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Title: Mechanistic Investigation of the Time-Dependent Aldehyde Oxidase Inhibitor Hydralazine

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Running Title: Mechanism of AO Inactivation by Hydralazine

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Abbreviations: AO, aldehyde oxidase; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; HPLC, high performance liquid chromatography; LC/MS/MS, liquid chromatography tandem mass spectrometry; MAO-B, monoamine oxidase B; MBEI,

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mechanism-based enzyme inactivator; MoCo, molybdenum-containing tetracyclic pteridine complex; N-Ac Cys, N-acetylcysteine; ROS, reactive oxygen species; XO, xanthine oxidase

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Abstract

The anti-hypertensive agent hydralazine is a time-dependent inhibitor of the cytosolic drug metabolizing enzyme aldehyde oxidase (AO). Glutathione (GSH) was found to suppress the inhibition of AO by hydralazine in multiple enzyme sources (human liver and kidney cytosol, human liver S9, rat liver S9, and recombinant human AO) and with different AO substrates (zoniporide, O^6-benzylguanine, and dantrolene). Hydralazine-induced AO inactivation was unaffected when GSH was added to the incubation mixture after pre-incubation of hydralazine with AO (rather than during the pre-incubation), suggesting that GSH traps a hydralazine reactive intermediate prior to enzyme inactivation. Consistent with previous reports of 1-phthalazylmercapturic acid formation when hydralazine was incubated with N-acetylcysteine, we detected a metabolite producing an MS/MS spectrum consistent with a 1-phthalazyl-GSH conjugate. O^6-Benzylguanine, an AO substrate, did not protect against hydralazine-induced AO inactivation, implying that hydralazine does not compete with O^6-benzylguanine for binding to the AO active site. Catalase also failed to protect AO from hydralazine-induced inactivation, suggesting that hydrogen peroxide is not involved. However, an allosteric AO inhibitor (thioridazine) offered some protection, indicating a catalytic role for AO in the bioactivation of hydralazine. AO inhibition by phthalazine (a substrate and inhibitor of AO and a metabolite of hydralazine) was unaffected by the presence of GSH. GSH also prevented hydralazine from inhibiting the nitro-reduction of dantrolene by AO. Furthermore, the GSH-hydralazine combination stimulated dantrolene reduction. Phthalazine inhibited only oxidation reactions, not reduction of dantrolene. Together these results support the hypothesis that hydralazine is converted to a reactive intermediate that inactivates AO.

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Significance Statement: These studies suggest that a reactive intermediate of hydralazine plays a primary role in the mechanism of aldehyde oxidase (AO) inactivation. Inactivation was attenuated by glutathione and unaffected by catalase. Phthalazine (hydralazine metabolite) inhibited AO regardless of the presence of glutathione; however, phthalazine inhibited only oxidation reactions, while hydralazine inhibited both oxidation and reduction reactions. This report advances our mechanistic understanding of hydralazine as an AO inhibitor and provides information to facilitate appropriate use of hydralazine when probing AO metabolism.

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Introduction

Aldehyde oxidase (AO) is a cytosolic molybdo-flavoenzyme that catalyzes the oxidation of a broad array of aromatic azaheterocycles, aromatic and aliphatic aldehydes, and iminium ions and catalyzes the reduction of nitro-containing compounds, sulfoxides, N-oxides, and certain heterocycles (Pryde et al., 2010). AO functions as a homodimer, with two identical subunits of approximately 150 kDa (Terao et al., 2016). Each subunit comprises 3 separate domains, which each contain a different co-factor required for catalytic activity: the 20 kDa N-terminal domain contains two iron-sulfur clusters, the 40 kDa central domain contains a flavin-adenine dinucleotide (FAD), and the 85 kDa C-terminal domain holds the molybdenum-containing tetracyclic pteridine complex (MoCo) within the enzyme active site (Terao et al., 2016). The catalytic mechanism by which AO oxidizes aromatic azaheterocyles has been proposed to proceed via the concerted nucleophilic attack of an electrophilic carbon (most often adjacent to a nitrogen) and hydride transfer to the sulfur of the MoCo, followed by the consequent reduction of the molybdenum atom (Alfaro and Jones, 2008). The oxidized product is released upon hydrolysis of the reaction intermediate, and the MoCo is reoxidized by shuttling the reducing equivalents to FAD via the iron-sulfur clusters, producing $FADH_2$. $FADH_2$ then transfers electrons to molecular oxygen, generating hydrogen peroxide or superoxide.

Several inhibitors of AO have been identified, including the antihypertensive drug hydralazine (Johnson et al., 1985; Obach et al., 2004; Strelevitz et al., 2012). Hydralazine is a time-dependent irreversible inhibitor of AO (K_i = 83 μM, k_{inact} = 0.063 min^{-1}), and it is commonly used in reaction phenotyping experiments to identify drugs as AO substrates (Strelevitz et al., 2012; Zientek and Youdim, 2015). In particular, hydralazine is useful in distinguishing AO-mediated metabolism from metabolism mediated by xanthine oxidase (XO), a

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related molybdo-flavoenzyme that is also capable of catalyzing the oxidation of aromatic azaheterocycles, but is not inhibited by hydralazine (Zientek and Youdim, 2015). Hydralazine is converted to a hydralazyl radical via one-electron oxidation in the presence of metal ions, liver microsomes, and activated leukocytes (Sinha and Motten, 1982; Sinha, 1983; Streeter and Timbrell, 1983; LaCagnin et al., 1987; Hofstra et al., 1991; Yamamoto and Kawanishi, 1991; Reilly and Aust, 1997; Sinha et al., 2014). In addition, covalent binding of hydralazine-related reactive species to microsomal protein has been reported, and this covalent binding was reduced in the presence of glutathione (GSH) (Streeter and Timbrell, 1983; LaCagnin et al., 1987). Furthermore, the oxidation of hydralazine by activated leukocytes and subsequent reaction with N-acetylcysteine to produce 1-phthalazylmercapturic acid has also been reported (Hofstra and Uetrecht, 1993). While these reports suggest the likelihood that hydralazine inactivates AO via formation of a reactive metabolite, the mechanism of AO inactivation is not fully understood.

While conducting an experiment to evaluate the formation of GSH conjugates of the drug lapatinib, which is an AO substrate, we discovered that GSH suppresses the inhibitory activity of hydralazine towards AO (Crouch et al., 2021). GSH is a cellular thiol that is used as a nucleophilic trapping agent to identify reactive metabolites formed by oxidative drug metabolism reactions (Evans et al., 2004). Additionally, in the process of characterizing an enzyme inhibitor as a mechanism-based enzyme inactivator (MBEI), GSH is commonly used to evaluate whether the inhibitor meets the MBEI criteria of inactivating the enzyme prior to release of the reactive species from the enzyme active site (Silverman, 1995). If enzyme inactivation occurs after release of the reactive species, then the presence of GSH is expected to reduce or prevent inactivation of the enzyme by trapping the released reactive species. Upon these findings, we sought to further evaluate the interaction between GSH and hydralazine with regard to the

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inhibition of AO by hydralazine. Because we observed a reduction in the inhibitory activity of hydralazine toward AO in the presence of GSH, we hypothesized that inactivation of AO by hydralazine occurs by means of a reactive species that can be neutralized by GSH prior to inactivation of the enzyme.

Methods and Materials

Chemicals and Reagents. Reduced glutathione (GSH), N-acetylcysteine (N-Ac Cys), hydralazine hydrochloride, raloxifene, phthalazine, O^6-benzylguanine, and bovine liver catalase were purchased from Sigma Aldrich (St. Louis, MO). Zoniporide dihydrochloride was purchased from Tocris Bioscience (Bio-Techne, Minneapolis, MN). 2-Oxo-zoniporide, 8-oxo-benzylguanine, and thioridazine were purchased from Toronto Research Chemicals (Toronto, ON). HPLC-grade acetonitrile and formic acid were purchased from Fisher Scientific. HPLC water was obtained from a Milli-Q plus purification system (Millipore, Bedford, MA). Substrate stock solutions were prepared in dimethyl sulfoxide (DMSO), and working solutions were diluted into 1:1 water/acetonitrile (v/v). Stock solutions of hydralazine were prepared in water and stored at -20 °C prior to use.

Tissue Fractions and Recombinant Human Aldehyde Oxidase. Commercially available human liver S9 fraction (catalog number H0606.S9(AX), lot number 1710129) and cytosol (catalog number H0606.C(AX), lot numbers 1810291 and 2010170) pooled from 20 donors (mixed sex) were purchased from Sekisui XenoTech (Kansas City, KS). Human kidney cytosol (catalog number H0610.RC, lot number 1310121) pooled from four donors (mixed sex) was purchased from Xenotech. Male rat liver cytosol (catalog number R1000.C, lot number 1410273) pooled from 454 animals was purchased from XenoTech. A cytosolic extract of *Escherichia coli*

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expressing recombinant human aldehyde oxidase was purchased from XenoTech (catalog number CYP150, batch number 150011B).

Effect of GSH on AO inhibition by Hydralazine in Human Liver and Kidney Cytosol, Rat Liver Cytosol, and Recombinant Human AO. Zoniporide (10 μM) was incubated at 37 ^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver cytosol, human kidney cytosol, human liver S9, rat liver cytosol (2 mg/ml protein) or recombinant human AO (0.5 mg/ml) in the presence or absence of hydralazine (50 μM) and in the presence or absence of GSH (5 mM). Reaction mixtures were pre-incubated in the presence or absence of hydralazine and GSH for 25 minutes prior to the addition of substrate. Reactions were then initiated with the addition of substrate (zoniporide). Total incubation volumes were 0.1 ml. After 60 minutes, reactions were terminated with 4 volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 μl of the resulting supernatants were removed and diluted into 100 μl of water in preparation for LC/MS/MS analysis of 2-oxo-zoniporide formation.

Inhibition of AO by Hydralazine and Raloxifene in the Presence and Absence of GSH. O^6-Benzylguanine (10 μM) was incubated at 37 ^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver cytosol (2 mg/ml protein) in the presence or absence of hydralazine (50 μM) or raloxifene (1 μM) and in the presence or absence of GSH (5 mM). Reaction mixtures were pre-incubated in the presence or absence of hydralazine or raloxifene and GSH for 25 minutes prior to the addition of substrate. Reactions were then initiated with the addition of substrate (O^6-benzylguanine). Total incubation volumes were 0.1 ml. At 60 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal

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standard (carbamazepine, 50 nM). After centrifugation at 3500 x gfor 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of 8-oxo-benzylguanine formation.

Inhibition of AO Activity by Hydralazine with GSH Present During Hydralazine Preincubation or After Preincubation. Experiments were also conducted as described above with simultaneous pre-incubation of 5 mM GSH and hydralazine or with the addition of GSH after hydralazine pre-incubation (GSH added at the time of substrate addition). Zoniporide (10 µM) was incubated at 37 ^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver cytosol (2 mg/ml protein) in the presence or absence of hydralazine (50 µM) and in the presence or absence of GSH (5 mM). Prior to addition of zoniporide, mixtures were pre-incubated with hydralazine (25 min) either in the presence of 5 mM GSH, or 5 mM GSH was added after the 25-min hydralazine pre-incubation (at the time of substrate addition). Reactions were initiated with the addition of substrate (zoniporide). Total incubation volumes were 0.1 ml. At 60 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of 2-oxo-zoniporide formation.

Inhibition of AO Activity by Hydralazine in the Presence and Absence of GSH or N-Ac Cys. *O*^{6}-Benzylguanine (10 µM) or zoniporide (10 µM) were incubated at 37^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing human liver cytosol (2 mg/ml protein) or recombinant human AO (0.5 mg/mL protein) with or without 50 µM hydralazine in the presence or absence of 5 mM GSH or N-Ac Cys. Reaction mixtures were pre-incubated in the presence or

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absence of hydralazine and GSH or N-Ac Cys for 25 minutes prior to the addition of substrate. Reactions were then initiated with the addition of substrate (O^6-benzylguanine or zoniporide). Total incubation volumes were 0.1 ml. At 60 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of 8-oxo-benzylguanine or 2-oxo-zoniporide formation.

Concentration Dependence of GSH and N-Ac Cys on AO Inhibition by Hydralazine in Human Liver Cytosol. To evaluate the concentration dependence of GSH and N-Ac Cys on the AO-inhibitory activity of hydralazine, experiments were conducted as described above using a range of GSH concentrations. O^6-benzylguanine (10 µM) was incubated at 37 ^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver cytosol (2 mg/ml protein) in the presence or absence of the AO inhibitor hydralazine (50 µM) and in the presence or absence of GSH (5 µM – 5 mM) or N-Ac Cys (50 µM – 50 mM). Reaction mixtures were pre-incubated in the presence or absence of hydralazine and GSH/N-Ac Cys for 25 minutes prior to the addition of substrate. Reactions were then initiated with the addition of substrate (O^6-benzylguanine). Total incubation volumes were 0.1 ml. At 60 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of 8-oxo-benzylguanine formation.

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Substrate Protection Experiments in Human Liver Cytosol. To determine if hydralazine competes with substrate binding at the AO active site, experiments in human liver cytosol were conducted using O^6-benzylguanine to protect the AO active site against hydralazine binding. Conversion of zoniporide to 2-oxo-zoniporide was monitored as a measure of AO activity. Hydralazine (25 μM) or vehicle was incubated at 37 °C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver cytosol (5 mg/ml) in the presence or absence of O^6-benzylguanine (10, 30, and 100 μM). After incubation for 0, 10, or 30 minutes, 2 μl aliquots of each mixture were removed and transferred to a secondary incubation mixture containing potassium phosphate buffer and zoniporide (100 μM), resulting in a 50-fold dilution of O^6-benzylguanine, hydralazine, and cytosol (0.1 mg/ml final concentration) and a total incubation volume of 0.1 ml. After 5 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 μl of the resulting supernatants were removed and diluted into 100 μl of water in preparation for LC/MS/MS analysis of 2-oxo-zoniporide formation.

Thioridazine Protection Experiment in Human Liver Cytosol. Thioridazine, an inhibitor of AO, is reported to bind at an allosteric site located at the dimer interface, separate from the substrate binding site (Coelho et al., 2015). In order to determine the requirement of AO catalysis on the bioactivation of hydralazine, experiments were conducted in human liver cytosol. Hydralazine (25 μM) or vehicle was incubated at 37 °C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver cytosol (5 mg/ml) in the presence or absence of thioridazine (10, 30, and 100 μM). After incubation for 0, 10, or 30 minutes, 2 μl aliquots of the mixture were removed and transferred to a secondary incubation mixture containing potassium phosphate buffer and zoniporide (100 μM), resulting in a 50-fold dilution of

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thioridazine, hydralazine and cytosol (0.1 mg/mL final concentration) and a total incubation volume of 0.1 ml. After 5 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of 2-oxo-zoniporide formation.

Effect of Catalase on the Inhibitory Activity of Hydralazine on AO. In order to evaluate a potential role of reactive oxygen species (ROS) in the inhibitory mechanism of hydralazine, hydralazine was incubated with recombinant human AO in the presence and absence of catalase prior to addition of zoniporide. Zoniporide (10 µM) was incubated at 37 ^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing recombinant human AO (0.5 mg/ml) in the presence or absence of hydralazine (50 µM) and in the presence or absence of catalase (800 units/ml). Reaction mixtures were pre-incubated in the presence or absence of hydralazine for 25 minutes prior to the addition of substrate. Reactions were then initiated with the addition of substrate (zoniporide). Total incubation volumes were 0.1 ml. At 60 minutes, reactions were terminated with 4 volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of 2-oxo-zoniporide formation.

Inhibition of AO Activity by Phthalazine in the Presence and Absence of GSH. Zoniporide (10 µM) was incubated at 37^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver cytosol (2 mg/ml protein) in the presence or absence of phthalazine (50 µM) and in the presence or absence of GSH (5 mM). Reaction mixtures were

pre-incubated in the presence or absence of phthalazine and GSH for 25 minutes prior to the addition of substrate. Reactions were then initiated with the addition of substrate (zoniporide). Total incubation volumes were 0.1 ml. After 60 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of 2-oxo-zoniporide formation.

Inhibition of AO Reductive Activity by Hydralazine or Phthalazine in the Presence and Absence of GSH. Dantrolene (10 µM) was incubated at 37 ^{o}C in potassium phosphate buffer (100 mM, pH 7.4) containing pooled human liver post-mitochondrial supernatant (S9) (2 mg/ml protein) in the presence or absence of hydralazine or phthalazine (50 µM) and in the presence or absence of GSH (5 mM). Reaction mixtures were pre-incubated in the presence or absence of hydralazine or phthalazine and GSH for 25 minutes prior to the addition of substrate. Reactions were then initiated with the addition of substrate (dantrolene). Total incubation volumes were 0.1 ml. After 60 minutes, reactions were terminated with four volumes of ice-cold acetonitrile containing an internal standard (carbamazepine, 50 nM). After centrifugation at 3500 x g for 10 minutes, 50 µl of the resulting supernatants were removed and diluted into 100 µl of water in preparation for LC/MS/MS analysis of aminodantrolene formation.

Metabolite Quantitation. Concentrations of 2-oxo-zoniporide and 8-oxobenzylguanine were quantified by LC/MS/MS using a matrix-matched standard curve composed of 6-8 concentrations (1 nM – 5000 nM). Standards used for quantification of metabolite formation from tissue fractions were prepared in DMSO, diluted into 1:1 water/acetonitrile, further diluted into 100 mM potassium phosphate buffer (pH 7.4), and matrix-matched with human liver

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cytosolic protein. Standards used for quantification of metabolite formation from experiments with recombinant AO were prepared in DMSO, diluted into 1:1 water/acetonitrile, and further diluted into potassium phosphate buffer alone. Standards were then prepared identically to the samples, as described above, with the addition of acetonitrile containing internal standard, centrifugation, and dilution of the resulting supernatants into water. Sciex Analyst 1.7 software was used to fit curves via linear regression analysis of the analyte/internal standard peak area ratio with $1/x^2$ weighting.

Liquid Chromatography-Mass Spectrometry Analysis. The extent of AO product formation was determined employing LC-MS/MS analysis with an electrospray ionization enabled Sciex QTRAP 6500 mass spectrometer (Sciex, Foster City, CA) coupled to Shimadzu LC-20ADXR HPLC pumps (Columbia, MD) and a CTC PAL autosampler (Leap Technologies, Carrboro, NC). Analytes were separated by gradient elution using a Kinetex C18 column (2.1 × 50 mm, 1.7 μm; Phenomenex, Torrance, CA) at 40 ^{o}C. Mobile phase A was 0.1% formic acid in water (v/v, pH unadjusted), and mobile phase B was 0.1% formic acid in acetonitrile. For analysis of 8-oxo-benzylguanine and 2-oxo-zoniporide, the gradient started at 5% B after a 0.2-minute hold and was linearly increased to 95% B over 1.0 minute, held at 95%B for 0.2 minute, and returned to 5% B in 0.1 minute, followed by a re-equilibration period (0.5 minute). The total run time was 2.0 minutes, and the HPLC flow rate was 0.5 ml/min. Mass spectral analyses were performed using multiple reaction monitoring, with transitions and voltages specific for each analyte using electrospray ionization (source temp 650^{o}C) in positive ionization mode (5500 V spray voltage). Multiple reaction monitoring transitions were the following: 2-oxo-zoniporide (m/z 337→278), 8-oxo-benzylguanine (m/z 258→91), and carbamazepine (m/z 237→194). Data were analyzed using Sciex Analyst 1.7 software.

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For analysis of aminodantrolene, analytes were separated by gradient elution using a Kinetex C18 column (2.1 x 50 mm, 1.7 μm; Phenomenex, Torrance, CA) warmed to 50°C. The gradient started at 10% B, and after a 0.3-minute hold was linearly increased to 90%B over 0.7 minute, held at 90%B for 0.5 minute, and returned to 10% B in 0.1 minute, followed by a re-equilibration period (0.5 minute). The total run time was 2.1 minutes, and the HPLC flow rate was 0.4 ml/min. Mass spectral analyses were performed using multiple reaction monitoring, with transitions and voltages specific for each analyte using electrospray ionization (source temp 550°C) in positive ionization mode (4000 V spray voltage). Multiple reaction monitoring transitions were the following: aminodantrolene (m/z 285→186) and carbamazepine (m/z 237→194). Data were analyzed using Sciex Analyst 1.7 software.

For analysis of the hydralazine-GSH conjugate, analytes were separated by gradient elution using a Luna Omega Polar C18 column (2.1 × 100 mm, 1.6-μm; Phenomenex, Torrance, CA) warmed to 40 °C. Mobile phase A was 0.1% formic acid in water (pH unadjusted), and mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 2% B, and after a 2-minute hold, was linearly increased to 80% B over 4 minutes, held at 80% B for 2 minutes, and returned to 2% B in 1 minute, followed by a re-equilibration period (1 minute). The total run time was 10 minutes, and the HPLC flow rate was 0.3 ml/min. Mass spectral analyses were performed using multiple reaction monitoring, with transitions and voltages specific for each analyte using electrospray ionization (source temp 650 °C) in positive ionization mode (5500 V spray voltage). Multiple reaction monitoring transitions were the following: GSH conjugate (m/z 436→307) and carbamazepine (m/z 237→194). Data were analyzed using Sciex Analyst 1.7 software.

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Results

Effect of GSH on Inhibition of AO Activity by Hydralazine in Human Liver and Kidney Cytosol or S9, Rat Liver Cytosol, and Recombinant Human AO. The AO substrate zoniporide (Dalvie et al., 2010) was incubated with multiple different sources of AO (human liver cytosol, human kidney cytosol, human liver S9, and rat liver cytosol) in the presence and absence of hydralazine and GSH in order to evaluate the effect of GSH (an electrophile trapping agent) on the inhibitory activity of hydralazine. GSH had a protective effect against enzyme inactivation by hydralazine in incubations with all five sources of AO (Figure 1A-D, Figure 2). 2-Oxo-zoniporide formation was increased in human liver cytosol pre-incubated with hydralazine plus GSH (31.6 ± 1.6 pmol/min/mg protein) vs. hydralazine alone (0.11 ± 0.02 pmol/min/mg protein), as well as in human kidney cytosol (0.30 ± 0.03 vs. 0.03 ± 0.01 pmol/min/mg protein), in human liver S9 fraction (27.0 ± 0.6 vs. 0.06 ± 0.02 pmol/min/mg protein), and in rat liver cytosol (24.1 ± 0.2 vs. 0.22 ± 0.02 pmol/min/mg protein).

Zoniporide was incubated with recombinantly expressed human AO in the presence and absence of hydralazine and GSH (Figure 2). GSH had a protective effect against enzyme inactivation by hydralazine, with an increase in 2-oxo-zoniporide formation observed in AO mixtures pre-incubated with hydralazine + GSH (0.34 ± 0.04 pmol/min/mg protein) vs. hydralazine alone (0.007 ± 0.002 pmol/min/mg protein).

The effect of GSH on the inhibitory activity of hydralazine was also evaluated using a different AO substrate, O^6-benzylguanine (Roy et al., 1995). O^6-Benzylguanine was incubated with human liver cytosol following pre-incubation with hydralazine in the presence or absence of GSH (Figure 3). As observed with zoniporide, 8-oxo-benzylguanine formation was also increased in human liver cytosol pre-incubated with hydralazine plus GSH (13.1 ± 0.2

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pmol/min/mg protein) vs. hydralazine alone (0.28 ± 0.02 pmol/min/mg protein). Raloxifene, a potent AO inhibitor that does not require bioactivation for its inhibitory activity(Obach, 2004), was unaffected by the presence of GSH, with similar 8-oxo-benzylguanine formation in incubations containing raloxifene plus GSH (1.6 ± 0.1 pmol/min/mg protein) vs raloxifene alone (2.2 ± 0.1 pmol/min/mg protein).

Zoniporide or O^6-benzylguanine was also incubated with human liver cytosol following pre-incubation with hydralazine either in the presence of GSH, or GSH was added to the mixture after hydralazine pre-incubation (at the time of substrate addition). Adding GSH after pre-incubating human liver cytosol with hydralazine resulted in the retention of inhibitory activity in incubations with both zoniporide (Figure 4A) and O^6-benzylguanine (Figure 4B).

Inhibition of AO activity by hydralazine in the presence and absence of GSH or *N*-acetycysteine (N-Ac Cys). The AO substrates O^6-benzylguanine or zoniporide were incubated with human liver cytosol or recombinant human AO, respectively, in the presence and absence of hydralazine and GSH or *N*-Ac Cys, another thiol-based trapping agent, in order to confirm the thiol-based mechanism of GSH in reducing the inhibitory activity of hydralazine. Both GSH and *N*-Ac Cys had a protective effect against enzyme inactivation by hydralazine in incubations with both human liver cytosol (Figure 5A) and recombinant human AO (Figure 5B). 8-Oxo-benzylguanine formation was increased in human liver cytosol pre-incubated with hydralazine + GSH (20.8 ± 1.0 pmol/min/mg protein) or hydralazine + *N*-Ac Cys (4.1 ± 0.2 pmol/min/mg protein) vs. hydralazine alone (0.17 ± 0.03 pmol/min/mg protein), although the protective effect was less pronounced with *N*-Ac Cys. 2-Oxo-zoniporide formation was also increased in incubations with recombinant human AO pre-incubated with hydralazine plus GSH (0.10 ± 0.01

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pmol/min/mg protein) or hydralazine plus *N*-Ac Cys (0.028 ± 0.001 pmol/min/mg protein) vs. hydralazine alone, in which case 2-oxo-zoniporide formation was below detection.

The concentration dependence of GSH and *N*-Ac Cys on reducing the inhibitory activity of hydralazine was also evaluated (Figure 6A-B). GSH concentrations of 5 and 50 μM had little to no protective effect against hydralazine (8-oxo-benzylguanine formation of 0.15 ± 0.0 and 0.23 ± 0.01 pmol/min/mg protein, respectively). However, AO activity was increased in a concentration dependent manner when GSH concentrations of 0.5 mM and 5 mM were included (8-oxo-benzylguanine formation of 4.1 ± 0.2 and 22.7 ± 0.3 pmol/min/mg protein, respectively), as compared to incubations containing hydralazine alone (0.16 ± 0.01 pmol/min/mg protein). Likewise, *N*-Ac Cys concentrations of 50 and 500 μM had little to no protective effect against hydralazine (8-oxo-benzylguanine formation of 0.19 ± 0.02 and 0.25 ± 0.01 pmol/min/mg protein, respectively), whereas an increase in AO activity was observed in incubations containing *N*-Ac Cys concentrations of 5 mM (1.7 ± 0.02 pmol/min/mg protein) and 50 mM (2.1 ± 0.2 pmol/min/mg protein) vs incubations containing hydralazine alone (0.20 ± 0.02 pmol/min/mg protein).

Reactive Metabolite Trapping by GSH and *N*-Ac Cys. Hofstra et al. previously identified a hydralazine reactive metabolite produced by activated leukocytes (Hofstra et al., 1991). The authors reported formation of 1-phthalazylmercapturic acid in the presence of *N*-Ac Cys and proposed 1-diazenylphthalazine as the reactive intermediate. We detected a peak with a protonated molecular ion MH^+ at *m/z* 292 in extracts from an incubation of human liver cytosol with hydralazine in the presence of *N*-Ac Cys. This *m/z* 292 ion produced an MS/MS spectrum consistent with the previously reported 1-phthalazylmercapturic acid (Supplemental Figure 1).

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Product ions at *m/z* 130 and 163 correspond to cleavage on either side of the *N*-Ac Cys thiol moiety, liberating the phthalazyl and thiophthalazyl fragments, respectively.

Accordingly, a peak with a protonated molecular ion (MH^+) at *m/z* 436 was detected in extracts from an incubation of human liver cytosol with hydralazine in the presence of GSH. The MS/MS spectrum produced by the *m/z* 436 ion was consistent with a GSH conjugate of 1-diazenylphthalazine (Supplemental Figure 2). A product ion at *m/z* 307 may be derived from the characteristic neutral loss of 129 Da, corresponding to loss of the γ-glutamyl portion of GSH or to the loss of the phthalazyl moiety, liberating GSH as the product ion.

Dependence of formation of the GSH conjugate on hydralazine concentration was also evaluated (Figure 7). Peak area ratios of the GSH conjugate were approximately 6-fold and 28-fold higher in human liver cytosol extracts from incubations with 50 μM and 500 μM hydralazine, respectively, relative to those from incubations with 5 μM hydralazine.

Substrate/Inhibitor Protection Experiments in Human Liver Cytosol. Substrate protection of AO inactivation by hydralazine was evaluated by including the AO substrate O^6-benzylguanine in primary incubations that contained human liver cytosol and hydralazine. The presence of O^6-benzylguanine did not protect AO from inactivation by hydralazine (Figure 8A).

Attenuation of AO inactivation by hydralazine was also evaluated using the allosteric AO inhibitor thioridazine. Inclusion of thioridazine in primary incubations containing human liver cytosol and hydralazine resulted in a concentration dependent protection of AO from inactivation by hydralazine (Figure 8B).

Effect of Catalase on the Inhibitory Activity of Hydralazine on AO. In order to evaluate a potential role of reactive oxygen species (ROS) in the inhibitory mechanism of hydralazine,

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hydralazine was incubated with recombinant human AO in the presence and absence of catalase prior to addition of zoniporide (Figure 9). Catalase offered no protection against AO inactivation by hydralazine.

Inhibition of AO Activity by Phthalazine in the Presence and Absence of GSH. Phthalazine, which is both a substrate and inhibitor of AO, is also a metabolite of hydralazine (Lesser et al., 1974; LaCagnin et al., 1987; Beedham et al., 1995; Abbasi et al., 2019; Garrido and Leimkühler, 2021). Therefore, we evaluated the effect of GSH on the ability of phthalazine to inhibit AO. Zoniporide was incubated with human liver cytosol in the presence and absence of phthalazine and GSH (Figure 10). The ability of phthalazine to inhibit AO was unaffected by the presence of GSH, as similar peak area ratios for 2-oxo-zoniporide were observed in incubations with phthalazine plus GSH and incubations with phthalazine alone.

Inhibition of AO Reductive Activity by Hydralazine or Phthalazine in the Presence and Absence of GSH. Dantrolene is a substrate of AO that undergoes nitro-reduction to aminodantrolene (Amano et al., 2018). In order to evaluate the effect of GSH on the inhibitory activity of hydralazine and phthalazine toward the reductive activity of AO, dantrolene was incubated with human liver cytosol following pre-incubation with hydralazine (Figure 11A) or phthalazine (Figure 11B) in the presence or absence of GSH. As observed with zoniporide and O^6-benzylguanine, aminodantrolene formation was also increased in human liver cytosol pre-incubated with hydralazine plus GSH (11-fold increase vs. hydralazine alone). Unlike observations with zoniporide and O^6-benzylguanine as substrates, however, formation of aminodantrolene was activated by the combination of GSH and hydralazine beyond the level of control activity (3.5-fold increased aminodantrolene formation vs. control).

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When human liver cytosol was pre-incubated with phthalazine alone, aminodantrolene formation was increased relative to control incubations (3.5-fold increase vs control). Aminodantrolene formation was further increased when human liver cytosol was pre-incubated with both GSH and phthalazine (4.5-fold increase vs. control).

Discussion

GSH was found to diminish the inhibitory activity of hydralazine toward AO. The protective effect of GSH was observed across multiple different sources of AO and occurred in a concentration-dependent manner. This observation, coupled with the detection of GSH and N-Ac Cys conjugates that are consistent with previous reports of a phthalazinyl intermediate, suggested that a reactive hydralazine metabolite plays a role in the mechanism of AO inactivation by hydralazine.

These findings imply that inactivation of AO occurs after the bioactivated hydralazine species has been released from the enzyme, or alternatively, that another cytosolic enzyme may bioactivate hydralazine, and the activated species subsequently diffuses away from that enzyme and binds to AO. For example, Hosogi et al. previously reported the irreversible inhibition of AO by an iminium ion metabolite of KW-2449 that is generated by monoamine oxidase B (MAO-B) (Hosogi et al., 2018). The bioactivation of hydralazine has been reported to occur in activated leukocytes and liver microsomes (i.e., via myeloperoxidase and P450 enzymes) (LaCagnin et al., 1987; Hofstra et al., 1991). However, because hydralazine inhibited AO not only from cytosolic and S9 fractions but also recombinant AO, it is likely that a separate enzyme is not required for bioactivation. (It should be noted, though, that the source of recombinant AO used in these studies was not purified enzyme, but rather a fraction of the cytosolic extract obtained from *E. coli* cells recombinantly expressing AO.)

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We also found that O^6-benzylguanine failed to protect AO from inactivation by hydralazine. However, the lack of substrate protection does not altogether preclude AO as the bioactivating enzyme, but rather it implies that hydralazine and O^6-benzylguanine do not compete for a binding site (presumably the active site). Accordingly, the allosteric AO inhibitor thioridazine appeared to have some protective effect against hydralazine, supporting the likelihood that AO participates in the bioactivation.

Aside from enzymatic bioactivation, hydralazine is reportedly bioactivated by metal ions (Cu^{2+} and Fe^{3+}) (Sinha and Motten, 1982; Sinha et al., 2014). Consequently, metal ion contamination within our experiments could potentially contribute to the formation of AO-inactivating reactive metabolites. We detected the GSH conjugate in control samples containing no enzyme (Supplemental Figure 3), and therefore the possibility of metal ion contamination contributing to hydralazine bioactivation cannot be ruled out. In addition, formation of the GSH conjugate in the absence of enzyme was reduced in the presence of EDTA, which is a chelator of metal cations (Supplemental Fig 3). While we did not supplement our cytosol, S9, and recombinant AO experiments with a metal ion chelator, these incubations did contain EDTA because the storage solutions for each enzyme source include EDTA, per the manufacturer-provided product specifications. Of note, GSH itself is capable of interacting with metal ions (Dimitrova et al., 2010), but any potential involvement of this interaction was not explored in our studies.

Raloxifene is a potent AO inhibitor, which reportedly inhibits AO via both a competitive and uncompetitive mechanism that is not time-dependent (Obach, 2004; Barr and Jones, 2013; Foti et al., 2016; Mota et al., 2021). As expected, the presence of GSH had no influence on the inhibitory activity of raloxifene. Alternatively, in the presence of phthalazine, which is an

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oxidative metabolite of hydralazine and a substrate of AO, a time-dependent loss of AO activity has been reported (Abbasi et al., 2019; Garrido and Leimkühler, 2021). However, unlike hydralazine, we found that the ability of phthalazine to inhibit AO was unaffected by the presence of GSH. AO produces the reactive oxygen species (ROS) hydrogen peroxide and superoxide as biproducts of its catalytic cycle, and a recent report by Garrido and Leimkuehler suggests that AO is inactivated by the ROS produced as a consequence of substrate turnover (Garrido and Leimkühler, 2021). Notably, Garrido and Leimkuehler reported the time-dependent inactivation of AO by phthalazine and concluded that AO is inactivated by the ROS produced during phthalazine turnover. Accordingly, because phthalazine is an oxidative metabolite of hydralazine, it is possible that phthalazine participates in the mechanism of hydralazine-mediated AO inactivation via ROS production during the oxidation of phthalazine to phthalazone. Furthermore, the lack of substrate protection observed in experiments where hydralazine was preincubated in the presence of O^6-benzylguanine may potentially be explained by the fact that O^6-benzylguanine turnover by AO would also be expected to produce ROS, thus contributing to AO inactivation (and therefore masking an observation of substrate protection by O^6-benzylguanine).

The literature, however, presents conflicting reports on the notion that AO is inactivated by ROS generated during substrate turnover. Abbasi et al., for example, did not find that ROS scavengers protect AO against time-dependent inactivation during substrate turnover (Abbasi et al., 2019). Alternatively, Garrido and Leimkuehler concluded that the ROS-mediated inactivation of AO is substrate-dependent and proposed that H_2O_2 production with rapidly metabolized substrates such as phthalazine (k_{cat} 123 min^{-1}) outpaces the reaction of H_2O_2 with catalase (Garrido and Leimkühler, 2021). In our studies, catalase offered no protection against

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AO inactivation by hydralazine. This observation would seem to indicate that conversion of hydralazine to a reactive metabolite does not involve ROS. However, assuming that phthalazine may inactivate AO via an ROS-dependent mechanism that is insensitive to catalase, these data cannot rule out the possibility that ROS production contributes to the mechanism of hydralazine-mediated inactivation of AO.

Interestingly, when dantrolene was used as the AO substrate, GSH not only attenuated AO inhibition by hydralazine, but it had an activating effect on AO activity. This activating effect was further amplified by the combination of GSH and hydralazine. Additionally, while phthalazine inhibited the oxidation of O^6-benzylguanine and zoniporide, phthalazine had no inhibitory effect on the reduction of dantrolene, but rather had an activating effect. This result is not surprising, as phthalazine oxidation would result in donation of electrons to AO, thus facilitating the reduction of dantrolene. Likewise, the oxidation of hydralazine by AO would also be expected to result in electron donation to the enzyme. Therefore, the combination of GSH and hydralazine may increase the conversion of dantrolene to aminodantrolene by 1) oxidation of hydralazine by AO, which promotes dantrolene reduction by providing electrons to the enzyme, and 2) trapping of the reactive hydralazine metabolite by GSH, which prevents the metabolite from reacting with AO. In addition, the presence of GSH may also serve as an H_2O_2 scavenger to further decrease AO inactivation via the action of cytosolic GSH peroxidase, as GSH appeared to consistently have a mild activating effect on AO activity in our experiments using tissue fractions as the AO source, but not in incubations with the recombinant enzyme, which would be expected to lack GSH peroxidase. However, as previously mentioned, the role of ROS in the inactivation of AO is unclear and warrants further investigation. Because phthalazine does not inhibit the reduction of dantrolene, it is possible that the mechanism by which phthalazine

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inhibits oxidation of O^6-benzylguanine and zoniporide is at least partly competitive in nature, rather than exclusively ROS-mediated.

These findings have implications regarding appropriate experimental design when using hydralazine as an AO inhibitor. For example, hydralazine may not be the most appropriate AO inhibitor to employ in experiments that require the presence of GSH (e.g. reactive metabolite trapping studies). However, a sufficient pre-incubation period of hydralazine with AO to permit enzyme inactivation prior to the addition of GSH may enable the use of hydralazine in these studies (Figure 4). Alternatively, phthalazine may serve as a potential substitute for hydralazine, as phthalazine-mediated inhibition of AO was unaffected by GSH. The usefulness of phthalazine, however, would be limited to inhibition of oxidation reactions, as reduction reactions are activated by phthalazine.

Interestingly, hydralazine has been reported to effectively inactivate AO in cryopreserved human hepatocytes (Hutzler et al., 2012; Strelevitz et al., 2012), despite our understanding that hepatocytes contain cytosolic GSH concentrations ranging from 1 – 10 mM (Forman et al., 2009). Likewise, hydralazine has been demonstrated to inhibit rabbit AO *in vivo* (Johnson et al., 1985). In their investigation of hydralazine as a selective AO inhibitor, Strelevitz et al. evaluated two different batches of hepatocytes and found that a sufficient level of AO inhibition was achieved with 25 μM hydralazine for one batch, whereas the second batch required a concentration of 50 μM (Strelevitz et al., 2012). Accordingly, the authors recommended that investigators conduct preliminary experiments to optimize the hydralazine concentration for each individual hepatocyte preparation. Among the range of GSH concentrations evaluated in our studies, we found those below 0.5 mM to have no effect on hydralazine-mediated AO inhibition. Consequently, it is possible that variability in endogenous GSH concentrations among

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hepatocyte donors could influence the effectiveness of AO inactivation by hydralazine in this system. The influence of endogenous GSH on the inhibitory activity of hydralazine in hepatocytes will be an important focus of future investigations, as hepatocytes represent a common system for reaction phenotyping studies, particularly when non-P450 metabolism is suspected.

In summary, we propose that hydralazine inactivates AO via the formation of a phthalazinyl radical intermediate, which can be neutralized by GSH (Scheme 1). Although the role of ROS in the inactivation of AO remains unclear, the conversion of the phthalazinyl radical to phthalazine may also contribute to the inhibitory mechanism of hydralazine. While hydralazine is able to inhibit both oxidation and reduction reactions mediated by AO, phthalazine appears to only inhibit oxidation reactions. As hydralazine is commonly used in reaction phenotyping experiments to identify AO substrates, these studies highlight the importance of understanding the mechanism of inhibition of hydralazine so that inappropriate use of the inhibitor does not confound experimental results (Zientek and Youdim, 2015).

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The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

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Authorship Contributions

Participated in research design: Barnes, Yang, Thompson, Pleasant, Guevarra, Do, Crouch

Conducted experiments: Barnes, Yang, Thompson, Pleasant, Guevarra, Do, Crouch

Contributed new reagents or analytic tools: N/A

Performed data analysis: Barnes, Yang, Thompson, Pleasant, Guevarra, Do, Crouch

Wrote or contributed to the writing of the manuscript: Barnes, Yang, Crouch

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Figure Legends

Figure 1. Inhibition of AO activity by hydralazine in the presence and absence of GSH. Zoniporide (10 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 2 mg/mL **(A)** human liver cytosol, **(B)** human kidney cytosol, **(C)** human liver S9, or **(D)** rat liver cytosol in the presence or absence of 5 mM GSH and/or 50 μM hydralazine. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Figure 2. Inhibition of recombinant human AO activity by hydralazine in the presence and absence of GSH. Zoniporide (10 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 0.5 mg/mL recombinant human AO in the presence or absence of 5 mM GSH and/or 50 μM hydralazine. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Figure 3. Inhibition of AO by hydralazine and raloxifene in the presence and absence of GSH. *O*^{6}-Benzylguanine (10 μM) was incubated at 37^{O}C for 60 min with human liver cytosol (2 mg/ml) in potassium phosphate buffer (100 mM, pH 7.4) with or without hydralazine (50 μM) or raloxifene (1 μM) in the presence or absence of 5 mM GSH. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Figure 4. Inhibition of AO activity by hydralazine when GSH is present during hydralazine pre-incubation or after pre-incubation. **(A)** Zoniporide (10 μM) or **(B)** *O*^{6}-benzylguanine (10 μM) was incubated for 60 minutes at 37^{O}C with human liver cytosol (2 mg/ml) in potassium phosphate buffer (100 mM, pH 7.4) with or without hydralazine (50 μM). Prior to addition of

substrate, mixtures were pre-incubated with hydralazine (25 min) either in the presence of 5 mM GSH ("co-incubation"), or 5 mM GSH was added after the 25-min hydralazine pre-incubation ("post-incubation"). Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Figure 5. Inhibition of AO activity by hydralazine in the presence and absence of GSH or N-Ac Cys. O^6-Benzylguanine or zoniporide (10 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 2 mg/mL **(A)** human liver cytosol or **(B)** 0.5 mg/mL recombinant human AO with or without 50 μM hydralazine in the presence or absence of 5 mM GSH or N-Ac Cys. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Figure 6. Concentration dependence of GSH and N-Ac Cys on the inhibitory activity of hydralazine. O^6-Benzylguanine (10 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 2 mg/mL human liver cytosol in the presence or absence 50 μM hydralazine and 0-50 mM of **(A)** GSH or **(B)** N-Ac Cys. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD. 8-Oxo-benzylguanine formations in control incubations were **(A)** 36.4 ± 0.520 pmol/min/mg protein and **(B)** 37.3± 0.912 pmol/min/mg protein.

Figure 7. Concentration dependent formation of the hydralazine-GSH conjugate. Hydralazine (5, 50, or 500 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 2 mg/mL human liver cytosol in the presence of 5 mM GSH. Relative metabolite levels were determined based on the peak area ratio of analyte to internal standard. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Figure 8. O^6-Benzylguanine or thioridazine protection against inactivation of AO by hydralazine. Human liver cytosol (5 mg/mL) was pre-incubated at 37^{O}C for 0, 10 or 30 minutes with potassium phosphate buffer (100 mM, pH 7.4) in the presence or absence of 25 μM hydralazine and 10-100 μM **(A)** O^6-benzylguanine or **(B)** thioridazine. After the designated pre-incubation period, mixtures were diluted 50 fold into buffer containing zoniporide (final concentration = 100 μM) and incubated for an additional 5 minutes. Data represent the means ± SD of a single experiment performed in triplicate. **Panel B:** Two of the three replicates for the "hydralazine alone" data point at 30 min were below the limit of quantitation (1 nM, which corresponds to 2 pmol/min/mg cytosol). These two replicates were designated a rate of 2 pmol/min/mg and averaged with the third replicate (4.62 pmol/min/mg).

Figure 9. Inhibition of recombinant human AO activity by hydralazine in the presence and absence of catalase. Zoniporide (10 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 0.5 mg/mL recombinant human AO in the presence or absence of 800 units/mL catalase and/or 50 μM hydralazine. Relative metabolite levels were determined based on the peak area ratio of analyte to internal standard. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Figure 10. Inhibition of AO activity by phthalazine in the presence and absence of GSH. Zoniporide (10 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 2 mg/mL human liver cytosol in the presence or absence of 5 mM GSH and/or 50 μM phthalazine. Relative metabolite levels were determined based on the peak area ratio of analyte to internal standard. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

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Figure 11. Inhibition of AO reductive activity by hydralazine or phthalazine in the presence and absence of GSH. Dantrolene (10 μM) was incubated at 37^{O}C for 60 minutes in potassium phosphate buffer (100 mM, pH 7.4) with 2 mg/mL human S9 in the presence or absence of 5 mM GSH and/or **(A)** 50 μM hydralazine or **(B)** 50 μM phthalazine. Relative metabolite levels were determined based on the peak area ratio of analyte to internal standard. Data points represent individual replicates from a single experiment performed in triplicate. The bars represent the means ± SD.

Scheme 1. Proposed pathway for hydralazine-mediated inactivation of aldehyde oxidase and mitigation of the inactivation by glutathione.

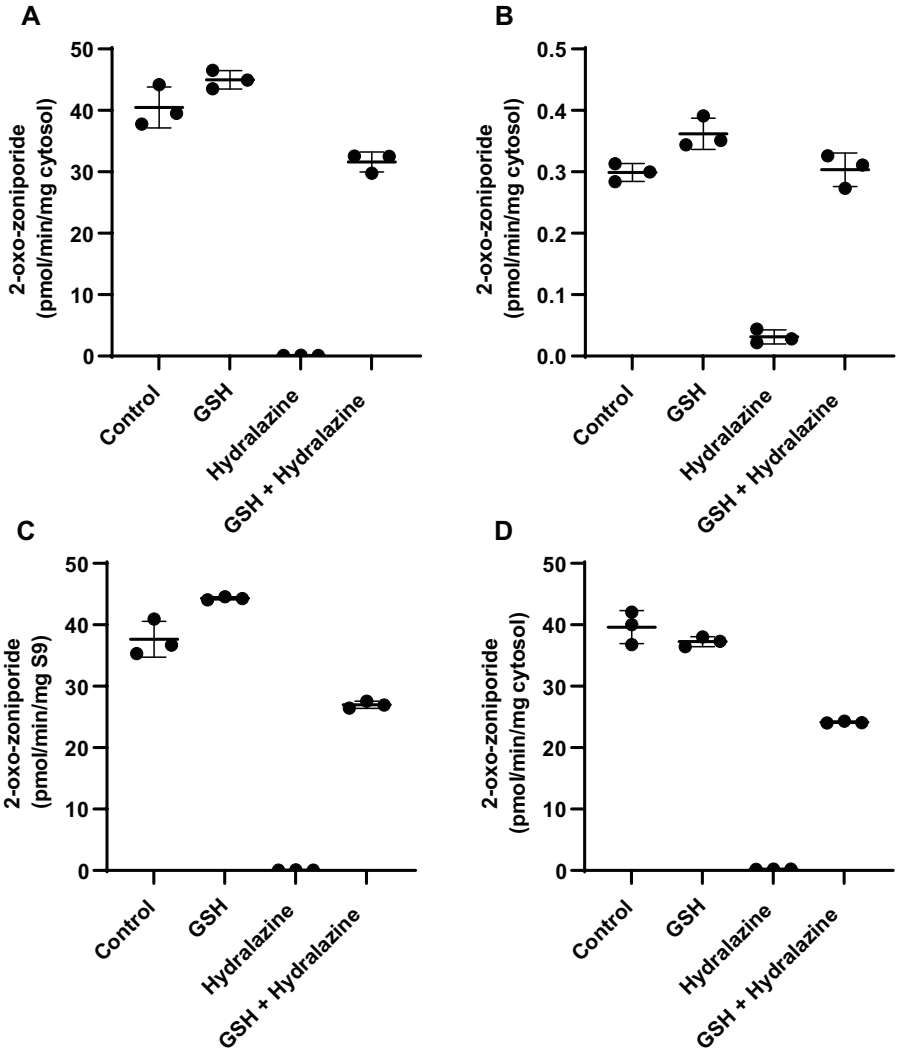

A
2-oxo-zoniporide (pmol/min/mg cytosol)
50
40
30
20
10
0
Control
GSH
Hydralazine
GSH + Hydralazine
B
2-oxo-zoniporide (pmol/min/mg cytosol)
0.5
0.4
0.3
0.2
0.1
0.0
Control
GSH
Hydralazine
GSH + Hydralazine
C
2-oxo-zoniporide (pmol/min/mg S9)
50
40
30
20
10
0
Control
GSH
Hydralazine
GSH + Hydralazine
D
2-oxo-zoniporide (pmol/min/mg cytosol)
50
40
30
20
10
0
Control
GSH
Hydralazine
GSH + Hydralazine

Figure 1

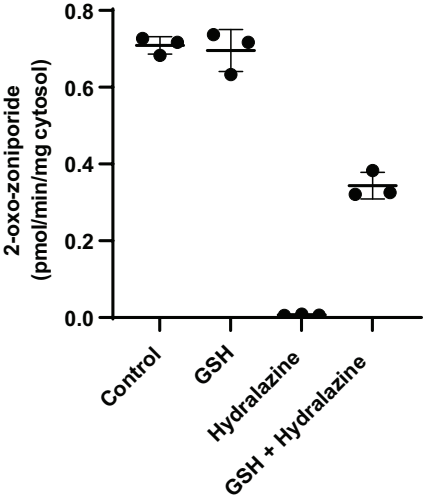


Figure 2

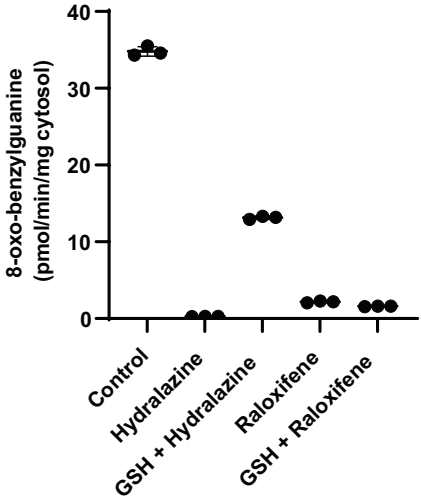
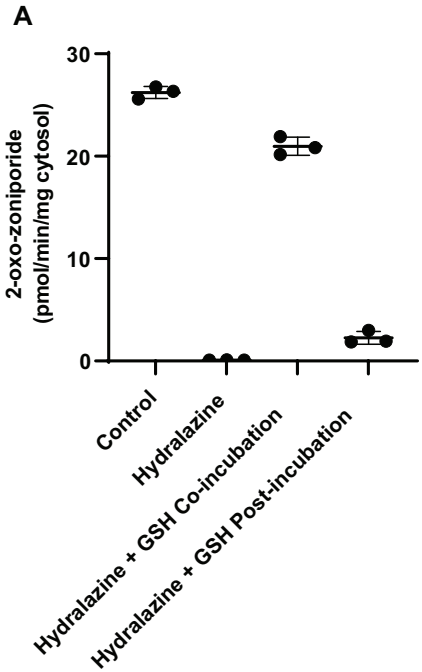

8-oxo-benzylguanine
(pmol/min/mg cytosol)
40
30
20
10
0
Control
Hydralazine
GSH + Hydralazine
Raloxifene
GSH + Raloxifene

Figure 3


A
2-oxo-zoniporide
(pmol/min/mg cytosol)
30
20
10
0
Control
Hydralazine
Hydralazine + GSH Co-incubation
Hydralazine + GSH Post-incubation

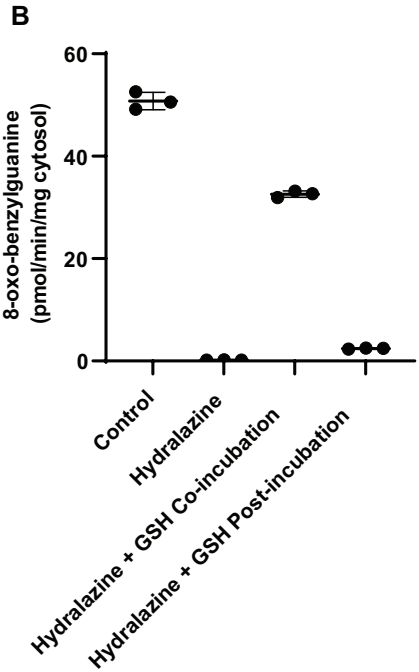

B
8-oxo-benzylguanine
(pmol/min/mg cytosol)
60
40
20
0
Control
Hydralazine
Hydralazine + GSH Co-incubation
Hydralazine + GSH Post-incubation

Figure 4

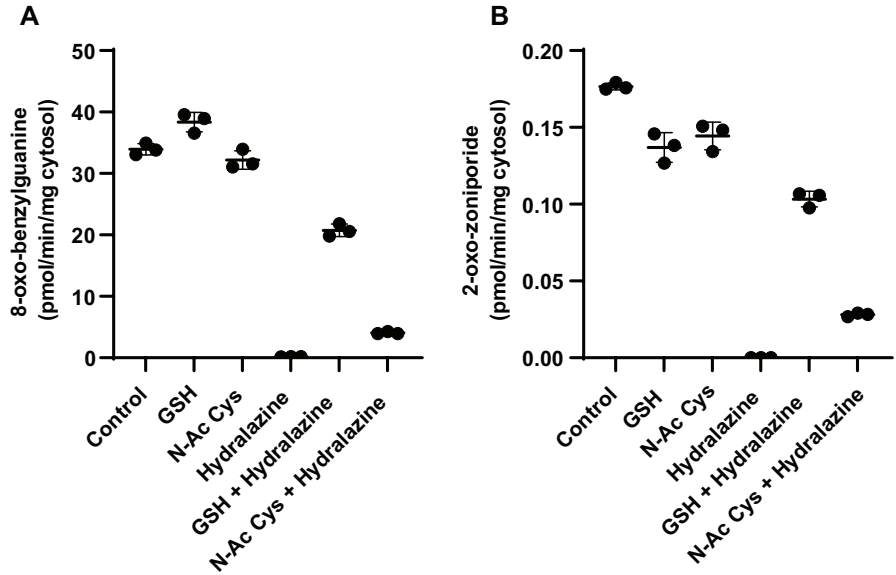

A
8-oxo-benzylguanine
(pmol/min/mg cytosol)
50
40
30
20
10
0
Control
GSH
N-Ac Cys
Hydralazine
GSH + Hydralazine
N-Ac Cys + Hydralazine
B
2-oxo-zoniporide
(pmol/min/mg cytosol)
0.20
0.15
0.10
0.05
0.00
Control
GSH
N-Ac Cys
Hydralazine
GSH + Hydralazine
N-Ac Cys + Hydralazine

Figure 5

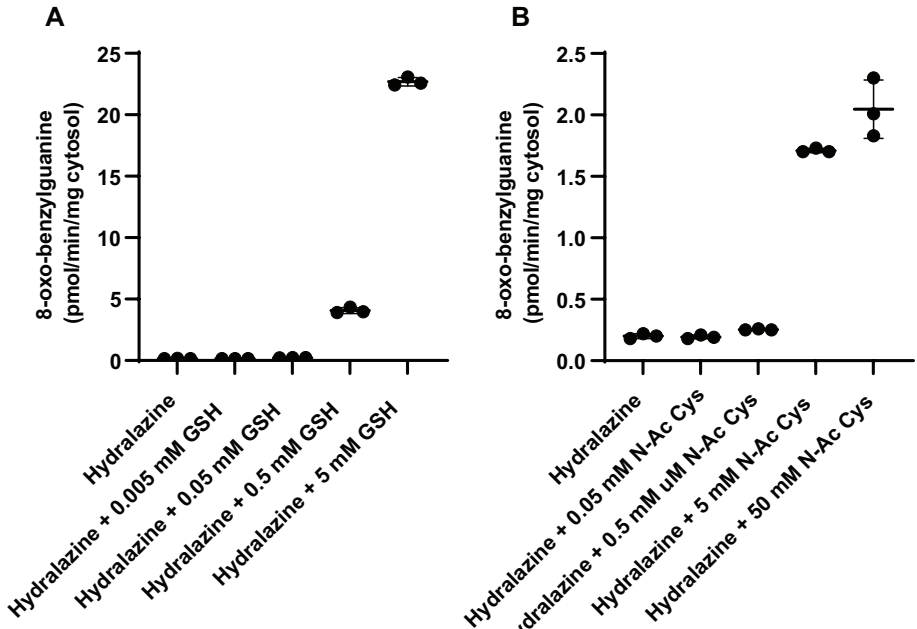

A
8-oxo-benzylguanine (pmol/min/mg cytosol)
25
20
15
10
5
0
Hydralazine
Hydralazine + 0.005 mM GSH
Hydralazine + 0.05 mM GSH
Hydralazine + 0.5 mM GSH
Hydralazine + 5 mM GSH
B
8-oxo-benzylguanine (pmol/min/mg cytosol)
2.5
2.0
1.5
1.0
0.5
0.0
Hydralazine
Hydralazine + 0.05 mM N-Ac Cys
Hydralazine + 0.5 mM uM N-Ac Cys
Hydralazine + 5 mM N-Ac Cys
Hydralazine + 50 mM N-Ac Cys

Figure 6

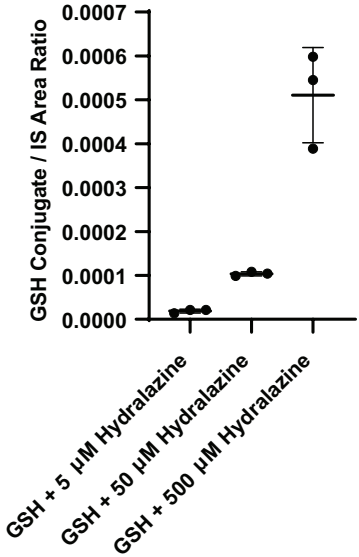

GSH Conjugate / IS Area Ratio
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
GSH + 5 µM Hydralazine
GSH + 50 µM Hydralazine
GSH + 500 µM Hydralazine

Figure 7

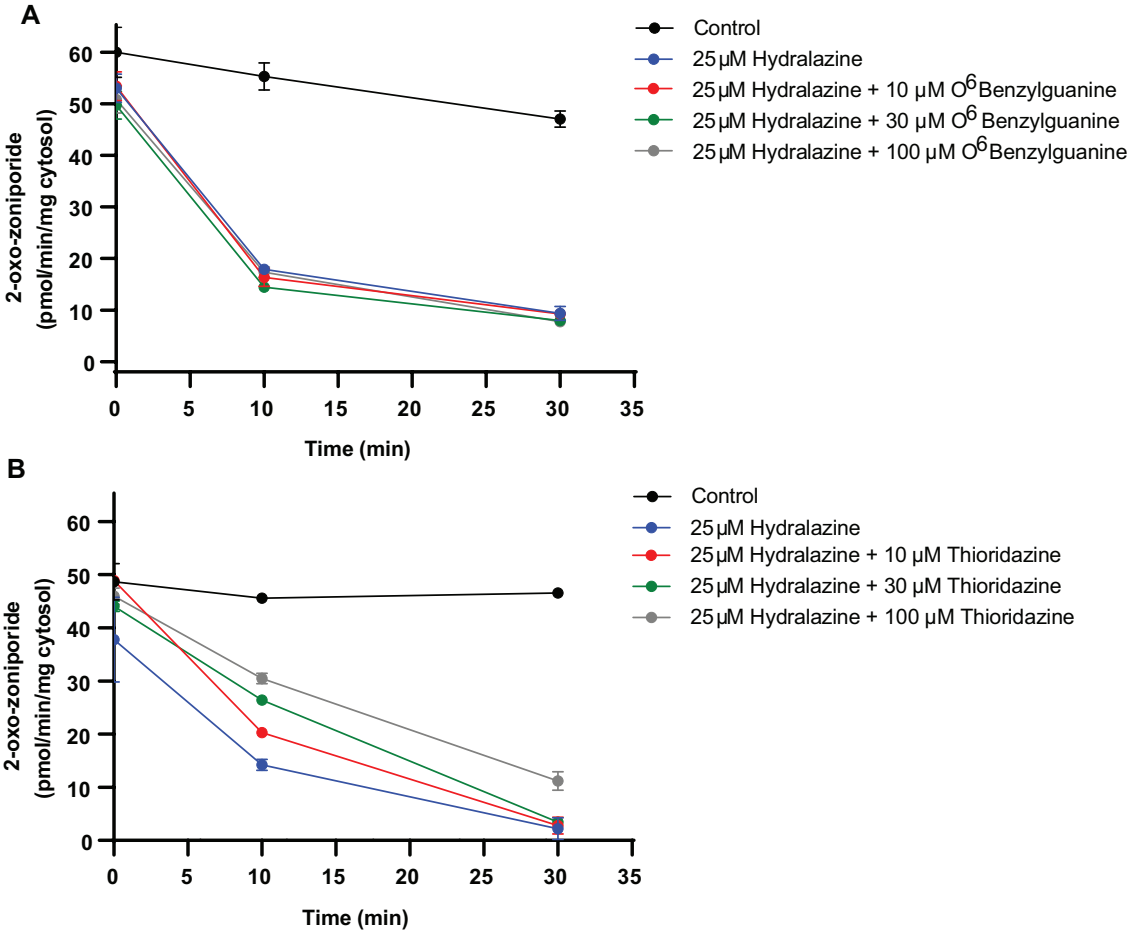

A
2-oxo-zoniporide (pmol/min/mg cytosol)
Time (min)
Control
25 µM Hydralazine
25 µM Hydralazine + 10 µM O6Benzylguanine
25 µM Hydralazine + 30 µM O6 Benzylguanine
25 µM Hydralazine + 100 µM O6Benzylguanine
B
2-oxo-zoniporide (pmol/min/mg cytosol)
Time (min)
Control
25 µM Hydralazine
25 µM Hydralazine + 10 µM Thioridazine
25 µM Hydralazine + 30 µM Thioridazine
25 µM Hydralazine + 100 µM Thioridazine

Figure 8

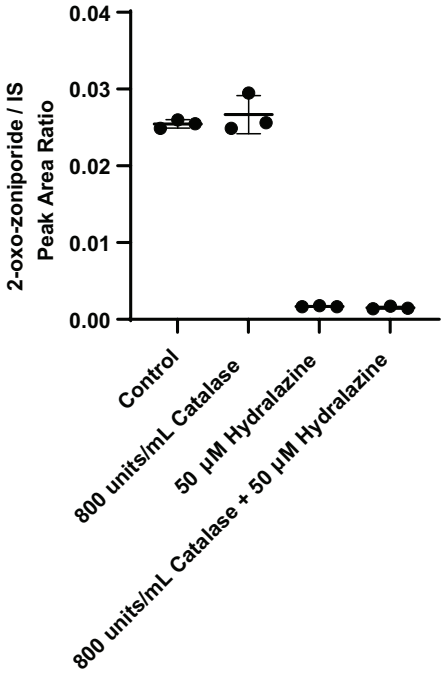

2-oxo-zoniporide / IS
Peak Area Ratio
0.04
0.03
0.02
0.01
0.00
Control
800 units/mL Catalase
50 µM Hydralazine
800 units/mL Catalase + 50 µM Hydralazine

Figure 9

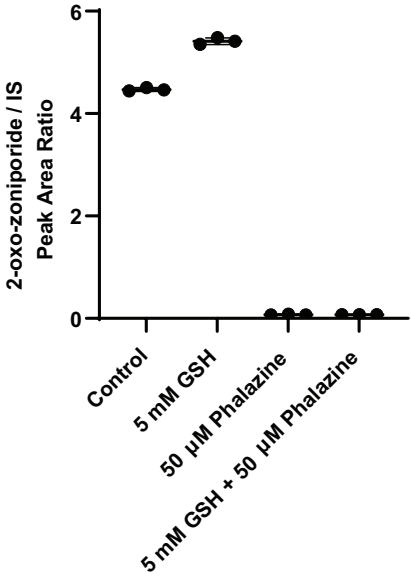
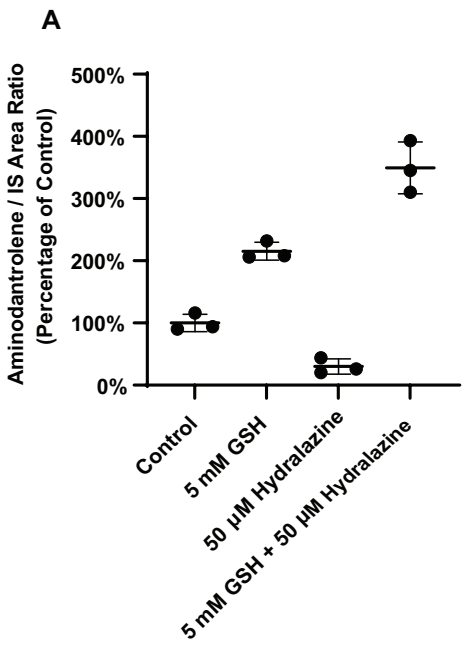

2-oxo-zoniporide / IS
Peak Area Ratio
6
4
2
0
Control
5 mM GSH
50 µM Phalazine
5 mM GSH + 50 µM Phalazine

Figure 10


A
Aminodantrolene / IS Area Ratio
(Percentage of Control)
500%
400%
300%
200%
100%
0%
Control
5 mM GSH
50 µM Hydralazine
5 mM GSH + 50 µM Hydralazine

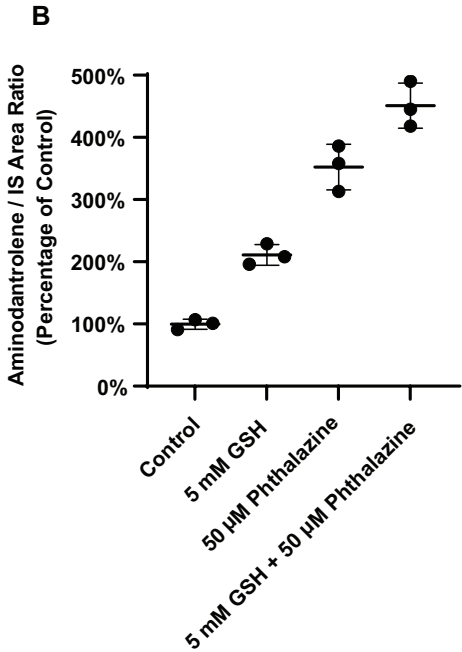
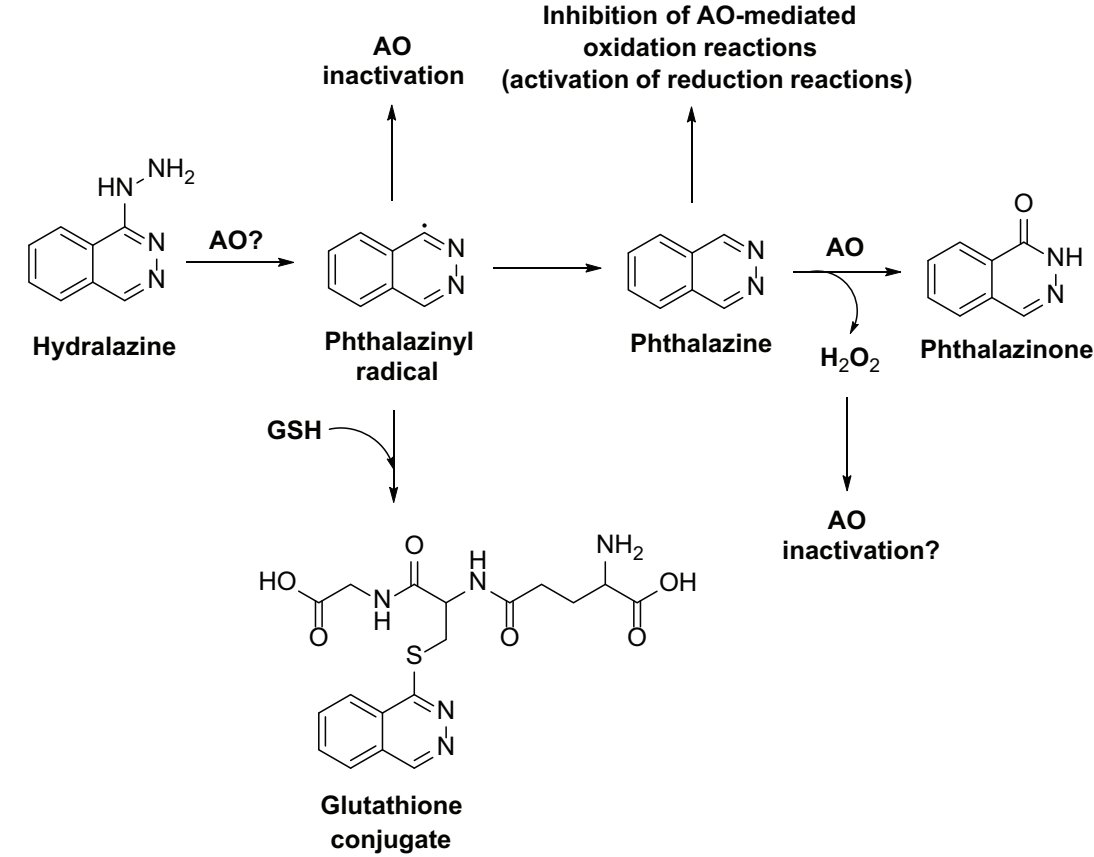

B
Aminodantrolene / IS Area Ratio
(Percentage of Control)
500%
400%
300%
200%
100%
0%
Control
5 mM GSH
50 µM Phthalazine
5 mM GSH + 50 µM Phthalazine

Figure 11


AO inactivation
Inhibition of AO-mediated oxidation reactions (activation of reduction reactions)
AO?
AO
Hydralazine
Phthalazinyl radical
Phthalazine
Phthalazinone
H_2O_2
GSH
AO inactivation?
Glutathione conjugate

Scheme 1