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Metabolism of Vesnarinone by Activated Neutrophils: Implications for Vesnarinone-Induced Agranulocytosis[1]

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ABSTRACT

Vesnarinone is an important new drug that significantly decreases mortality rates in severe congestive heart failure; however, its use is associated with a relatively high incidence (~1%) of agranulocytosis. The authors studied its metabolism by activated neutrophils, the target for this toxicity, and evidence pointed to a pathway that involved a reactive iminium ion. Hydrolysis of the iminium ion led to a reactive quinone imine. The same pathway was observed with a combination of myeloperoxidase/hydrogen peroxide/chloride, the major oxidizing system of neutrophils, or hypochlorous acid, which is generated by this system. Activation of the neutrophils could be achieved by phorbol ester or by influenza vaccine and there is evidence to suggest that the administration of influenza vaccine during vesnarinone therapy may increase the risk of agranulocytosis. Incubation of radiolabeled vesnarinone with activated neutrophils led to covalent binding of almost 5% of the drug to the cells. Both the iminium ion and quinone imine generated by hypochlorous acid could be trapped with glutathione. It was proposed that these reactive metabolites, generated by neutrophils or neutrophil precursors in the bone marrow, may be responsible for the vesnarinone-induced agranulocytosis. Factors such as infection or vaccination that activate neutrophils may increase the risk of agranulocytosis.

Congestive heart failure is a major cause of death and vesnarinone appears to represent a significant advance in its treatment, with a reported decrease in the mortality rate of 62% (Feldman *et al.*, 1993). Although vesnarinone has a clearly beneficial risk *versus* benefit ratio, its use is associated with agranulocytosis, which is a potentially fatal adverse reaction. In studies of toxicity in animals, no significant bone marrow toxicity was noted. In the initial clinical trials in Japan that involved 256 patients, no significant toxicity was observed. By contrast, when clinical trials were initiated in the United States, in 4 of the first 28 patients, agranulocytosis developed (Uetrecht *et al.*, 1989). This observation suggested that there might be a racial difference in the risk of vesnarinone-induced agranulocytosis. One other difference in the two populations was that none of the Japanese patients had received influenza vaccine, although seven of the patients in the United States had. All four cases of agranulocytosis occurred in patients who had received influenza vaccine, one just before the initiation of vesnarinone therapy and the other three during therapy with vesnarinone. Since these early studies, vesnarinone has been marketed in Japan, which resulted in more than 100 cases of agranulocytosis, with an approximate incidence of 0.1 to 1%. Further clinical trials in the United States, in which the administration of influenza vaccine was excluded, also resulted in additional cases of agranulocytosis; however, the incidence, which was initially 4 of 28, dropped to 1 to 2%. Thus, if there is a racial difference in the risk of agranulocytosis, it is not as large as it first appeared. During these latter studies, which included weekly neutrophil counts, there did not appear to be any deaths caused by the agranulocytosis but there were several deaths of cardiac failure associated with discontinuation of the drug. Although the four cases of agranulocytosis in patients who received influenza vaccine could have been due to chance, it represented an interesting lead to the possible risk factors for drug-induced agranulocytosis.

The incidence of idiosyncratic drug reactions appears to be increased by some viral infections. For example, the incidence of ampicillin-induced rash is greatly increased in patients who have mononucleosis (Pullen *et al.*, 1967) and the incidence of adverse reactions to drugs, especially sulfonamides, is greatly increased in patients with acquired immunodeficiency syn-

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ABBREVIATIONS: MPO, myeloperoxidase; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NMR, nuclear magnetic resonance; LC/MS, liquid chromatography interfaced with mass spectroscopy; s, singlet; d, doublet; t, triplet; m, multiplet; m/z, mass over charge; M, molecular ion; PMA, phorbol myristate acetate.

drome (Gordin *et al.*, 1984; Mitsuyasu and Groopman, 1983). The basis for these virus-associated increases in the incidence of adverse drug reactions is unknown. We have demonstrated that several drugs that are associated with agranulocytosis are metabolized to reactive intermediates by activated neutrophils and monocytes (Uetrecht, 1990, 1992). When activated, neutrophils release MPO and generate hydrogen peroxide (Klebanoff, 1968; Weiss, 1989). This combination can oxidize drugs but the major product results from the oxidation of chloride to hypochlorous acid. It is possible that one risk factor for drug-induced agranulocytosis is an infection that activates neutrophils or neutrophil precursors in the bone marrow so that they form reactive metabolites. It is also possible that influenza vaccine could activate leukocytes. We set out to determine whether neutrophils metabolize vesnarinone to reactive intermediates, to identify those reactive metabolites that were formed and to determine whether influenza vaccine could activate neutrophils.

Materials and Methods

Materials. Vesnarinone and ^{14}C-vesnarinone (specific activity, 13.8 mCi/mmol) in which the radiolabel was on the carbonyl of the dihydroquinolinone ring were obtained from Otsuka America (Rockville, MD). Its radiochemical purity was 98% and there were no other peaks on HPLC. The chemical ^{14}C-vesnarinone, in which the radiolabel was on the veratric carbonyl (specific activity, 1.6 mCi/mmol), was a custom synthesis by Dr. Peter Pekos (York University, Toronto, Ontario, Canada). Its radiochemical purity was lower than reported and it was further purified by TLC (silica gel, developed with ethyl acetate, ethanol, 95:5, v/v, Rf 0.6, to give a radiochemical purity of 95%). MPO was obtained from Alpha Therapeutics (Los Angeles, CA). The MPO activity was determined by the method of Chance and Maehly (1955).

Synthesis of veratrylpiperazinamide. Veratric acid (1 g; Fluka, Buchs, Switzerland) was added to thionyl chloride (2 ml) with a drop of dimethylformamide and refluxed for 1 hr. The thionyl chloride was removed under vacuum and piperazine (0.86 g) in anhydrous ether was added. After 1 hr, the solvent was evaporated, which left an oil that was about 85% pure by HPLC. Purification by silica gel chromatography was unsuccessful but the crude product was sufficient for further studies.

Synthesis of reactive intermediate glutathione adducts. Sodium hypochlorite (1 ml of a 12 mM aqueous solution) was added to vesnarinone (10 ml of a 4 mM solution in 60% ethanol with the pH adjusted to 4 with acetic acid) with rapid stirring. The solution immediately became dark purple and glutathione (1 ml of a 4 mM aqueous solution) was immediately added, which rapidly turned the color to a light yellow. The ethanol was removed on a rotary evaporator and more ethanol was added to remove the water as an azeotrope. The residue was dissolved in methanol and purified on preparative reverse-phase TLC (Whatmann LKC18F, Maidstone, England) with a solvent that consisted of 1% aqueous ammonium acetate, acetonitrile and acetic acid (60:40:1, v/v). A band with a Rf of 0.61 contained the glutathione adducts. The conjugates were further purified by HPLC with a solvent that consisted of water, acetonitrile, acetic acid and triethylamine (84:16:1:0.05, v/v).

Analytical methods. The HPLC of vesnarinone metabolites, glutathione conjugates and procainamide and its hydroxylamine were performed with a 15-cm, 5-μm Spherisorb ODS2 column (Jones Chromatography, Mid Glamorgan, UK) and a solvent of water, acetonitrile, acetic acid and triethylamine (80:20:1:0.05, v/v) at a flow rate of 1 ml/min. Under these conditions, the retention time of procainamide was 3.6 min and that of its hydroxylamine was 2.7 min. A linear standard curve was obtained for the hydroxylamine of procainamide and veratrylpiperazinamide. However, we did not have sufficient material to produce a standard curve for the other metabolites of vesnarinone and we were forced to assume that the extinction coefficient for these metabolites was the same as for the parent drug. This of course is not strictly accurate but, because the aromatic rings of the two major metabolites, other than veratrylpiperazinamide, do not appear to be altered, this approximation is probably reasonably close.

MS in the electron impact mode was performed with a VG-Analytical (Manchester, England) ZAB-SE mass spectrometer. Samples of metabolites for MS were obtained by repeated collections from the HPLC column.

The mass spectra of the reactive intermediates produced by the reaction of vesnarinone with hypochlorite were obtained with a continuous-flow system in which the reactants were mixed and continuously fed into a Sciex API III mass spectrometer (Thornhill, Ontario). A vesnarinone solution (2.5 mM dissolved in 60% ethanol and adjusted to pH 4 with acetic acid) and a hypochlorite solution (4 mM aqueous solution) were pumped by Harvard (South Natick, MA) pumps into an Upchurch Mixing Tee (Upchurch Scientific, Oak Harbor, WA) with a dead volume of 3.1 μl. The flow rate was 60 μl/min for vesnarinone and 40 μl/min for hypochlorite. The product then flowed through a fused silica capillary to the mass spectrometer with a splitter just before the mass spectrometer inlet to decrease the flow rate to 10 μl/min. IonSpray was used as the ionization mode.

The Sciex mass spectrometer was also used for LC/MS. The HPLC column used was an Ultracarb ODS (30) 2 × 100-mm column (Phenomenex, Torrance, CA) and it was eluted with a solvent of water, acetonitrile and acetic acid (80:20:1, v/v) at a flow rate of 0.2 ml/min. A splitter was used to decrease the flow into the mass spectrometer to 10 μl/min. The Ultracarb column was used for this application because it did not require triethylamine to prevent tailing of peaks and triethylamine markedly inhibits ionization in the mass spectrometer. For the minor metabolites described under Results, the percent acetonitrile in the HPLC solvent was increased from 20% to 30%.

Both ^1H and ^{13}C NMR spectra were recorded at 500 (125, ^{13}C) MHz with a Brucker (Brucker Canada; Milton, Ontario) AM500 spectrometer at the NMR Spectrometry Laboratory of the Carbohydrate Research Centre, University of Toronto. Spectra were obtained either in CD_3OD:$CDCl_3$, 2:1, which contained a trace of tetramethylsilane (0 ppm, ^1H and ^{13}C) as the internal standard with the *delta* (ppm) scale. Aromatic protons of vesnarinone and its adducts were assigned by conventional 1D decoupling assignments and for the parent drug by ^1H-^{13}C correlation. The ^1H-^1H and ^1H-^{13}C correlation experiments used the standard manufacturer's software.

Neutrophil isolation. Blood was drawn into a heparinized syringe from normal white subjects. Neutrophils were isolated by differential centrifugation on Ficoll-Paque (#17-0840-03; Pharmacia, Uppsala, Sweden) by the method of Boyum (1984). Cell viability was greater than 95%, as determined by trypan blue exclusion.

Oxidation of vesnarinone by activated neutrophils. Vesnarinone (5 mM in 10 μl of 60% ethanol; final concentration, 0.1 mM) was added to 2 × 10^6 neutrophils in 0.5 ml of Hanks' balanced salt solution. Metabolism was initiated by the activation of the neutrophils with 20 ng of PMA dissolved in 10 μl of dimethyl sulfoxide. After incubation for various lengths of time, the cells were spun down and 100 μl of the supernatant was injected into the HPLC.

Oxidation of vesnarinone by the MPO system. Vesnarinone (5 μl, 4 mM in 60% ethanol) was added to 185 μl of phosphate-buffered saline (0.1 M, pH 6) to give a final concentration of 100 μM. MPO as added and then the reaction was initiated by adding hydrogen peroxide (5 μl; final concentration, 0.2 mM). After incubation for various lengths of time, 15-μl aliquots were injected directly into the HPLC without further preparation. Some incubations were carried out in the absence of added chloride.

Oxidation of vesnarinone by hypochlorous acid. The oxidation of vesnarinone was studied in the flow system coupled with the mass spectrometer, as described earlier. In addition, equal volumes of vesnarinone (80 μM in 60% ethanol) and hypochlorite (70 μM in buffer) were mixed with a Pasteur pipet as rapidly as possible in a cuvette and the spectra were scanned in a Hewlett Packard (Fort Collins, CO) diode array spectrophotometer.

Activation of neutrophils by influenza vaccine. Procainamide (0.1 mM) and ascorbic acid (1 mM) were added to neutrophils (4.8 × 10^6/ml). The cells were activated with either influenza vaccine [Connaught (Willowdale, Ontario) trivalent type A+B, 40 μl/ml] or phorbol ester (PMA, 40 ng/ml dissolved in 20 μl of dimethyl sulfoxide) and incubated in Hanks' buffer (0.5 ml) for 45 min at 37°C. The influenza vaccine was either used from the bottle or it was opsonized by incubation for 45 min with an equal volume of serum from the same donor. After incubation, the cells were sedimented by centrifugation and the supernatant was analyzed by HPLC to quantify the amount of hydroxylamine metabolite produced.

Covalent binding of vesnarinone to neutrophils. Human neutrophils (2.4 × 10^6) were suspended in 0.5 ml of Hanks' buffer and 0.25 μCi of ^{14}C-vesnarinone (labeled on the dihydroquinolinone ring, dissolved in 10 μl of 60% ethanol) was added. The cells were then activated with either influenza vaccine, opsonized influenza vaccine or phorbol ester, as described earlier, and incubated at 37°C for 45 min. At the end of the incubation, the cells were collected on a filter (GF/B, 2.4 cm, Whatmann) and washed repeatedly with ethanol until the activity of the alcohol washes was similar to that of the background. The filters were then suspended in scintillation fluid and counted in a scintillation counter.

To obtain more information about the metabolite responsible for covalently binding, we compared the covalent binding of two different forms of radiolabeled vesnarinone, one in which the radiolabel was on the dihydroquinolinone ring and the other in which it was on the veratric carbonyl. For this experiment, the specific activity of the two radiolabeled drugs was matched by a dilution of the dihydroquinolinone-labeled form with unlabeled vesnarinone to a specific activity of 1.6 mCi/mmol. Both forms of radiolabeled vesnarinone were dissolved in 60% ethanol and 10 μl of each, which contained 0.071 μCi, was added to the neutrophils, activated with PMA, incubated, washed and counted as before.

Covalent binding of vesnarinone to albumin. Albumin (0.2 mg), MPO (1 unit) and ^{14}C-vesnarinone (0.25 μCi, dihydroquinolinone label)] were dissolved in 0.2 ml of phosphate-buffered saline (pH 6) and hydrogen peroxide was added to make the final concentration 0.2 mM. After incubation at 25° for 45 min, the protein was precipitated with four volumes of acetone and washed with acetone. The protein was then redissolved, precipitated and washed two more times. The protein was dissolved again and the protein content was determined by the Bradford method with a kit from Sigma (St. Louis, MO). The radioactivity was determined with a scintillation counter.

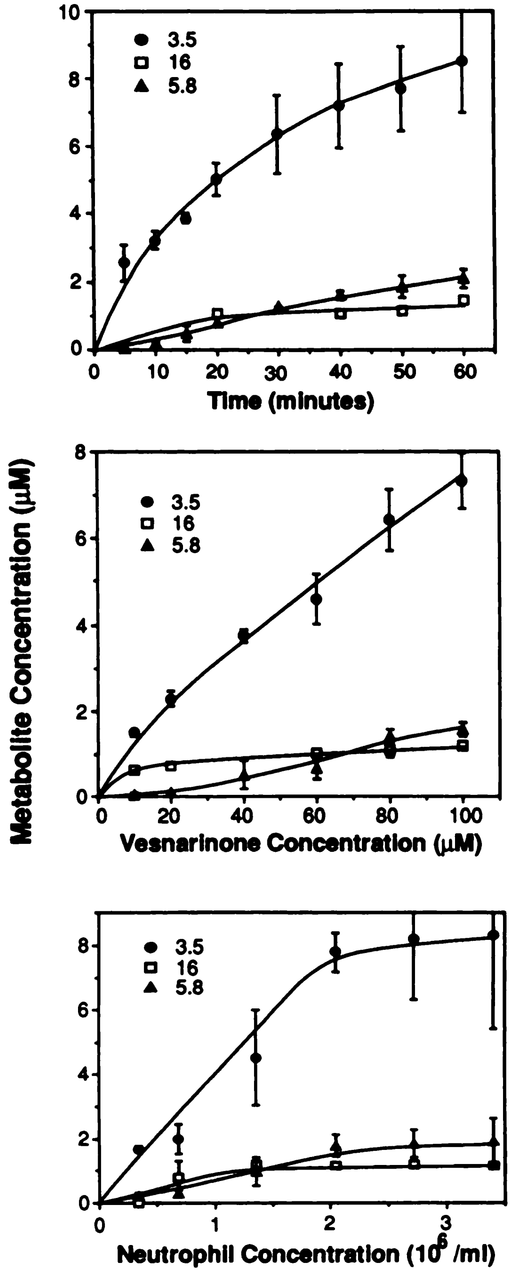

Fig. 1. Metabolism of vesnarinone to three metabolites with retention times of 3.5, 5.8 and 16 min as a function of time, vesnarinone concentration and neutrophil concentration. The concentration of the metabolites 5.8 and 16 are approximate because no standards were available to determine a standard curve. The data represent the mean ± S.E. from four determinations.

Results

Metabolism of vesnarinone by neutrophils. Vesnarinone was metabolized by neutrophils to three major metabolites with retention times of 3.5, 5.8 and 16 min on HPLC. No significant metabolism occurred in the absence of activation of the cells. The metabolites increased with time, vesnarinone concentration and cell number (fig. 1).

The mass spectrum of the metabolite with a retention time of 3.5 min was obtained in the electron impact mode and it consisted of a molecular ion at m/z 250 (100%) with fragment ions at m/z 195 (13%), 194 (11%), 182 (48%) and 165 (100%). This mass spectrum suggested that the metabolite was veratrylpiperazinamide and synthesis, as described under Materials and Methods, produced a product with the same retention time on HPLC and mass spectrum as the metabolite.

The mass spectrum of the metabolite with a retention time of 5.8 min was also obtained in the electron impact mode with high resolution. The molecular ion was at m/z 411.1184 (31%, $C_{22}H_{25}N_3O_5$) and fragment ions at m/z 383.1870 (13%, $C_{21}H_{25}N_3O_4$, -CO), 230.1057 (66%, $C_{13}H_{14}N_2O_2$), 202.1100 (44%, $C_{12}H_{14}N_2O$), 189.1028 (50%, $C_{11}H_{13}N_2O$), 165.0556 (100%, $C_9H_9O_3$).

The mass spectrum of the metabolite with a retention time of 16 min was obtained in the electron impact mode. The molecular ion was at m/z 397 (60%) with fragment ions at m/z 369 (58%), 216 (71%), 188 (58%) and 165 (100%). The same product was obtained (as determined by its mass spectrum and retention time on HPLC) from the oxidation by hypochlorite of a known hepatic metabolite of vesnarinone with two hydroxy groups on the same side of the piperazine ring (OPC-8677; Miyamoto *et al.*, 1988)).

Later, when HPLC was interfaced with the Sciex API III mass spectrometer, two additional minor metabolites were observed with M + 1 ions at m/z 430 and 440. There were at least two isomers of the metabolite at m/z 430, with retention times of 17.5 and 20.5 min, respectively (the retention time of the 440 metabolite was 29.5 min). The two 430 isomers both had a chlorine isotope peak at m/z 432 and major fragment ions at m/z 165. This result indicated that they contained chlorine and that the chlorine could not be on the veratryl ring. The same products were produced by oxidation of vesnarinone by hypochlorous acid. The isomer of the 430 metabolite with a retention time of 17.5 min was purified by TLC in sufficient quantity to obtain its proton NMR spectrum ($CDCl_3$). This was similar to that of vesnarinone, except for the aromatic region, which consisted of δ 6.78 (1H, s), 6.85 (1H, s), 6.88 (1H, d, J = 7.8 Hz), 7.02 (2H, m). The two triplets at δ 2.6 and 2.9 indicate the chlorine could not be on positions 2 or 3. We believe that the chlorine is on the 5 position for the reasons outlined in the interpretation of the NMR spectra of the glutathione conjugates.

The concentrations of veratrylpiperazinamide formed by neutrophils from two patients with a history of vesnarinone-induced agranulocytosis were 1.1 and 2.0 μM. This was not higher than that produced by neutrophils from four normal controls, 1.9 ± 0.2 μM (95% confidence interval, 1.5–2.2 μM). There was also no difference in the formation of the other major metabolites (data not shown).

Oxidation of vesnarinone by MPO/hydrogen peroxide. A similar pattern of products was obtained when vesnarinone was oxidized by the MPO system, except the metabolism was more extensive and additional products were observed. After 70 min, no parent drug remained (data not shown). A major metabolite observed in this system, but not in the neutrophil system, had a retention time of 21 min. It could be extracted into chloroform under acidic conditions and was rapidly reduced to veratrylpiperazinamide with N-acetylcysteine or more slowly on standing without the addition of any reducing agent. The EI mass spectrum of this product had a molecular ion at m/z 284 (2%) with a chlorine isotope peak at m/z 286 and fragments at m/z 249 (20%, loss of chlorine), 248 (9%, loss of HCl) and a peak at m/z 165 (100%, for the veratryl fragment). The large fragment at 165 indicated that the chlorine could not be on the aromatic ring. The loss of basic character and reactivity indicated that the chlorine was on the basic nitrogen of the piperazine ring and it was concluded that this product is simply N-chloroveratrylpiperazinamide.

The rate of oxidation was greatly decreased in the absence of added chloride ion (fig. 2). Although no chloride was added to the incubation, the MPO contained a trace of chloride and, therefore, a small amount of chloride was present in the incubation. There was also a large decrease in the formation of other products in the absence of added chloride (data not shown).

Oxidation of vesnarinone by hypochlorous acid. Vesnarinone was rapidly oxidized by hypochlorous acid. The major metabolites were the same as those observed with MPO and hydrogen peroxide, although many minor products were also observed. At pH 5 or lower, a deep purple color resulted. At a higher pH, rapid oxidation occurred to produce the same pattern of metabolites but no purple color was observed. Scanning of the absorption spectrum of this mixture at pH 4 at 4-sec intervals resulted in the pattern shown in figure 3. New peaks

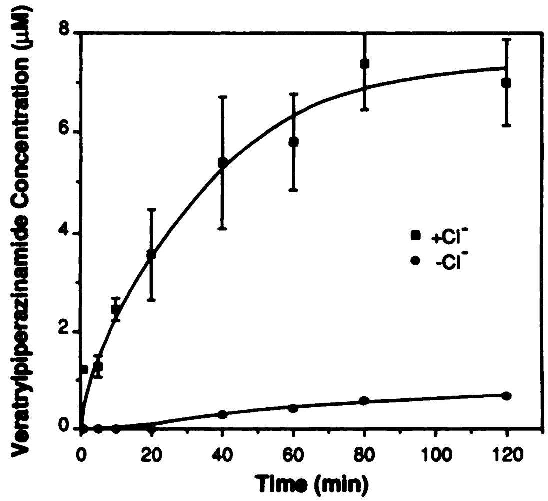

Fig. 2. Comparison of formation of veratrylpiperazinamide by MPO/H_2O_2 in the absence of added chloride ($-Cl^-$) or in the presence of added chloride ($+Cl^-$, 0.15 M). The data represent the mean ± S.E. from four determinations.

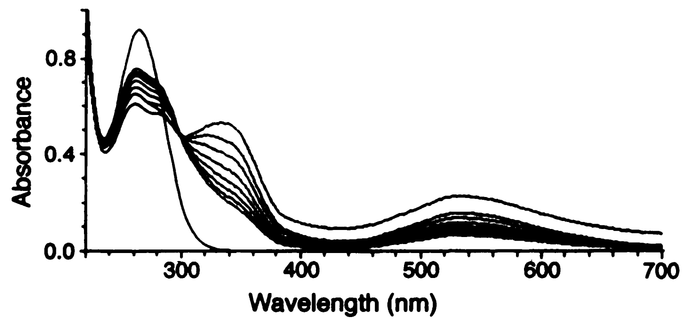

Fig. 3. Repetitive absorption spectra from the reaction of vesnarinone with hypochlorous acid at pH 4. The concentration of vesnarinone was 40 μM and that of hypochlorous acid was 35 μM. The one scan with no absorption at wavelengths greater than 350 nm was that of 40 μM vesnarinone alone. The first spectrum after mixing the reagents was the one with the greatest absorption at 340 and 525 nm. The time between subsequent spectra was 4 sec.

were observed at 340 and 525 nm, which rapidly decreased in parallel. At pH 6, a similar pattern was seen at 340 nm but there was no absorption at 525 nm (not shown).

Oxidation of vesnarinone in the continuous-flow system coupled with the Sciex mass spectrometer gave major ions at m/s 165 (100%), 307 (49% with a chlorine isotope peak at 309), 394 (59%), 395 (22%), 396 (4%), 412 (100%) and 440 (11%). Using the MS/MS mode, the major fragment ion of all ions, except 307, was at m/z 165. The largest fragment of the peak at m/z 307 was 23, which indicated that it is a sodium adduct ion; therefore, this appears to represent the sodium adduct ion of N-chloroveratrylpiperazinamide.

At lower flow rates without a splitter, there was a decrease in the size of the 394 and 395 peaks, presumably because the flow rate was slower and there was more time for the reaction of these species with water.

Activation of neutrophils by influenza vaccine. Initial studies to determine whether influenza vaccine could activate neutrophils were done with procainamide rather than vesnarinone because of our experience with the metabolism of procainamide to a relatively stable reactive metabolite. We found that some activation of neutrophils occurred when influenza vaccine was added to neutrophils, as evidenced by the production of the hydroxylamine of procainamide, but the activation was significantly less than that observed with phorbol ester, which is the standard agent that we use to activate neutrophils. However, when the vaccine was opsonized by incubation with human serum, which more closely mimics *in vivo* conditions, its ability to activate cells was increased to almost equal that of the phorbol ester (fig. 4).

Covalent binding of vesnarinone to activated neutrophils and albumin. When radiolabeled vesnarinone was incubated with neutrophils and the cells were activated, under optimal conditions, almost 5% of the drug became covalently bound to the cells. In this case, although the degree of binding of cells activated by opsonized influenza vaccine was higher than the control, it was significantly less than that observed when the cells were activated by phorbol ester (fig. 5). Although covalent binding of vesnarinone to activated neutrophils occurred when the radiolabel was either on the right or the left side of the molecule, the binding observed when the radiolabel was on the dihydroquinolinone ring was approximately double that of the other isomer (fig. 6).

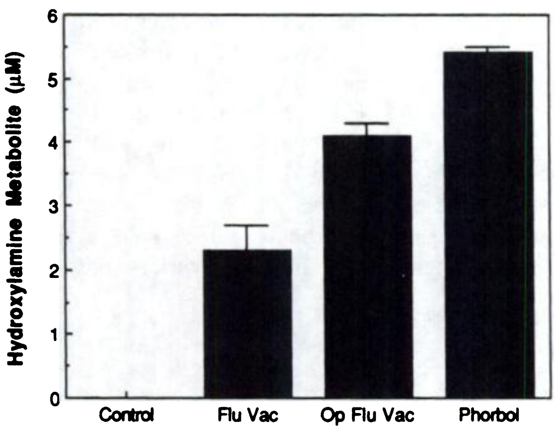

Fig. 4. Oxidation of procainamide to its hydroxylamine by neutrophils activated by different stimuli (control, no activation; Flu Vac, influenza vaccine; Op Flu Vac, opsonized influenza vaccine; Phorbol, PMA). The data represent the mean ± S.E. from four determinations.

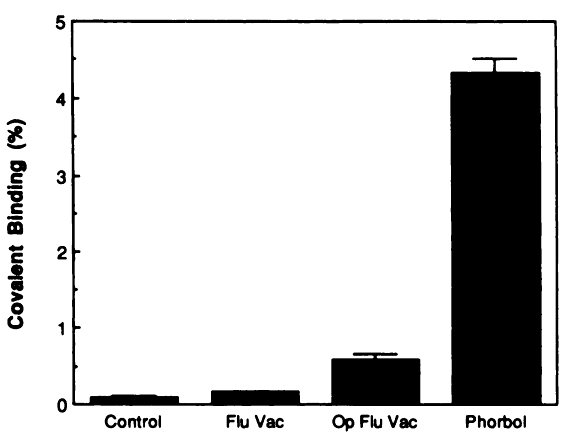

Fig. 5. Covalent binding of radiolabeled vesnarinone to neutrophils that had been activated by different stimuli (control, no activation; Flu Vac, influenza vaccine; Op Flu Vac, opsonized influenza vaccine; Phorbol, PMA). The data represent the mean percent of the added drug that became bound to the neutrophils ± S.E. from three determinations. Using the alternate one-tailed Welch test, Flu Vac was significantly different from control, with $P < .05$; Op Flu Vac, with $P < .01$; and Phorbol, with $P < .001$.

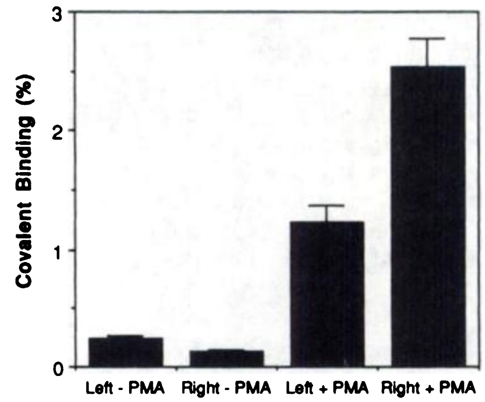

Fig. 6. Dependence of covalent binding of radiolabeled vesnarinone to neutrophils on the position of the radiolabel. Left indicates binding of the isomer in which the radiolabel is on the left side of the molecule (veratryl carbonyl). Right indicates binding of the isomer in which the radiolabel is on the right side of the molecule (dihydroquinolinone ring). −PMA indicates absence of activation of the neutrophils by phorbol ester and +PMA indicates activation of the cells by phorbol ester. The data represent the mean percent of the added vesnarinone that covalently bound to the neutrophils ± S.E. from four determinations.

The covalent binding to albumin of reactive intermediates of vesnarinone generated by MPO was determined to be 6.60% ± 0.15%. Little binding was observed in the absence of hydrogen peroxide or MPO (< 0.2%), which again indicated the formation of reactive intermediates by this system.

Glutathione adducts. When the reactive metabolite was reacted with glutathione, the product was analyzed on HPLC/MS and m/z 701 was monitored, two peaks at 11 and 12.2 min were observed; the first had about twice the area of the second. The mass spectrum at these peaks consisted almost entirely of the M + 1 peak at m/z 701; this finding was consistent with the proposed glutathione adducts. A decrease in the concentration of acetonitrile in the solvent on analytic HPLC (Jones ODS2 column) to 16% resolved the glutathione adducts into three peaks, with retention times of 12.6, 14.6 and 16.9 min. This analytical system was used to prepare samples for NMR. These samples were dissolved in a mixture of deuterated chloroform and methanol at a ratio of 2:1. The proton NMR spectra of the three glutathione adducts were complex but, except for the aromatic region, they were similar and consisted of δ 2.04 (1H, broad m) and 2.19 (1H, broad m) GluCH$_2\beta$, 2.50 (2H, broad m) GluCH$_2\gamma$, 2.57 (2H, t, J = 7 Hz) and 2.90 (2H, t, J = 7 Hz) CH$_2$'s dihydroquinolinone, 3.00 (4H, broad m), 3.15 (2H, m) and 3.55 (1H, dd, J = 4.4, 13.2 Hz) CysCH$_2\beta$, 3.80 (5H, broad m) and 3.93 (6H, 2 X s) OCH$_3$ and 4.40 (1H, dd, J = 4.4, 8.5 Hz) CysCHα. The Cys protons showed the most variation. For example, for the adduct with a retention time of 12.6 min, the CH2β protons were found at δ 3.29 (1H, dd, J = 4.2, 13.5 Hz) and δ 3.07 (1H, dd, J = 10.1, 13.4 Hz). The number of hydrogens is approximate and there are many small and overlapping signals. The signal for the piperazine protons at δ 3.0 and 3.8 were broad and this was also observed in the spectrum of vesnarinone itself. The glutathione protons of the adducts were broadened relative to those in pure glutathione.

Compared with the NMR spectrum of vesnarinone, there was a change in the aromatic region. The aromatic region of the isomer with a retention time of 12.6 min consisted of δ 6.82 (1H, d, J = 1.6 Hz), 6.95 (1H, d, 8.1 Hz) and 7.05 (3H, m); that of the isomer with a retention time of 14.6 min consisted of δ 6.83 (1H, d, J = 8.5), 6.95 (2H, d, J = 8.0) and 7.04 (2H, m);

and that of the isomer with a retention time of 16.9 min consisted of δ 6.87 (1H, s), 6.94 (2H, skewed d, J = 8.2) and 7.03 (2H, m). In contrast, the same region of vesnarinone contained δ 6.80 (3H, m), 6.95 (1H, d, J = 8.2) and 7.03 (2H, m). These spectra suggest that the adduct with a retention time of 12.6 min had glutathione substituted on the 6 position of vesnarinone (fig. 7 for numbering system). The adduct with a retention time of 14.6 min had glutathione substituted in the 4 position and the adduct with a retention time of 16.9 min had glutathione substituted in the 5 position. This is based on the interpretation that the singlet at 6.87 (16.9 adduct) is due to the lack of splitting of the proton in the 4 position by the *para* hydrogen, the small splitting of the doublet at 6.82 (12.6 adduct) caused by splitting by the *meta* hydrogen and the large splitting of the doublet at 6.83 (14.6 adduct) that resulted from the *ortho* hydrogen (*i.e.*, *Jortho* ≫ *Jmeta* > *Jpara*).

Enough of the adduct with a retention time of 16.9 min was isolated to obtain a ^{13}C NMR spectrum (not shown). The results indicated that the only major perturbations were to the dihydroquinolinone aromatic carbons, which strongly supported this ring as the site of adduct formation. The substitution at position 4 for adduct with the retention time of 14.6 min is unambiguous because of the large *Jortho* coupling. It is further substantiated by down-field shift of the dihydroquinolinone CH_2 protons to δ 3.15 from δ 2.90. The distinction between the assignments for the substitution at positions 5 and 6 is less certain because it is based on the assumption that *Jmeta* > *Jpara* and could be reversed. Further evidence for the assigned structures is that the piperazine CH_2 protons are at δ 3.0 in the isomers with retention times of 14.6 and 16.9 min and δ 3.15 in the adduct with a retention time of 12.6 min.

We also observed a peak in the LC/MS close to the solvent front at m/z 469, which presumably represents an adduct between glutathione and the quinone imine of hydroxydihydroquinolinone. With the Sciex mass spectrometer in the MS/MS mode, the daughter ions of the peak at m/z 469 were 340 (24%), 220 (100%) and 194 (88%).

Similar adducts were produced when N-acetylcysteine was used in place of glutathione, as indicated by LC/MS, which contained major peaks at m/z 557. However, it was more difficult to separate the isomers and the NMR spectrum was typical of a mixture of isomers.

Discussion

Vesnarinone was metabolized by activated neutrophils and the major metabolite observed was veratrylpiperazinamide. This is significantly different from the hepatic metabolism of vesnarinone (Miyamoto *et al.*, 1988). It represents N-dealkylation of an aromatic ring, which is unusual because there is no hydrogen on the carbon involved. It can be explained by a mechanism in which the nitrogen is chlorinated followed by loss of HCl to form an iminium ion, as shown in figure 7. This reactive species can either hydrolyze to form the major metabolite or react with other nucleophiles, such as glutathione. Hydrolysis also leads to another reactive intermediate, a quinone imine, which also appears to react with glutathione. Oxidation of vesnarinone by $MPO/H_2O_2/Cl^-$ and hypochlorous acid gave similar metabolites. This finding suggests that hypochlorous acid was the major neutrophil-derived oxidant responsible for this metabolism. The observation that the rate of

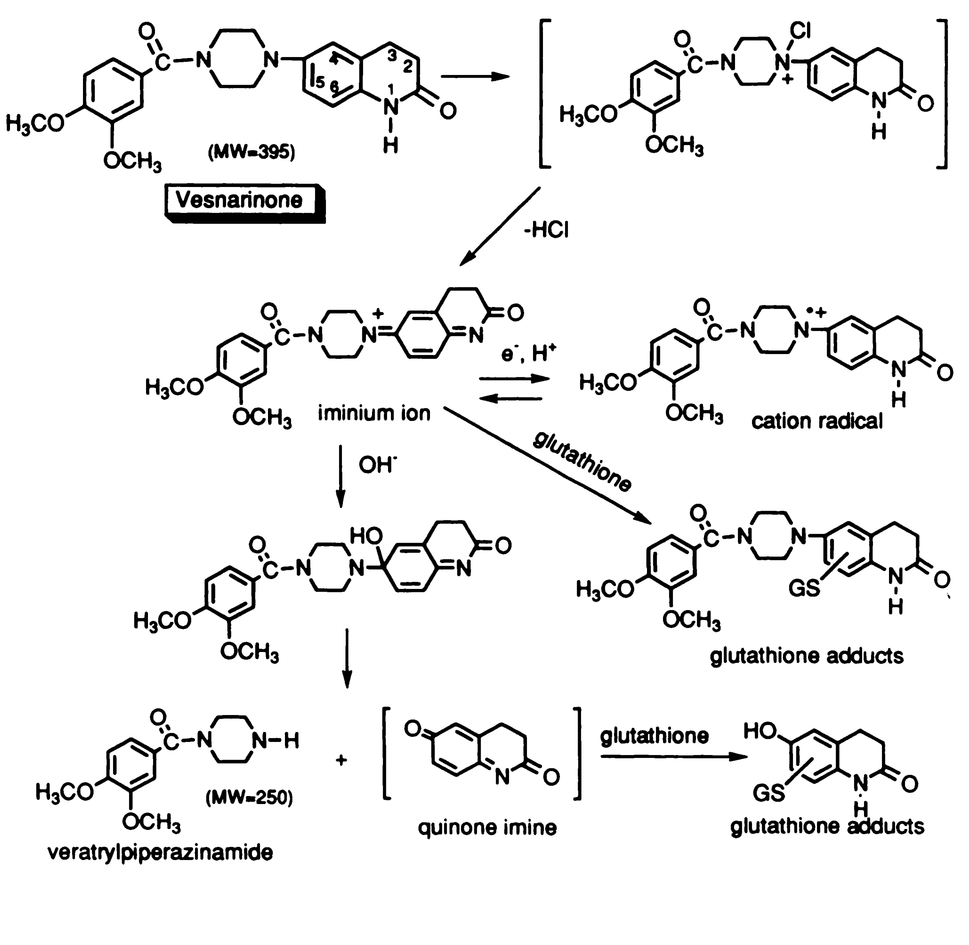

Fig. 7. Proposed pathway of oxidation of vesnarinone by activated neutrophils, $MPO/H_2O_2/Cl^-$ or HOCl followed by trapping of the reactive species with glutathione. The structures in brackets are those for which an ion was not observed in the mass spectrum of the reaction of vesnarinone with HOCl.

oxidation by MPO/H_2O_2 was greatly decreased in the absence of added chloride is also consistent with this hypothesis.

We hypothesize that the new absorption peak at 340 nm observed in figure 2 during the oxidation of vesnarinone with hypochlorous acid represents the iminium ion and the peak at 525 nm represents a cation radical the structure of which is shown in figure 7. It is likely that such a radical cation would be relatively stable, similar to the blue cation radical formed by peroxidase oxidation of aminopyrine, another drug associated with a high incidence of agranulocytosis (Eling *et al.*, 1985; Palva and Mustala, 1970). We postulate that it would be formed by the reaction of the iminium ion with another molecule of vesnarinone and a hydrogen ion to form two cation radicals (coproportionation). Unlike the peak at 340, which was also observed at neutral pH, the 525-nm peak was only observed under acidic conditions.

When the products of the reaction between vesnarinone and hypochlorous acid were analyzed by MS in the flow system so that short-lived intermediates could be detected, ions were observed at most of the values suggested by the reaction scheme depicted in figure 7. The exceptions are the initial N-chloro derivative, which probably lost chloride ion too rapidly to be detected in this system, and the quinone imine, which is more difficult to ionize by IonSpray because it does not contain a basic nitrogen. The formation of the quinone imine can be inferred by the adduct formed when it was trapped with glutathione. Some of the ions formed can be explained by other products. For example, some of the ions at m/z 412 may represent the product that has a hydroxy group on the piperazine ring (the metabolite with a retention time of 16 min) rather than the partially hydrolyzed iminium ion. Likewise some of the ion at m/z 394 may represent an iminium ion formed by the loss of a proton from the piperazine ring rather than the dihydroquinolinone nitrogen. Although the mass spectrum that demonstrated a transient intermediate with a molecular weight of 394 does not prove that the proposed iminium ion intermediate was produced, the combination of the mass spectrum, the stable products formed and the structure of the glutathione adducts formed by trapping the reactive intermediates produce a consistent picture that strongly supports the proposed pathway.

We do not have enough data to identify the metabolites conclusively with retention times of 5.8 and 16 min; however, based on the fragmentation patterns of the mass spectra, we propose the structures in figure 8. The minor metabolites with the M + 1 ion at m/z 430 appear to represent chlorinated vesnarinone and, from the NMR spectrum of one isomer, the chlorine must be on the aromatic portion of the dihydroquinolinone ring. The structure of the minor metabolite at m/z 440 is unknown.

The covalent binding studies indicated the formation of a reactive intermediate and the difference in covalent binding, which depended on the location of the radiolabel, suggested that both the initial iminium ion and the quinone imine make a contribution to the binding. Specifically, the binding observed when the radiolabel is on the dihydroquinolinone ring was about twice that observed when the radiolabel was on the veratryl carbonyl. This can be explained if about one-half of the covalent binding involves binding of the quinone imine in which the radiolabel has been lost from the molecule if it was on the veratryl portion of the molecule but not if it was on the dihydroquinolinone ring. In addition, both the iminium ion and quinone imine were trapped by the addition of glutathione or N-acetylcysteine when these metabolites were generated by hypochlorous acid. The amount of veratrylpiperazinamide formed and the amount of covalent binding are similar and we believe that most of the observed covalent binding is due to these two reactive species. In contrast, it is unlikely that the pathway that leads to the minor metabolites plays a significant role in covalent binding. The proposed cation radical is somewhat reactive and could be responsible for covalent binding and toxicity; however, it is likely to be much less electrophilic than the iminium ion. Furthermore, it was only observed at a pH of less than 5 and covalent binding occurred at neutral pH.

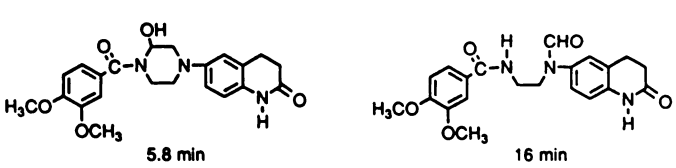

Fig. 8. Proposed structures of the minor metabolites with HPLC retention times of 5.6 and 16 min.

The observation that influenza vaccine was able to activate neutrophils to initiate covalent binding is intriguing because it suggests a mechanism by which a vaccine or viral infection might act as a risk factor for an adverse drug reaction; however, such *in vitro* activation is unlikely to mimic the events exactly that can occur *in vivo*. The observation that the influenza vaccine was nearly as effective as phorbol ester for the activation of neutrophils, as measured by the oxidation of procainamide, but was less effective when measured by covalent binding of vesnarinone was puzzling. One possible explanation is that when the influenza vaccine is opsonized by incubation with serum, the serum protein is not separated later from the vaccine. We showed that covalent binding of the metabolites of vesnarinone to a protein, such as albumin, also occurs; therefore, it is likely that, in the cells activated with opsonized vaccine, binding of reactive metabolite with serum protein would compete with binding to the neutrophils. Binding to serum protein would not be detected by this procedure because it is based on the retention of the covalently bound material by a filter and serum protein would go through such a filter.

One possible risk factor for vesnarinone-induced agranulocytosis could be a higher degree of metabolism of vesnarinone by neutrophils (or neutrophil precursors). We did not find a significant difference in the degree of metabolism of vesnarinone by maximally activated neutrophils from two patients with a history of vesnarinone-induced agranulocytosis. Although the results are in no way conclusive, because only two patients were studied, they are not surprising because it is more likely that differences in the degree of vesnarinone metabolism reflect differences in the degree of *in vivo* activation of the cells than in the intrinsic ability of cells to metabolize the drug when maximally activated. We have used the cells from a great number of volunteers and we did not find large differences in the ability of cells from different subjects to metabolize other drugs. (Obviously, patients with chronic granulomatous disease in whose cells the ability to generate hydrogen peroxide is lacking would be an exception.)

In short, we explored the neutrophil-mediated metabolism of vesnarinone, which is associated with a high incidence of agranulocytosis. We found that reactive metabolites were generated and this led to a high degree of covalent binding of the drug to neutrophils. Strong evidence for the identity of these reactive intermediates was obtained. It is possible that such reactive metabolites are responsible for the relatively high incidence of agranulocytosis that is associated with this drug but further clinical studies will be needed to test this hypothesis. This drug is of obvious clinical benefit and it is hoped that a better understanding of the mechanism of its toxicity will lead its safer use.

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