triple quadrupole mass spectrometer (Perkin-Elmer Sciex, Concord, Ontario) equipped with an Ion-Spray interface. Analyses were performed with an ionizing voltage of 5 kV, and high-purity air was used as the nebulizing gas at an operating pressure of 40 psi. The orifice voltage was 60 V. Collision-induced dissociation of selected precursor ions was performed in the radio frequency-only quadrupole region, and used argon as the target gas at an energy of 26 V. A splitter was used just before the mass spectrometer inlet to decrease the flow rate from 200 to about 20 μl/min.

1H NMR spectra were recorded at 500 MHz with a Varian Unity Plus 500 spectrometer (Varian Associates Inc., Palo Alto, CA). The standard was tetramethylsilane and the solvent was CD3OD.

**Metabolism of Ticlopidine by Activated Neutrophils.** Venous blood was collected from normal subjects with a heparinized syringe after obtaining informed consent. Neutrophils were isolated by a modification of the method of Klebanoff (Klebanoff et al., 1992; Liu and Uetrecht, 1995) and suspended in Hanks’ balanced salt solution. Metabolism was initiated by the activation of the neutrophils with 40 ng of PMA dissolved in 20 μl of dimethyl sulfoxide (20 μl dimethyl sulfoxide alone was added to the control), and the suspension was incubated in a shaking water bath at 37°C. Some incubations were carried out in the presence of azide, catalase, or GSH. Standard incubation conditions were: 1) 100 μM ticlopidine, 2) 4.5 × 10^6 cells/ml, and 3) a 45 min incubation time. After incubation, the suspension was centrifuged at 13,000g for 5 min, and 20 μl of the supernatant was analyzed by liquid chromatography/mass spectrometry (LC/MS). For GSH conjugates, the supernatant was collected and the solvent removed with a stream of nitrogen at 55°C. The samples were redissolved in water and analyzed by LC/MS using selective ion monitoring of m/z 569.

**Metabolism of Ticlopidine by MPO System.** Ticlopidine (5 mM in 20 μl of methanol; final concentration, 0.1 mM) was added to 0.1 M pH 6 phosphate-buffered saline. MPO was then added to give a final concentration of 5 IU/ml, and the reaction was initiated by adding hydrogen peroxide (final concentration 0.4 mM). Incubations were done at 37°C for 5 min in a shaking water bath. In control experiments hydrogen peroxide, PPO, or chloride was replaced with phosphate buffer. Aliquots (20μl) of the solution were analyzed by HPLC. For the quantitative study of the metabolism as functions of ticlopidine concentration and incubation time, the standard conditions were 1 IU/ml for MPO, 0.1 mM for hydrogen peroxide, and 5 min at 37°C. Some incubations were done in the presence of GSH (1 or 5 mM) to trap the reactive intermediate.

**Oxidation of Ticlopidine by Hypochlorous Acid.** Mass spectra of the reactive intermediate of ticlopidine were obtained using a flow system coupled to a mass spectrometer. A ticlopidine solution (0.2 mM in water and adjusted to pH 6 with acetic acid) and a HOCl solution (0.2 mM aqueous solution) were fed into an Upchurch Mixing Tee (dead volume of 3.1 μl; Upchurch Scientific, Oak Harbor, WA) by two Harvard syringe pumps (Harvard Apparatus Inc., South Natick, MA). The flow rate was 40 μl/min for both ticlopidine and HOCl. From the mixing chamber the products flowed through a fused silica capillary to the mass spectrometer in ~10 s with a splitter just before the mass spectrometer inlet to decrease the flow rate to ~15 μl/min.

A Hewlett-Packard diode-array spectrophotometer (HP8452A; Hewlett-Packard Company, Palo Alto, CA) was used to determine the rate of oxidation of ticlopidine by HOCl. Scanning of the reaction mixture was initiated immediately after the addition of sodium hypochlorite (80 μl, 5 mM aqueous solution) to ticlopidine (2 ml, 0.2 mM in pH 6, 0.1 M phosphate buffer) with rapid stirring. Reactions were monitored at 10-s intervals for 300 s over a wavelength range of 190 to 490 nm.

Stable ticlopidine oxidation products were analyzed by LC/MS. In one experiment, an excess of sodium borohydride was added to the products to determine whether any of the products were readily reduced. In particular, we were looking for evidence that the dehydro-metabolite might be an imine. For NMR study, a sodium hypochlorite solution (1.75 ml of 0.1 M aqueous solution) was added dropwise to ticlopidine (10 ml of 20 mM aqueous solution in 60% ethanol with the pH adjusted to pH 6 with 2.5 ml of acetic acid) with rapid stirring. The solution became pale yellow. The products were purified by HPLC using a Phenomenex 10 × 150 mm column with a 10 × 60 mm guard column and a mobile phase of water/acetoniitrile/acetic acid (60:40:1, v/v) at a flow rate of 4 ml/min, and the fraction with the retention time of 8.6 min was collected. HPLC analysis showed that the purity for the product was greater than 95%.

**Results**

**Metabolism of Ticlopidine by Activated Neutrophils.** Ticlopidine was metabolized by activated human neutrophils to a major metabolite with a retention time of 10 min on LC/MS. The metabolite increased almost linearly with ticlopidine concentration and cell number (Fig. 1, a and b), respectively. Sodium azide, an inhibitor of MPO, inhibited the metabolism of ticlopidine by the activated cells (~70% at 1 mM). Catalase, which catalyzes the breakdown of hydrogen peroxide, also inhibited the metabolism of ticlopidine (maximal
65% inhibition at ~3000 U/ml). The tandem mass spectrometry (MS/MS) spectrum of the major metabolite consisted of a M + 1 ion at m/z 262 (100%) with a fragment ion at m/z 125 (24%). The MS/MS spectrum of ticlopidine had a M + 1 ion at m/z 264 (100%) with fragment ions at m/z 125 (15%) and 154 (44%). We suspect that the lack of a fragment at m/z 154 in the spectrum of the metabolite is due to the formation of a double bond as shown in Fig. 2. This metabolite is referred to as dehydro-ticlopidine.

On incubation of ticlopidine with activated neutrophils and GSH, a major ticlopidine-GSH conjugate was found using LC/MS in the selective ion monitoring mode. The adduct had a molecular weight of 568, and with a solvent containing 15% acetonitrile, the retention time was 2 min, which was the same as the major conjugate produced by oxidation of ticlopidine by hypochlorous acid followed by reaction with GSH. The LC/MS/MS of the chemically synthesized (reactive intermediate generated by HOCl) GSH conjugate (M + 1 ion at m/z 569) is shown in Fig. 3. An analogous adduct, with an M + 1 ion at m/z 425 by mass spectrometry, was formed when N-acetylcysteine was added instead of GSH immediately after oxidation of ticlopidine with HOCl (spectrum not shown).

Metabolism of Ticlopidine by MPO System. The oxidation of ticlopidine by the MPO system was extensive and no parent drug remained after 30 min. The extent of oxidation was greatly decreased in the absence of hydrogen peroxide, MPO, or chloride (Fig. 4). Ticlopidine was oxidized by the MPO system to two major products; the same products were formed by oxidation with HOCl. LC/MS and LC/MS/MS analysis indicated that the first product was the same as the metabolite generated in activated neutrophils, i.e., dehydro-ticlopidine, whereas the second product had a molecular ion at m/z 298 with an isotopic pattern of two chlorines. The MS/MS spectrum of the second product was similar to that of ticlopidine except in the thiophene region. The thiophene region of the product consisted of a singlet at δ 6.59 ppm (1H); in contrast, that of ticlopidine consisted of δ 6.69 ppm (1H, d, J = 5.13 Hz) and δ 7.11 ppm (1H, d, J = 5.13 Hz). Compared with the spectra of ticlopidine, the changes in the aromatic region were minimal. These spectra suggested that the second product had a chlorine substituted on the 2-position of ticlopidine (Silverstein and Bassler, 1991). The production of dehydro-ticlopidine and 2-chloroticlopidine as a function of ticlopidine concentration is shown in Fig. 5.

Oxidation of Ticlopidine by Hypochlorous Acid. Ticlopidine was readily oxidized by hypochlorous acid. The diode-array spectrophotometric data (Fig. 6) first demonstrated an increase in absorption at 270 nm, which then decreased with a half-life of greater than 1 min. (Although hypochlorous acid also absorbs at 270 nm, the extinction coefficient at the λ_{max} of 290 is only 41 M^{-1} cm^{-1} under these conditions, and it does not make a significant contribution to the absorbance.) Analysis of the products by HPLC indicated that the major products were 2-chloroticlopidine and dehydro-ticlopidine. The dehydro-ticlopidine peak was not affected by the addition of an excess of sodium...
borohydride, thus providing additional evidence for the structure proposed in Fig. 7 rather than an imine, which would also be consistent with the MS data. Oxidation of ticlopidine in the flow system interfaced with the mass spectrometer gave major products at \( m/z \) 262 (18%), 264 (100%), 266 (64%), 298 (22%), and 300 (13%) as shown in Fig. 8. The ion at \( m/z \) 262 corresponded to the dehydro-ticlopidine, and the ion at \( m/z \) 264 consisted of the protonated molecular ion of ticlopidine and the chlorine isotopic peak of the dehydro-ticlopidine. The MS/MS spectrum of the ion at \( m/z \) 298 was essentially the same as that of 2-chloroticlopidine. In one experiment, a 0.1 mM solution of ticlopidine was oxidized with an equimolar amount of HOCl, and then an excess of GSH was immediately added. This led to an immediate 65% decrease in the ion at \( m/z \) 298 followed by a very slow decrease with subsequent injections. This suggests that about 65% of the \( m/z \) 298 ion consisted of a reactive chlorinated species and the rest was 2-chloroticlopidine. We attempted to purify the dehydro-ticlopidine and the GSH conjugate by preparative HPLC so that they could be analyzed by NMR; however, they appeared to break down so that we were never able to obtain reasonable NMR spectra.

**Discussion**

Ticlopidine was metabolized by activated neutrophils, and the major observed metabolite was a dehydro-ticlopidine. This is significantly different from the hepatic metabolism of ticlopidine (Tuong et al., 1981; Picard-Fraire, 1984). It can be explained by a mechanism in which the sulfur is chlorinated to a reactive intermediate, thiophene-S-chloride, followed by loss of HCl to form the proposed dehydro-ticlopidine, as shown in Figs. 7 and 8. In the oxidation of ticlopidine by MPO/H\(_{2}\)O\(_2\)/Cl\(^-\) and HOCl, the same reactive intermediate can also react with nucleophiles (chloride ion) or rearrange to form the
2-chloroticlopidine. This reactive intermediate was also trapped by GSH to form a GSH conjugate in the MPO and HOCl systems as well as in the neutrophil incubations.

We hypothesize that at least part of the decrease in absorption at 270 nm observed in Fig. 6 was due to the decay of the reactive intermediate. This intermediate had a protonated molecular ion at m/z 298 as detected by the flow system coupled with a mass spectrometer. The flow system could not differentiate the stable 2-chloroticlopidine (m/z 298) from the proposed intermediate; however, the very rapid 65% decrease in the 298 ion on addition of excess GSH followed by a very slow decrease strongly suggests that about two-thirds of this ion is due to the reactive intermediate.

The oxidation products of ticlopidine by MPO/H₂O₂/Cl⁻ and HOCl systems were very similar (2-chloroticlopidine and dehydroticlopidine). However, only dehydro-ticlopidine was observed in the neutrophil incubations, and we suspect that this is due to the nucleophilic environment of the cells in which the reactive intermediate selectively reacts with “soft” nucleophiles (GSH and other protein sulfhydryl groups), which probably eliminate its reaction with chloride to form the 2-chloroticlopidine. In addition, although as much as 8% of ticlopidine was metabolized by activated human neutrophils to the dehydro-metabolite, this metabolic pathway did not account for all the loss of ticlopidine. However, we did not have any radiolabeled ticlopidine to conduct covalent binding studies. The major GSH conjugate found in all three oxidative systems (activated cells, MPO/H₂O₂/Cl⁻, and HOCl) is proposed to be 2-GSH-ticlopidine based on LC/MS/MS data and by analogy with the structure of 2-chloroticlopidine. This leads to the proposed pathway as shown in Fig. 7. The observation that the metabolism of ticlopidine by activated neutrophils was inhibited by low concentrations of azide and catalase is consistent with the involvement of MPO and the observation that HOCl produced a similar pattern of products suggests that the major oxidant was HOCl. The observation that the same GSH conjugate was formed by all three systems suggests that the same reactive intermediate was formed. We have demonstrated that other drugs that are associated with a high incidence of agranulocytosis are extensively metabolized to reactive intermediates by activated neutrophils. These include clozapine (Liu and Uetrecht, 1995), aminopyrine (Uetrecht et al., 1995), propylthiouracil, dapsone, sulfonamides, and procainamide (Uetrecht, 1992). Others have found that amiodarone, another drug associated with a high incidence of agranulocytosis, is oxidized to a reactive metabolite by activated neutrophils (Clarke et al., 1990). It is likely that these reactive metabolites are responsible for drug-induced agranulocytosis; however, the mechanism is not clear. Formation of the reactive metabolites could occur in the bone marrow, either by neutrophils or neutrophil precursors that contain MPO. Alternatively, formation of reactive metabolites by neutrophils outside of the bone marrow could lead to modification of the cells and this could, in turn, lead to an immune response to the altered cells. Such an immune response could also injure cells in the bone marrow. The formation of a reactive metabolite may explain why ticlopidine reduces the clearance of theophylline (Colli et al., 1987), antipyrine (Knudsen et al., 1992), and phenytoin (Rindone and Bryan, 1996; Riva et al., 1996), which has been speculated to be due to the inhibition of cytochrome P450. Because the thiophene moiety is susceptible to S-oxidation (Valadon et al., 1996), ticlopidine may be oxidized to a reactive S-oxide in the liver and acts as a mechanism-based inhibitor for cytochrome P450. It is likely that similar reactive metabolites would be formed by clopidogrel, a newer agent with a very similar structure; however, clopidogrel is given at a much lower dose, and it is not associated with a high incidence of bone marrow toxicity.

In summary, we have found that ticlopidine is oxidized by activated neutrophils to a reactive intermediate, possibly a thiophene-S-chloride. Although this metabolic pathway is unlikely to make a significant contribution to the clearance of ticlopidine, we propose that this reactive intermediate may be responsible for ticlopidine-induced bone marrow toxicity. This observation is consistent with the pattern in which the drugs associated with the highest incidence of idiosyncratic agranulocytosis are oxidized by activated neutrophils to reactive metabolites.

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References