Published in final edited form as:
Chem Res Toxicol. 2007 July ; 20(7): 1038–1045.

Aminoglutethimide-Induced Protein Free Radical Formation on Myeloperoxidase: A Potential Mechanism of Agranulocytosis

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Abstract

Aminoglutethimide (AG) is a first-generation aromatase inhibitor used for estrogen-dependent breast cancer. Unfortunately, its use has also been associated with agranulocytosis. We have investigated the metabolism of AG by myeloperoxidase (MPO) and the formation of an MPO protein free radical. We hypothesized that AG oxidation by MPO/H_2O_2 would produce an AG cation radical that, in the absence of a biochemical reductant, would lead to the oxidation of MPO. We utilized a novel anti-DMPO antibody to detect DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) covalently bound to protein, which forms only by the reaction of DMPO with a protein free radical. We found that AG metabolism by MPO/H_2O_2 induced the formation of DMPO–MPO, which was inhibited by MPO inhibitors and ascorbate. Glutethimide, a congener of AG that lacks the aromatic amine, did not cause DMPO–MPO formation, indicating the necessity of oxidation of the aniline moiety in AG. When analyzed by electron spin resonance spectroscopy, we detected a phenyl radical adduct, derived from AG, which may be involved in the free radical formation on MPO. Furthermore, we also found protein–DMPO adducts in MPO-containing, intact human promyelocytic leukemia cells (HL-60). MPO was affinity-purified from HL-60 cells treated with AG/H_2O_2 and was found to contain DMPO. These findings were also supported by the detection of protein free radicals with electron spin resonance in the cellular cytosolic lysate. The formation of an MPO protein free radical is believed to be mediated by one of two free radical drug metabolites of AG, one of which was characterized by spin trapping with 2-methyl-2-nitrosopropane. These results are the first demonstration of MPO free-radical detection by the anti-DMPO antibody that results from drug oxidation. We propose that drug-dependent free radical formation on MPO may play a role in the origin of agranulocytosis.

Introduction

Aminoglutethimide (Cytadren, AG[1]) is a first-generation aromatase inhibitor used for estrogen-dependent breast cancer therapy or adrenal tumor suppression (1). Aromatase is a cytochrome P450 enzyme that hydroxylates androstenedione to form estrogen. Recent studies have shown that AG improves the outcome of prostate cancer patients (2). An idiosyncratic adverse drug reaction with AG use is agranulocytosis, a condition characterized by severe neutropenia where the absolute neutrophil count decreases below 1500/mm^3 (3–5). This condition greatly increases the risk of bacterial infection. In a case report, a breast cancer patient died from septicemia associated with AG-induced agranulocytosis (6). Young et al. reviewed hematologic complications arising from AG clinical trials and found a 0.9% incidence of neutropenia in 1,345 patients, with a combined incidence of thrombocytopenia, pancytopenia, and neutropenia of 1.6%. A study in the Netherlands between 1974 and 1994 revealed a similar incidence of AG-induced agranulocytosis (7). This incidence is quite similar to the ~1%

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[1]Abbreviations: AG, aminoglutethimide; MPO, myeloperoxidase; ESR, electron spin resonance; DTPA, diethylenetriaminepentaacetic acid; MNP, 2-methyl-2-nitrosopropane; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ABAH, 4-aminobenzoic acid hydrazide; G/GO, glucose/glucose oxidase.

incidence of clozapine-induced agranulocytosis (8). The annual risk of agranulocytosis to the general population has been reported as 3.4 per million, with 72% of all cases attributed to pharmaceutical drug use (4).

The mechanism of drug-induced agranulocytosis, however, is unknown, and there is no suitable screening assay for it in drug development. Because agranulocytosis is an unpredictable event during the course of drug therapy, any insight into the mechanism of drug-induced agranulocytosis would be significant.

It has been proposed that neutrophil myeloperoxidase activates xenobiotics to reactive intermediates that mediate further events to produce an adverse drug reaction (9). Arylamine drugs have been proposed to form *N*-hydroxylamine metabolites catalyzed by mixed function oxidases that lead to nitroso metabolites (9,10), which are proposed to bind covalently to proteins that may be involved in agranulocytosis. However, a free radical metabolite mediating protein radical formation has not been considered, nor evaluated. The mature neutrophil is estimated to contain ~5% myeloperoxidase (MPO) stored within azurophilic cytosolic granules (11). HL-60 cells derived from a patient with acute promyelocytic leukemia also contain similar amounts of MPO. Although the primary role of MPO is considered to be the formation of bactericidal hypochlorous acid from chloride and H_2O_2, this enzyme behaves similarly to other peroxidases in its metabolism of many organic xenobiotics, including phenols and anilines (12,13).

Native MPO comprises two heavy (~59 kDa) and two light (~14 kDa) polypeptide subunits with a weight of ~150 kDa (14). Each heavy-light set (referred to as hemi-MPO) contains catalytic activity just as the native enzyme does (15). Each hemi-MPO (~78 kDa) is linked via one disulfide bond, and the heavy and light subunits are also linked by one disulfide bond. The heme active site is located in each heavy subunit, thus having two heme active sites per native MPO (16).

AG is an aniline derivative and should be a peroxidase substrate (12,17), although this has never been reported. The first product of AG metabolism by MPO/H_2O_2 would presumably be a nitrogen-centered cation radical, which decomposes into a para-substituted phenyl radical. We hypothesize that one or both of these species attack MPO to form a protein free radical as outlined in Scheme 1. Because an immune response has been associated with certain instances of drug-induced agranulocytosis, it is possible that protein free radicals may trigger an autoimmune response against MPO or other neutrophil proteins. For example, propylthiouracil, an anti-thyroid drug that has a risk of agranulocytosis, has been shown to result in the formation of anti-MPO antibodies in patients (18) and in cats (19).

The crux of our approach to studying drug-induced protein radical formation is based on an anti-DMPO antibody that was developed in our laboratory. This antibody has been used for the detection of protein free radicals on myoglobin (20), hemoglobin (21), lactoperoxidase (22), thyroid peroxidase (23), and catalase (24). This spin-immuno assay (see (*25*) for a review) allows the detection of protein free radicals with one-thousandth the protein required by electron spin resonance (ESR) spectroscopy investigations. Herein we show that AG induced the formation of a protein free radical on MPO and demonstrate the detection of protein–DMPO adducts from intact HL-60 cells. Also, we have identified an AG free radical metabolite produced from metabolism by MPO/H_2O_2 that we believe mediated the protein radical formation on MPO.

Experimental Procedures

Chemicals

Human MPO and 4-aminobenzoic acid hydrazide (ABAH) were purchased from Calbiochem (San Diego, CA). MPO was dissolved in 0.1 M phosphate buffer at pH 7.4 and dialyzed overnight against the same buffer with Slide-A-Lyzer dialysis cassettes (10,000 MWCO; Pierce Biotechnology, Rockford, IL), and its concentration was determined by its extinction coefficient of 178 mM^{-1} cm^{-1} at 429 nm (26). AG was purchased from MP Biomedicals (Solon, OH). Diethylenetriaminepentaacetic acid (DTPA), 2-methyl-2-nitrosopropane (MNP), concanavalin A, and methyl α-D-mannopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO). The latter was dissolved in methanol, heated at 37 °C until blue, and wrapped in foil to prevent photodecomposition. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Alexis Biochemicals (San Diego, CA), purified twice by vacuum distillation at room temperature, and stored under argon atmosphere at −80 °C until use. Hydrogen peroxide (30% v/v, H_2O_2) was obtained from Fisher Scientific Co. (Fair Lawn, NJ) and was assayed by its extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm (27). Chelex-100 resin was purchased from Bio-Rad Laboratories (Hercules, CA).

Spectrophotometry

Spectrophotometry was performed with a Cary 100 spectrophotometer (Varian Inc., CA) using a 500 μL quartz cuvette. Reactions were carried out in 0.1 M phosphate buffer (Chelex-100 treated with 100 μM DTPA) at pH 7.4. The spectrophotometer was set to kinetic scan mode, where each scan was recorded every minute for 60 min. The scan rate was 600 nm/min between 400 and 500 nm for the measurement of the MPO Soret spectrum. The baseline was derived from the buffer alone. MPO (500 nM) was added first, followed by AG and/or H_2O_2. MPO peroxidase activity was also determined after treatment with AG 20 μM/H_2O_2 80 μM for 60 min. This solution was diluted 20-fold, and then 5 mM guaiacol and 100 μM H_2O_2 were added. The formation of the guaiacol tetramer was used as a measure of peroxidase activity (22).

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA plates (96-well, Greiner Labortechnik, Germany) were used to carry out the reaction and to detect the DMPO–MPO adduct. The reactants (final concentrations 100 μM H_2O_2, 200 μM AG, 100 mM DMPO, and 50 nM MPO) were added to Chelex-100-treated 0.1 M phosphate buffer (pH 7.4) containing 100 μM DTPA, mixed, and 100 μL of this reaction immediately added to the ELISA plate containing 200 μL water. The reaction was carried out for 2 h at 37 °C with mixing at 500 RPM. The plate was washed once with wash buffer (Tris buffered saline, 0.2% coldwater fish gelatin, and 0.05% Tween-20 at pH 7.4) and incubated overnight with blocking buffer (0.1 M $NaHCO_3$ and 4% coldwater fish gelatin at pH 9.6). The plate was subsequently washed once, and the anti-DMPO antibody was added to the plate and incubated for 1 h at 37 °C. After four washes, the secondary antibody (anti-Rabbit IgG (Fc), alkaline phosphatase; Pierce, IL) was added and incubated for 1 h at 37 °C. After four washes, a chemiluminescent reagent (CDP-Star, Roche Applied Science) was diluted to 25 μM in Tris-buffered saline at pH 9.6 and added to the plate. The light emitted was recorded in arbitrary units using a TECAN type GENios microplate reader using Xfluor software (Tecan US, NC). Optimal concentrations were determined by varying the concentrations of AG, H_2O_2, DMPO, and MPO one at a time (data not shown).

Electron Spin Resonance (ESR)

An AG free radical metabolite was detected by spin trapping (28), where the free radical metabolite covalently binds to the nitroso spin trap (MNP) to produce a relatively stable nitroxide adduct. We used MNP for these studies instead of DMPO because the DMPO

nitroxide can be further oxidized to an ESR-silent nitrone product, whereas the MNP nitroxide cannot be oxidized to a nitrone. Reactions were prepared by adding a final concentration of 25 mM AG and 25 mM MNP in a 200 μL volume of water to a micro test tube containing 25 μM MPO. Reactions were initiated by the addition of 1 mM H_2O_2 and were briefly vortexed prior to transfer to a quartz ESR flat cell for spectrum recording. ESR spectra were obtained with a Bruker EMX spectrometer (Billerica, MA) equipped with an ER 4122 SHQ cavity operating at 9.78 GHz and 100 kHz modulation field at room temperature with the following parameters: power = 20 mW, scan rate = 0.47 G/s, modulation amplitude = 0.4 G, and receiver gain = 6.32×10^5. Spectra were recorded four times (355 s/scan, time constant = 327 ms). Spectra were simulated using WinSim v1.0 software (http://epr.niehs.nih.gov/) running on a desktop PC.

For protein free radical detection, reaction mixtures contained 7 mg of protein/mL of dialyzed HL-60 cell cytosol (see Cell Culture below), 1 mM MNP, 1 and 5 mM AG, and 1 mM H_2O_2, which was added last to initiate the reaction. Inhibitors were added prior to the addition of AG and preincubated for 5 min. Reactions were premixed in micro test tubes and then transferred to a flat cell for recording ESR spectra. The dialyzed preparation of HL-60 cell cytosol was prepared by adding RIPA buffer (see Cell Culture) to 5×10^8 cells, followed by homogenization with a Dounce homogenizer. The cytosol was then dialyzed for 72 h with 0.1 M phosphate buffer in 10 kDa cutoff Slide-A-Lyzer dialysis cassettes (Pierce). The parameters for spectrum recording were identical to those for AG free radical metabolite detection with the exception of the modulation amplitude, which was set to 2.5 G.

Cell Culture

HL-60 cells were maintained in RPMI-1640 medium (Invitrogen, Gibco #11875119) containing 10% FBS, 31 μg/mL penicillin, 50 μg/mL streptomycin, 10 μL/mL fungizone, 27 mM $NaHCO_3$, 16 mM HEPES, and 8 mM MOPS in a water-jacketed incubator. Cells were centrifuged, washed once, resuspended in RPMI-1640 (no serum) to a concentration of 2×10^6 cells/mL, and plated in 12-well plates (Corning Inc., Corning, NY) for experiments. DMPO (100 mM) was added 10 min prior to adding 5 mM glucose and 50 mU/mL glucose oxidase (G/GO). ABAH (100 μM) was added 30 min prior to initiating the reaction. Other inhibitors, AG, and glutethimide were added 1 min before initiating the reaction. This concentration initially produced 5 μM H_2O_2 per minute but required mixing because the reaction is oxygen-dependent. At the desired time points, the cells were centrifuged at 50*g* for 5 min to remove the supernatant and washed twice. Cell viability was assessed by measuring relative ATP levels using Cell-Titer Glo Cell Viability Assay (Promega Corp., Madison, WI). The remaining cells were lysed with RIPA buffer (0.05 g of sodium deoxycholate, 100 μL of Triton X-100, and 10 μL of 10% SDS in 10 mL of 0.1 M PBS) containing protease inhibitors (Complete, Mini Protease Inhibitor Cocktail Tablets, Roche Applied Science, IN). After 30 min, the samples were centrifuged at 20,000*g* for 20 min. The supernatant (cytosol) was stored at 4 °C until used for Western blots.

Western Blots

Western blots were run under reducing conditions and were performed as described previously with minor changes (29). NuPAGE LDS 4X LDS Sample Buffer and NuPAGE Sample Reducing Agent (Invitrogen) were added to HL-60 cell cytosol, heated for 10 min at 70 °C, then loaded on NuPAGE Novex 4–12% Bis-Tris 1.0 mm Gel. After proteins were transferred to nitrocellulose membranes, they were blocked for at least 1 h with 4% gelatin from coldwater fish (Sigma) in Tris-buffered saline buffer at pH 7.4 and treated with either anti-DMPO or anti-MPO for antigen detection. A solution of 250 μM CDP-Star and Nitro-Block-II Enhancer (Applied Biosystems, Foster City, CA) in Tris-buffered saline at pH 9.6 was added to the membrane for 5 min before exposure of the membrane to CL-Xposure X-ray film (Pierce).

Affinity Purification of MPO from HL-60 Cells

Affinity purification was performed after treatment of the HL-60 cells with DMPO, G/GO, drugs, and inhibitors as described above. A volume of 5 mL of cells was used to ensure an adequate yield of MPO. We followed the method of Hope et al., which is based on using concanavalin A to bind MPO (which is glycosylated) to separate it from the protein mixture (30). We first determined from what fraction MPO was eluted from the concanavalin A resin by using anti-MPO in an ELISA, then pooled and concentrated the samples using Centricon Centrifugal Filter Units (100 kDa cutoff, Millipore Corp., Billerica, USA). Three to four samples were pooled, which resulted in a final protein concentration of 1.3–1.6 mg/mL, determined using the BCA protein assay (Pierce).

Results

MPO Compound II Accumulation Is Induced by AG/H_2O_2

As shown in Figure 1A, when 80 μM H_2O_2 was added to 500 nM MPO, there was a rapid formation of Compound II (λ = 455 nm), followed by a gradual return to the resting enzyme (λ = 429 nm). Although Compound I is initially formed, it cannot be detected in conventional spectrophotometry because of its rapid reduction to Compound II (31). However, in the presence of 20 μM AG and 80 μM H_2O_2 (Figure 1B), MPO Compound II accumulated and did not return back to the resting state. It appears that AG belongs to a class of substrates that are good substrates for Compound I, but poor substrates for Compound II (17). Such substrates have been shown to cause an inhibition of Cl^- oxidation, which is carried out only by MPO Compound I. Incubation of AG (20–2000 μM) with MPO/H_2O_2 did not result in irreversible inhibition of peroxidase activity as assayed by guaiacol tetramer formation (data not shown).

ESR Spectra of an AG-Derived Phenyl Radical MetaboLite

AG reacted with MPO/H_2O_2 in the presence of MNP to produce an ESR spectrum consisting of 21 lines arranged in three sets of seven lines (Figure 2A). This spectrum, characteristic of a small, rapidly rotating free radical, is predominantly the spectrum of an MNP radical adduct of an AG-derived radical. Computer simulation of this spectrum produced a close match (98.7% correlation) to the experimental spectrum in A (Figure 2B). This simulated spectrum consisted of two species. The first species, shown in Figure 2C, contained two hydrogen hyperfine splitting constants (a_H = 1.91 G and a_H = 0.93 G) each from two equivalent hydrogens ($a_{H(2)} = a_{H(6)}$ = 1.91 G; $a_{H(3)} = a_{H(5)}$ = 0.93 G), and a nitroxide nitrogen (a_N = 14.4 G). This species represents the adduct formed between the AG-derived free radical metabolite and MNP as proposed in Figure 2E. Controls without AG, H_2O_2, or MPO failed to form this radical adduct (data not shown). The second species containing three sharp lines, shown in Figure 2D, contained only a nitrogen (nitroxide) hyperfine splitting constant (a_N = 17.1 G). The latter has been previously characterized as the photodecomposition product of MNP (32). Even though these reactions were carried out in the dark as much as possible, some photodecomposition was unavoidable. Therefore, the composite spectrum in Figure 2B was a combination of the two spectra shown in Figure 2C and D. A large class of *t*-butyl-*para*-phenyl nitroxide radicals that have been detected in water have two equivalent hyperfine couplings of about 2 G arising from two ortho hydrogens and two equivalent hyperfine coupling constants of about 1 G arising from the two meta hydrogens (33–36). The structure of this phenyl radical metabolite of glutethimide is unambiguous.

ELISA Detection of DMPO–MPO Adducts and the Effect of an AG Congener, MPO Inhibitors, and Ascorbate

To test the involvement of the metabolic activation of AG in the formation of MPO protein free radicals, we used an ELISA and probed for DMPO–MPO using a polyclonal DMPO

antibody developed in our laboratory (20). Figure 3 shows that a significant signal was detected in the reaction system that contained DMPO, AG, H_2O_2, and MPO. The absence of AG resulted in a 10-fold loss of luminescence. Formation of the DMPO–MPO adduct was inhibited by the MPO inhibitors ABAH and azide and by the antioxidant ascorbate. If these inhibitors were added after the completion of the reaction, the signal was not affected (data not shown). When glutethimide, which lacks the aniline group, was substituted for AG (the chemical structures for each are shown), there was no significant increase in DMPO–MPO, and it was comparable to having no AG present. The inset shows the concentration dependence of AG on DMPO–MPO formation. A biphasic pattern is apparent, with the optimum concentration of AG in this system at 50 μM.

Western Blot Detection of Anti-DMPO from Intact HL-60 Cells

To investigate the production of protein–DMPO adducts in intact HL-60 cells, we incubated 2×10^6 cells/mL in serum free RPMI-1640 DMPO, AG, and G/GO (to generate H_2O_2). As shown in Figure 4A, this treatment produced band-specific anti-DMPO staining. Molecular weight markers showed the darkest anti-DMPO staining to occur on a protein between 50 and 75 kDa. Anti-MPO staining of the same samples showed that the heavy chain of MPO comigrated with the anti-DMPO cross-reacting band and that each lane contained approximately the same amount of MPO (anti-MPO, Figure 4A). ABAH, azide, ascorbate, and the absence of G/GO prevented DMPO–protein formation. Substitution of glutethimide for AG also did not result in significant DMPO–protein formation.

Relative ATP levels of these treatments (compared to those of DMPO treatment alone) were assayed in order to determine the effect on HL-60 cell ATP content: DMPO and G/GO (81 ± 4.3%); DMPO, G/GO, and AG (74 ± 4.5%); + ABAH (89 ± 4.7%); + azide (1 ± 0.1%); + ascorbate (44 ± 2.1%); DMPO and AG (91 ± 5.8%); DMPO, G/GO, and glutethimide (57 ± 2.9%).

Detection of DMPO–MPO from Affinity-Purified MPO from HL-60 Cells

Reactions were carried out as described above for Western blot analysis after affinity purification (Figure 4B). Only the complete system containing DMPO, G/GO, and AG produced immunoreactivity with anti-DMPO, demonstrating that DMPO was bound to the MPO heavy chain (~55kDa). A separate gel of the same samples shows that MPO was present in each lane in approximately equal amounts.

ESR Spectra of a Protein Radical from HL-60 Cytosolic Lysate

To support the results of the immunological detection of a DMPO–MPO adduct as seen with the ELISAs and Western blots, we recorded ESR spectra using a dialyzed preparation of HL-60 cytosol. Reactions contained 1 mM MNP to trap protein radicals, 1 and 5 mM AG, and 1 mM H_2O_2, which was added last to initiate the reaction. DMPO did not give useful spectra when MPO was present presumably because its nitroxide spin adducts can be oxidized to the nitrone by peroxidase. Therefore, we chose MNP as a spin trap because, unlike DMPO, its nitroxide spin adducts are not oxidizable to nitrones. Figure 5 shows an AG dose-dependent increase in protein free radical intensity (Figure 5A and B). The broad lined (partially anisotropic) spectrum was indicative of slowly tumbling macromolecule radical adducts (37). The same spectrum was attenuated in the presence of ABAH, azide, and ascorbate (Figure 5C, D, and E). The absence of either H_2O_2 (Figure 5F) or AG (Figure 5G) resulted in no protein radical formation. The substitution of glutethimide for AG did not result in significant protein radical formation. Attempts were made to digest or denature the protein (using Pronase, trypsin, and/or urea) in order to characterize the amino acid residue bearing the radical adduct, but were unsuccessful as such treatments caused the loss of the ESR spectrum because of increased radical adduct decay.

Discussion

In this study, we have shown that AG metabolism catalyzed by MPO resulted in the formation of a protein free radical on MPO, which was then trapped by DMPO and subsequently identified by a novel anti-DMPO antibody. Although the peroxidation of aromatic amines has been well established, there have been no previous reports in the literature of the peroxidase metabolism of AG. We found that AG caused the accumulation of MPO Compound II. Winterbourn et al. (17) have previously shown that certain NSAIDs also exhibit this activity, which resulted in the inhibition of MPO chlorination activity. It is likely that AG also inhibits chlorination.

The metabolism of AG to a substituted-phenyl radical was demonstrated by ESR using spin trapping with MNP. The phenyl structure of the MNP adduct is unambiguous (33–36). Previously, an almost identical spectrum was found with sulfanilamide after UV photolysis (34). This spectrum was assigned to 4-sulfamoylbenzene-*t*-butylnitroxide. As sulfanilamide and AG are both aromatic amines, their free radical chemistry appears to be similar on the basis of the similarity of their ESR spectra. It has been reported that ammonia evolved in solutions of sulfanilamide after UV photolysis (38). Therefore, on the basis of the ESR spectrum obtained and in combination with the analogy to sulfanilamide, we conclude that the AG free radical adduct with MNP results from the cleavage of the N–C bond of the aniline moiety, with subsequent trapping of the phenyl radical by MNP to form 3-(4-[*t*-butylnitroxide]phenyl)-3-ethyl-piperidine-2,6-dione (Figure 2E). This phenyl radical metabolite may be responsible for the formation of an MPO protein free radical by hydrogen abstraction or possibly its addition across an amino acid double bond. In addition, the parent AG cation radical will be a strong oxidant with an oxidation potential of about 1.03 V (39). As such, it may oxidize one or more aromatic amino acid residues of MPO.

In this study, MPO-catalyzed AG metabolism was immunochemically found to produce a positive response to our anti-DMPO antibody. To our knowledge, this is the first report of drug oxidation by a peroxidase/H_2O_2 system that resulted in a DMPO–protein adduct, which we propose occurs by the following reactions (eqs 1–6):

$$\mathrm{MPO(native)} + \mathrm{H_2O_2} \rightarrow \mathrm{MPO(Compound\ I)} + \mathrm{H_2O} \quad (1)$$

$$\mathrm{MPO(Compound\ I)} + \mathrm{AG} \rightarrow \mathrm{MPO(Compound\ II)} + \mathrm{AG}^{\bullet+} \quad (2)$$

$$\mathrm{AG}^{\bullet+} + {}^{-}\mathrm{OH} \rightarrow \mathrm{glutethimide}^{\bullet} + \mathrm{NH_2OH} \quad (3)$$

$$\mathrm{glutethimide}^{\bullet} + \mathrm{MPO} \rightarrow \mathrm{MPO}^{\bullet} + \mathrm{glutethimide} \quad (4)$$

$$\mathrm{AG}^{\bullet+} + \mathrm{MPO} \rightarrow \mathrm{MPO}^{\bullet} + \mathrm{AG} \quad (5)$$

$$\mathrm{MPO}^{\bullet} + \mathrm{DMPO} \rightarrow \mathrm{MPO-DMPO}^{\bullet} \rightarrow \mathrm{MPO-DMPO\,(nitrone)} \quad (6)$$

Interestingly, MPO, which itself catalyzed the peroxidation of the drug, was also the target of the resulting reactive drug metabolite. Because mechanism-based inhibitors of peroxidases have been proposed to form reactive free radical intermediates that bind to the heme active site and inhibit enzyme activity (40–43), we assayed remaining peroxidase activity after incubation with AG. However, we found no inhibition of MPO peroxidase activity after treatment with AG (data not shown).

The inhibition of anti-DMPO detection when MPO inhibitors were included in the reaction suggests that MPO catalytic activity is necessary for MPO protein free radical formation.

ABAH (42) and azide (43) are both inhibitors of MPO, and both attenuated DMPO–MPO adduct formation. Ascorbate is a substrate and also a reductant in that MPO/H_2O_2 can oxidize ascorbate, but, perhaps more importantly, ascorbate can rapidly reduce many radical intermediates formed by peroxidases (44). It is probable that ascorbate inhibited DMPO–MPO nitrone formation because of its reduction of the AG free radical metabolites back to the parent molecule, which prevented a radical attack on MPO. The evidence for the involvement of the AG cation radical in MPO radical formation was also proven with the use of glutethimide, a congener of AG that lacked the aromatic amine. The biphasic nature of DMPO–MPO formation (Figure 3, inset) could result from low concentrations of AG acting as a pro-oxidant and high concentrations of AG acting as an antioxidant (45,46).

HL-60 cells were used to investigate whether DMPO–protein adducts could be detected in intact cells because, similar to human neutrophils, HL-60 cells contain 47.5 μg MPO/10^7 cells (47). Neutrophils can be primed by many different agents, all resulting in the association of the NADPH oxidase complex that produces H_2O_2 (48). We used G/GO to generate H_2O_2 to simulate the respiratory burst in neutrophils or macrophages. The formation of H_2O_2 as a cosubstrate fueled the peroxidation of AG by MPO. The model we have used may illustrate a possible outcome of AG use under a condition of inflammation because neutrophils and macrophages both produce H_2O_2 during infection.

In addition to the immunological detection of protein–DMPO in HL-60 cells formed from AG metabolism, the ESR spectra of HL-60 cell cytosol unequivocally showed the presence of a protein free radical that was AG-dependent and attenuated in both systems with MPO inhibitors, further validating its immunological detection. However, these ESR experiments could not provide information regarding the identity of the protein target. The Western blot from HL-60 cell cytosol indicated a DMPO-containing band of between 50 and 75 kDa, which led us to consider that the target may be the heavy chain of MPO whose molecular weight has been reported from 55 to 62 kDa (15,47,49,50). Through the use of concanavalin A, an affinity purification resin for glycoproteins, we were able to partially purify MPO from the HL-60 cells and detect DMPO binding induced by AG.

We propose that the free radical chemistry of AG may be implicated in agranulocytosis. Female albino mice treated with AG (50 mg/kg/day for 21 days) were found to display a statistically lower leukocyte count (leucopenia), which included low neutrophil counts, compared to that of the control (51). This study also found no such blood dyscrasias using glutethimide, the AG congener that lacked the phenylic amine. Correspondingly, we did not detect an MPO radical with glutethimide, leading us to hypothesize that AG-induced MPO free radical formation may be involved in leucopenia and/or agranulocytosis.

It has been proposed, on the basis of liver microsomal incubations, that an *N*-hydroxylamine metabolite was responsible for leucopenia in mice because cimetidine, a cytochrome P450 inhibitor, attenuated the leucopenic effects of AG and inhibited microsomal protein binding (51). However, SKF525A, another cytochome P450 inhibitor, did not inhibit leucopenia, and cimetidine itself has been shown to interfere with MPO activity (52). Therefore, *N*-hydroxylation of AG and subsequent covalent binding to protein may not be the only mechanism of leucopenia in mice. We propose that free radical modification of MPO or other neutrophil proteins (see the high molecular weight smear in Figure 4A) *in vivo* may lead to anti-neutrophil antibody formation and granulocyte death via immune-mediated mechanisms.

In summary, we have shown that AG, a drug associated with a 1% incidence of agranulocytosis, induced the formation of an MPO free radical in a pure enzymatic system and in HL-60 cells. These findings warrant further study into the toxicological relevance of an MPO free radical *in vivo* and the possible role it may play in the pathogenesis of AG-induced agranulocytosis.

Acknowledgements

We would like to acknowledge Dr. Colin Chignell for the use of his cell culture facility, Dr. Dario Ramirez for his assistance with immunological techniques, Dr. Michelle Block for her technical assistance, and Dr. Ann Motten and Mary Mason for their editing of the manuscript. Also, we thank Jean Corbett for the purification of DMPO. This research was supported by the Intramural Research Program of the NIH and NIH/NIEHS.

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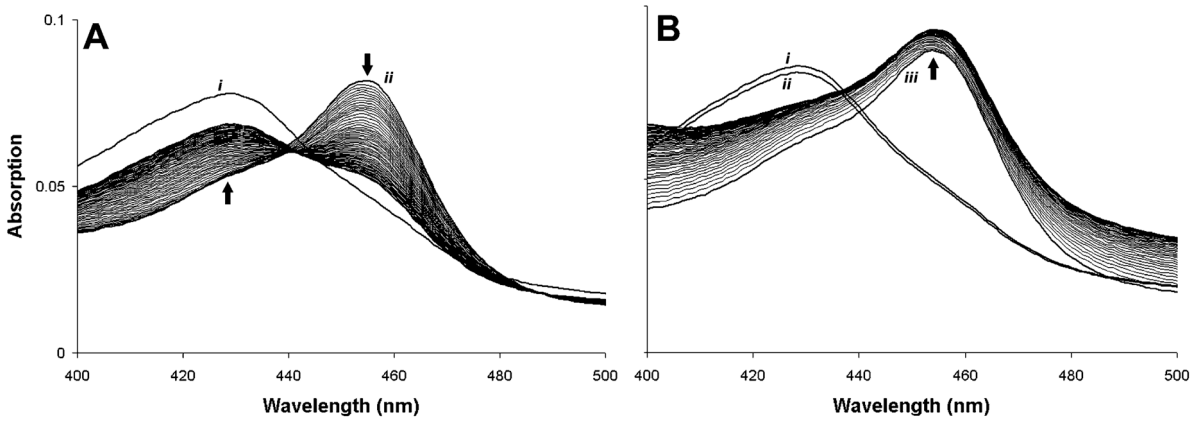


Figure 1.

AG induces MPO Compound II accumulation. The MPO Soret region was recorded once every minute in the absence (A) or presence (B) of AG for 60 min. In A, resting MPO (500 nM) was recorded first (*i*), followed by the addition of 80 μM H_2O_2 (*ii*). In B, resting MPO (500 nM) was recorded first (*i*), followed by the addition of 20 μM AG (*ii*), and finally 80 μM H_2O_2 (*iii*). The MPO resting spectrum could be restored after 60 min by the addition of ascrobate (not shown).

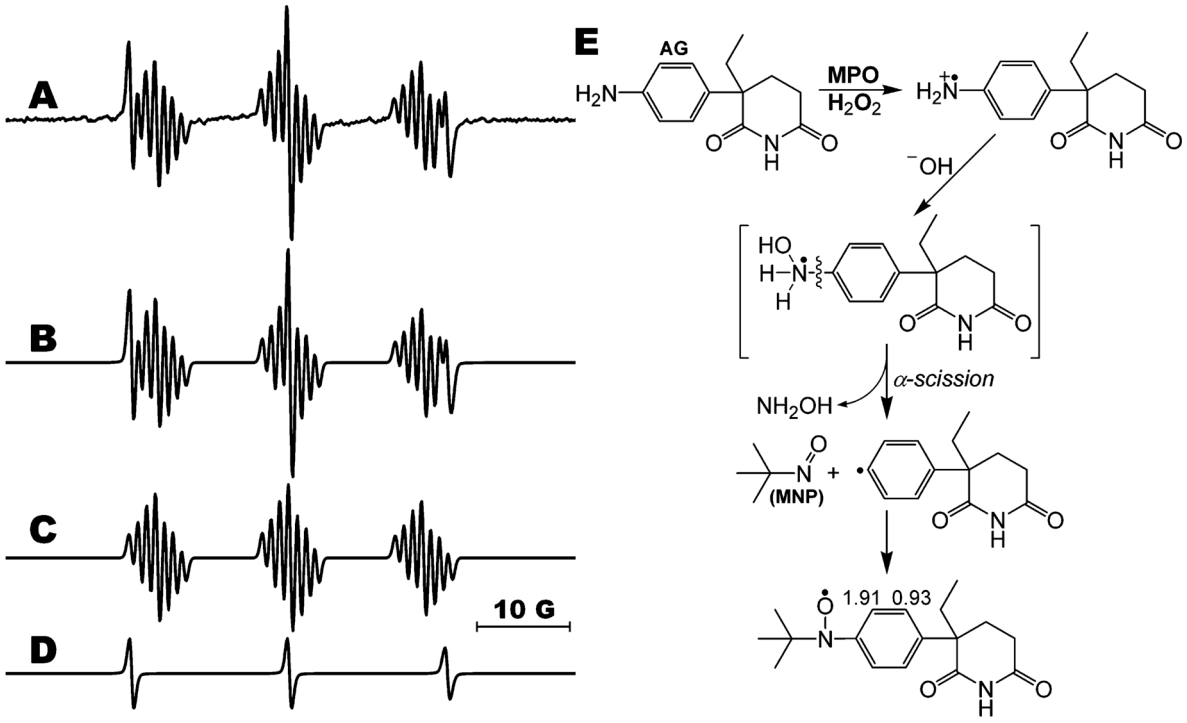


Figure 2.

ESR spin trapping of the AG free radical metabolite formed by MPO/H_2O_2. ESR spectra of MPO/H_2O_2-catalyzed oxidation of AG to a free radical metabolite. In spectrum A, AG (25 mM) and MNP (25 mM) were added to 200 μL of a solution of 25 μM MPO. The reaction was performed in deionized water and was initiated by the addition of 1 mM H_2O_2. ESR settings are described in Experimental Procedures, with the following changes: modulation amplitude = 0.4 G and receiver gain = 2×10^5. Spectrum B is a computer-simulated spectrum that closely matched the actual experimental spectrum (correlation of 0.987). Spectrum C was also simulated and shows that the major component consisted of a 21-line spectrum, in combination with the 3-line spectrum shown in D. Controls without AG, H_2O_2, or MPO did not form this radical adduct (data not shown). The one-electron oxidation mechanism of AG (E) results in a nitrogen-centered cation free radical which is delocalized into the phenyl ring. We propose that loss of the amino moiety occurs through loss of hydroxylamine, which is required to form the phenyl radical that we have unambiguously detected. The hydrogen splitting constants are shown in the radical adduct.

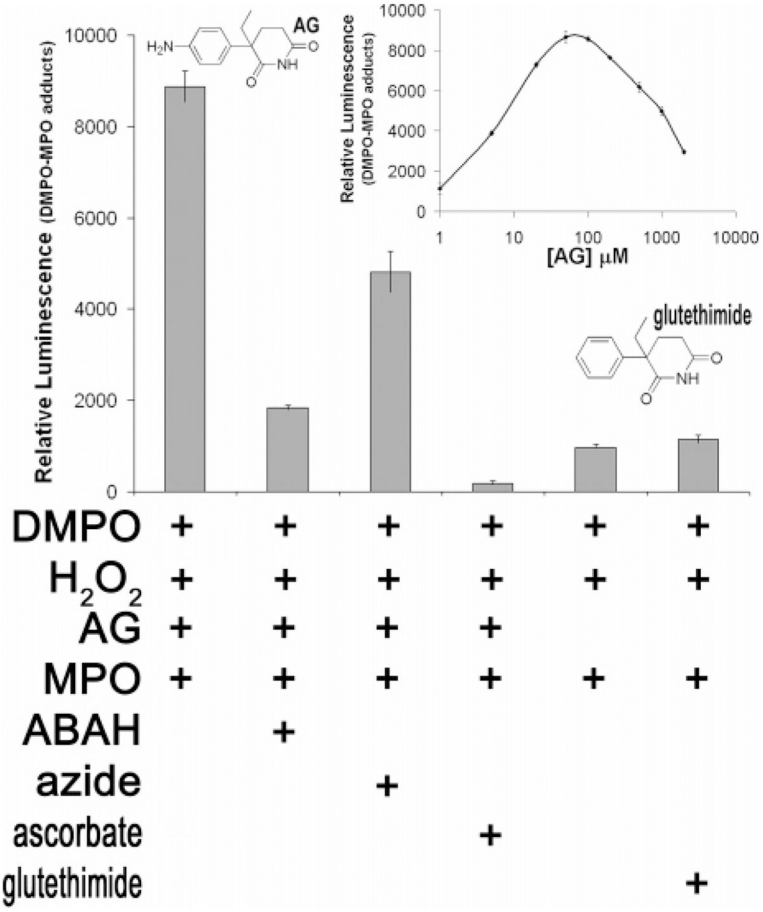


Figure 3.

ELISA of MPO–DMPO formation catalyzed by AG metabolism by MPO/H_2O_2. Reactions were performed in Chelex-100 treated 0.1 M phosphate buffer, pH 7.4, at 37 C. The incubation of DMPO (100 mM), H_2O_2 (100 μM), AG (50 μM), and MPO (50 nM, added last) resulted in anti-DMPO binding. The presence of ABAH (1 mM), azide (1 mM), or ascorbate (1 mM) attenuated the signal with variable efficacy. The absence of AG or its substitution with 50 μM glutethimide resulted in greatly decreased antibody recognition. MPO–DMPO detection was carried out using polyclonal anti-DMPO as described in Experimental Procedures. The error bars represent ±SD of three wells.

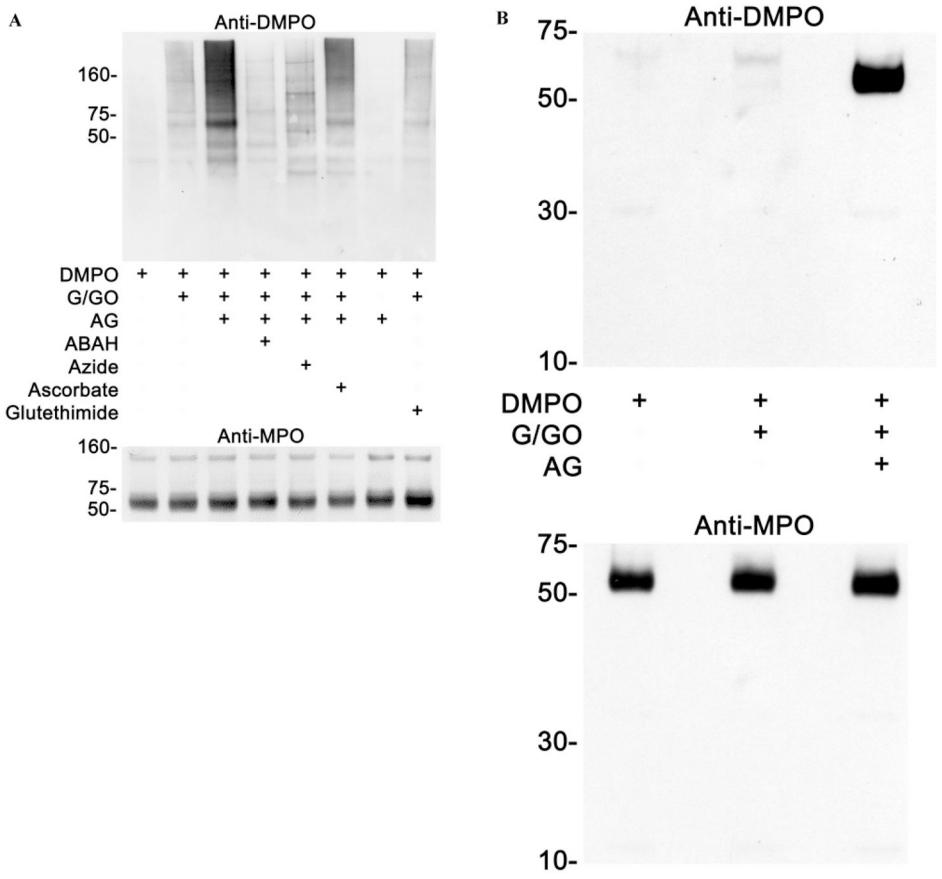


Figure 4.

(A) Western blot of HL-60 cell cytosol after incubation with AG and inhibitors. HL-60 cells (2×10^6/mL, 2 mL) were preincubated with DMPO (50 nM) for 10 min before the addition of AG. ABAH (100 μM) was preincubated for 30 min before the addition of DMPO. The reaction was initiated with 5 mM glucose/50 mU/mL glucose oxidase (G/GO), which was added last. The reaction was shaken for 5 min on a plate stirrer every 30 min and was maintained in a water-jacketed incubator at 37 °C and 5% CO_2 for 4 h; 10 μg/lane was loaded for anti-DMPO detection, and 1 μg/lane was loaded for anti-MPO. (B) Affinity purified MPO from HL-60 cells is bound to DMPO when incubated with AG. HL-60 cell incubations were carried out as described in A, except with 5 mL of cells in order to obtain sufficient MPO for affinity purification. In the anti-DMPO Western blot, 30 μg of protein was loaded into each lane, but in the anti-MPO Western blot, 3 μg of protein was loaded into each lane.

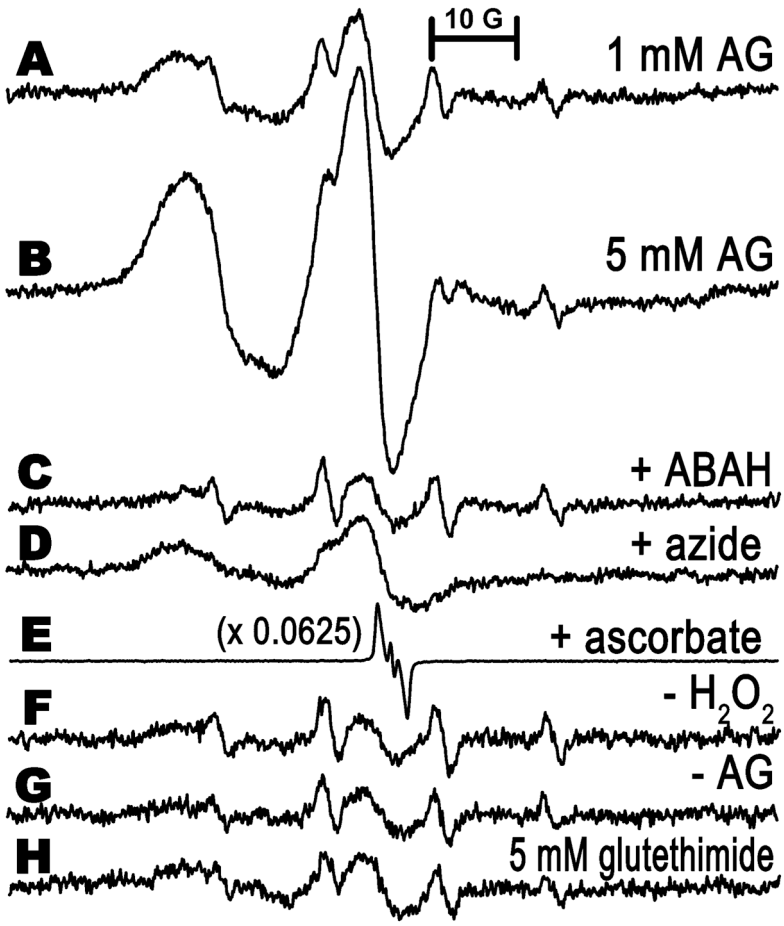
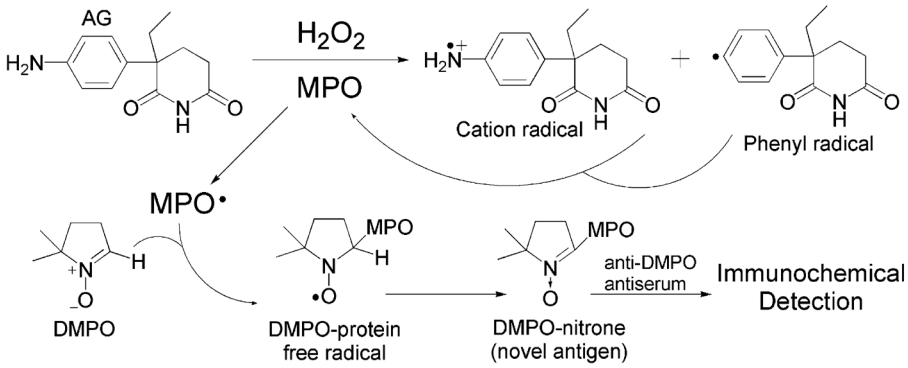


Figure 5.

ESR spectra showing the detection of a protein free radical from HL-60 cell lysates treated with AG and H_2O_2. Cell lysates were obtained as described in Experimental Procedures. The reactions contained 7 mg/mL of dialyzed lysate, 1 mM MNP (spin trap), variable concentrations of AG, and 1 mM H_2O_2 (added last). The reactions were heated at 37 °C and mixed for 2 min before spectra were recorded. Spectra A and B show an AG dose-dependent increase in protein free radical intensity. AG (5 mM) was used for the remaining experiments. The spectrum was attenuated in the presence of 1 mM ABAH (C), 25 mM azide (D), 1 mM ascorbate (E), and when either H_2O_2 (F) or AG (G) was omitted from the reaction. Ascorbate produced an intense ascorbyl radical. When 5 mM glutethimide (H) was substituted for AG, there was no significant protein radical spectrum recorded. ESR settings are described in Experimental Procedures.



Scheme 1. Postulated Mechanisms of Arylamine Reactive Metabolite Induced Modification of Proteins, Especially MPO[a]

[a] AG metabolism by MPO results in cation radical formation (and in phenyl radical formation) where one or both of these species attacks MPO, resulting in MPO protein free radical formation. The latter can be trapped by DMPO, resulting in a spin adduct that we detect by immunological methods.