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Relative inhibitory potency of molinate and metabolites with aldehyde dehydrogenase2: implications for the mechanism of enzyme inhibition

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Abstract

Molinate is a thiocarbamate herbicide used as a pre-emergent in rice paddy fields. It has two predominant sulfoxidation metabolites, molinate sulfoxide and molinate sulfone. Previous work demonstrated an *in vivo* decrease in liver aldehyde dehydrogenase (ALDH) activity in rats treated with molinate and motor function deficits in dogs dosed chronically with this compound. ALDH is an enzyme important in the catabolism of many neurotransmitters, such as dopamine. Inhibition of this enzyme may lead to the accumulation of endogenous neurotoxic metabolites such as 3,4-dihydroxyphenylacetaldehyde (DOPAL), a dopamine metabolite, which may account for the observed neurotoxicity. In this study, the relative reactivity of molinate and both of its sulfoxidation metabolites towards ALDH were investigated, as well as the mechanism of inhibition. ALDH activity was monitored in two different model systems, human recombinant ALDH (hALDH2) and mouse striatal synaptosomes. Molinate sulfone was found to be the most potent ALDH inhibitor, compared to molinate and molinate sulfoxide. The reactivity of these three compounds was also assessed, using *N*-acetyl Cys, model peptides, and hALDH2. It was determined that molinate sulfone is capable of covalently modifying Cys residues, including catalytic Cys302 of ALDH, accounting for the observed enzyme inhibition.

Introduction

Molinate (Ordram) is a thiocarbamate herbicide commonly used as a pre-emergent in rice paddy fields to control broad-leaved and grassy plants. In the United States, this herbicide was used primarily in California, and only for a six-week long season. Due to toxicity risks, the United States Environmental Protection Agency recently banned the use of this pesticide, in effect at the conclusion of the 2009 growing season (1).

Exposure risks for molinate include dermal and inhalation for field workers using this pesticide and oral consumption of contaminated water from field run-off in areas surrounding rice fields (2). Some of the adverse effects observed from molinate exposure include reproductive toxicity (3), as well as neurotoxic consequences, such as peripheral neuropathy (2). Reproductive effects observed include Leydig cell lesions and inhibition of testicular esterases (3–5). Treatment of rats with molinate has been shown to cause neurotoxicity, based upon neuronal cell necrosis in the pyriform cortex and dose-response

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relationship observed for both motor activity and sensory response (tail-flick) (6,7). Irreversible motor function deficits in dogs dosed chronically were also found upon treatment with molinate (7,8). The motor function deficits found in the latter study included splayed limbs, reduced motor activity, and tremors, accompanied by minor demyelination in the spinal cord. The irreversibility of the adverse effects observed in dogs is a very critical consideration, because even though molinate was used for short durations each year, the neurotoxic effects resulting from this exposure period are permanent, making the mechanism of this toxicity of significant importance. In mice dosed with molinate, peripheral nerve demyelination was observed in addition to eosinophilic bodies (swollen, degenerated axons) in the medulla and spinal cord, hindlimb adduction, splayed hindlimbs, and ataxia (7,9). It should be noted that no peripheral neuropathy was observed in rats dosed with molinate (10).

A previously identified target of molinate is the enzyme aldehyde dehydrogenase (ALDH)¹, indicated by an *in vivo* decrease in liver ALDH activity in rats treated with molinate (11–13). Elevated levels of acetaldehyde in the blood and brain of ethanol-challenged rats dosed with molinate were also found, indicative of ALDH inhibition (13). It is important to note that in humans, there are 19 genes attributed to ALDH enzymes (14,15). In addition to the biotransformation of acetaldehyde, ALDHs also play a critical role in the metabolism of many toxic aldehydes such as 3,4-dihydroxyphenylacetaldehyde (DOPAL) and 4-hydroxy-2-nonenal (4HNE) (15). DOPAL is the aldehyde metabolite of dopamine, the neurotransmitter important for motor activity, whereas 4HNE is a product of lipid peroxidation. When ALDH is inhibited, it can lead to the accumulation of these reactive electrophiles, which have been shown to modify proteins and lead to increased oxidative stress, mitochondrial dysfunction, and toxicity (15–21). Inhibition of ALDH, such as by pesticide exposure, has also been implicated in the development of neurodegenerative disorders such as Parkinson's Disease and Alzheimer's Disease (15,21,22).

It has been proposed that metabolites of molinate are primarily responsible for the toxic effects observed from exposure to this pesticide (3,23,24). Molinate is metabolized by two main pathways, 1.) hydroxylation of the ring or 2.) oxidation of the thiol moiety first to a sulfoxide then further oxidation to the sulfone (Scheme 1) (24). The ring hydroxylation of molinate is thought to be a detoxification pathway, and was found to be predominant at lower doses of molinate. At higher doses, molinate metabolism is thought to occur via the sulfoxidation route (24). These metabolites may then undergo glutathione conjugation, followed by excretion of the corresponding mercapturate product, but species differences in rates and routes of metabolism have been observed (25,26). Previous studies have shown that in humans, only 1–5% of the dose of molinate is excreted as the mercapturate, and 35–40% is excreted as hydroxymolinate or a comparable conjugate (25,27). Based upon these results, a recent report concluded that at the current recommended exposure limits, human

¹Abbreviations:

4HNE, 4-hydroxy-2-nonenal

ACN, acetonitrile

ALDH, aldehyde dehydrogenase

BCA, bichinchonic acid

DOPAC, 3,4-dihydroxyphenylacetic acid

DOPAL, 3,4-dihydroxyphenylacetaldehyde

DTNB, 5,5'-dithiobis(2-nitrobenzoic acid)

hALDH2, human recombinant mitochondrial aldehyde dehydrogenase

mCPBA, *m*-chloroperoxybenzoic acid

MS/MS, tandem mass spectrometry

Q-TOF, quadrupole time-of-flight

RT, retention time

TNBSA, 2,4,6-trinitrobenzenesulfonic acid

toxicity risk is minimized (23). However, the target of the remaining 60% of the initial dose that is not excreted is unknown.

A few studies have investigated the role of the sulfoxidation metabolites in the toxicity observed from molinate exposure. It has been shown that in rats and humans, molinate sulfoxide and molinate sulfone are both more potent testicular carboxylesterase inhibitors than molinate, resulting in the carbamylation of an active site Ser residue (3,23). This esterase inhibition is thought to contribute to the reproductive toxicity observed in rats and mice (5,23).

In addition to esterase inhibition, molinate sulfoxide has been shown to be capable of inhibiting liver ALDH (12), however, the relative inhibitory potency of both sulfoxidation metabolites of molinate towards ALDH has not been addressed. Also, the protein reactivity profile of these three compounds has not been investigated, nor the specific target of protein modification. The goal of this paper is to establish the mechanism of inhibition of ALDH by molinate and its sulfoxidation metabolites, by examining their reactivity and potency profiles.

Based upon the relative reactivity of other similar pesticides (28,29) and previously reported studies on molinate (5,23,24), it is hypothesized that molinate sulfone is a more potent inhibitor of ALDH than molinate or molinate sulfoxide, and that the mechanism of inhibition involves thiol modification. The relative reactivity of molinate and its metabolites was determined by comparing the inhibition kinetics of these three compounds for a human recombinant mitochondrial ALDH (hALDH2). The reactivity of each compound towards various amino acids and hALDH2 was demonstrated, identifying Cys as the target for modification. This work indicates the sulfoxidation of molinate leads to the production of a reactive metabolite, molinate sulfone, capable of inactivating ALDH2.

Experimental Procedures

Chemicals

Molinate was purchased from Chem Service (West Chester, PA), 2,4,6-trinitrobenzenesulfonic acid (TNBSA) was obtained from Pierce (Rockford, IL) and peptides (ANP(1–11) and RKRSRAE) were obtained from American Peptide (Sunnyvale, CA). All other reagents, unless otherwise noted, were purchased from Sigma Aldrich (St. Louis, MO).

Chemical Synthesis. Molinate Sulfone (S-Ethyl-hexahydro-1H-azepine-1-carbothioate sulfone)

Adapted from a literature method (10), molinate (54mg, 0.29mmol) was combined with *m*-chloroperoxybenzoic acid (*m*CPBA, 103mg, 0.59mmol) in dichloromethane and stirred in an ice bath overnight. The reaction was filtered and washed with saturated sodium bicarbonate twice. The product was purified using column chromatography on silica gel with a mobile phase consisting of hexanes (A) and ethyl acetate (B) (0–17% B). ¹H NMR (CDCl₃): δ 1.45 (t, 3H, CH₃), 1.64 (m, 4H, ring CH₂), 1.80 (m, 2H, ring CH₂), 1.89 (m, 2H, ring CH₂), 3.39 (q, 2H, CH₂), 3.56 (t, 2H, ring CH₂), 3.92 (t, 2H, ring CH₂); TOF MS (ESI) *m/z* calcd for C₉H₁₇NO₃S 219.1, found *m/z* 242.1 (M + Na)⁺.

Molinate Sulfoxide (S-Ethyl-hexahydro-1H-azepine-1-carbothioate sulfoxide)

Adapted from a literature method (30), molinate (112.7mg, 0.6mmol) was combined with iron trichloride (FeCl₃, 3mg, 0.02mmol) in 600μL of acetonitrile (ACN) and vigorously vortexed for 5min. Then, periodic acid (150mg, 0.66mmol) was added. After 2h, an excess

of sodium thiosulfate was added to quench the reaction, and then allowed to sit overnight. The product was extracted four times with dichloromethane (5mL), and the combined organic layers dried with magnesium sulfate. The product was dried by rotoevaporation and then purified by column chromatography on silica gel, with a mobile phase consisting of hexanes (A) and ethyl acetate (B) (10% B). ^1H NMR (CDCl_3): δ 1.38 (t, 3H, CH_3), δ 1.68 (m, 4H, ring CH_2), δ 1.80 (m, 4H, ring CH_2), δ 3.01 (q, 2H, CH_2), δ 3.40 (t, 4H, ring CH_2); TOF MS (ESI) m/z calcd for $\text{C}_9\text{H}_{17}\text{NO}_2\text{S}$ 203.0980, found 226.0895 ($\text{M} + \text{Na}$) $^+$.

hALDH2 Preparation

The preparation of wild-type hALDH2 and the C302S mutant of hALDH2 were conducted as described previously (20,31–33), and stored in 50% glycerol. Prior to use, the wild-type hALDH2 was dialyzed for 4h at 4°C with a 4000-fold excess of 50mM sodium phosphate buffer, pH 7.4, to remove glycerol. The protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce) and a Molecular Devices SpectraMax 190 plate reader (Sunnyvale, CA). The specific activity of the wild-type enzyme was measured by incubating hALDH2 (0.3–3.5 μg), propionaldehyde (1.4 μM), and NAD (1mM), and monitoring the conversion of NAD to NADH, measured as an increase in absorbance at 340nm ($\epsilon = 6220 \text{ M}^{-1}\text{cm}^{-1}$).

Kinetics of Aldehyde Dehydrogenase Inhibition

One microgram of hALDH2 was incubated and equilibrated with 1mM NAD, followed by the addition of 0–25 μM of molinate, molinate sulfoxide, or molinate sulfone in 50mM sodium phosphate buffer, pH 7.4, at 37°C. At time points between 0 and 15min, propionaldehyde in 50mM sodium phosphate buffer, pH 7.4, was added for a final concentration of 1mM. Upon addition of substrate, the activity of hALDH2 was measured as an increase in the production of NADH. Percent activity was determined by comparing the initial linear slopes of NADH production in treated wells to the initial slopes from control wells. To determine if this inhibition was reversible, hALDH2 (6 μg) was incubated with NAD (1mM) and then molinate sulfone (0–50 μM) at 37°C for 0–15min. This solution was then diluted 1:40 with NAD (1mM) and propionaldehyde (1mM), and absorbance readings taken as before, with initial linear slopes of NADH production monitored.

Determination of k_i Values

The bimolecular rate constants of inhibition (k_i) for each compound (inhibitor) were determined according to literature methods (34–36). The inhibitory reaction proceeded with $[\text{inhibitor}] > 10 [\text{enzyme}]$, and the k_i approximated using pseudo-first order kinetics. Linear regression was used to calculate the slopes of primary kinetic plots of $\ln (\% \text{ activity remaining})$ vs time (t) to obtain apparent first-order rate constants of inhibition (k') at each $[\text{inhibitor}]$ and to determine the k_i from the slopes of secondary plots of $-k'$ vs $[\text{inhibitor}]$. All slopes were linear for the duration of the experiment (900s), except for 25 μM molinate sulfone due to complete enzyme inhibition after 600s. Therefore, for this concentration, the slope (k') was determined only from 0–600s, whereas all other slopes were determined through 900s.

Preparation of Striatal Synaptosomes

Striatal synaptosomes were prepared from 20–25g Swiss Webster (CD1 IGS) female mice, as previously described (18,37,38). Briefly, mice were euthanized with an ip dose of 150mg/kg sodium pentobarbital, and the brains were quickly removed. The striatal tissue was removed and homogenized in media (0.32M sucrose, 10mM Tris, and 0.5mM EDTA, pH 7.5, 4°C) at 10% w/v. The homogenate was then centrifuged at $800 \times g$ for 10min, and the pellet discarded. The supernatant was mixed (1:1) with 1.2M sucrose and the solution

centrifuged at $18000 \times g$ for 25min. The pellet was resuspended to the original volume in HEPES-buffered synaptosomal media (136mM NaCl, 4.8mM KCl, 1.2mM MgSO₄, 1.4mM CaCl₂, 10mM glucose, 140μM EDTA, and 20mM HEPES, pH 7.4). Protein concentration was determined using the BCA assay (Pierce).

Treatment and Analysis of Striatal Synaptosomes

Synaptosomes (0.5mg/mL) were pre-incubated in 50mM sodium phosphate buffer, pH 7.4, with 0.1mM dopamine for 15min at 37°C. Then molinate, molinate sulfoxide, or molinate sulfone were added (50μM), or sodium phosphate buffer for vehicle controls. Aliquots were removed at various time points (0–60min) and combined with perchloric acid (5% v/v final concentration), to precipitate proteins and stop the reaction. Solutions were stored at –20°C, thawed, and centrifuged at $10,000 \times g$ for 5min prior to HPLC analysis. Samples were analyzed by an Agilent 1100 Series Capillary HPLC with Phenomenex C18 Luna column (1 × 150mm) to quantify dopamine metabolites: DOPAL, and 3,4-dihydroxyphenylacetic acid (DOPAC). Separation of these metabolites was achieved using isocratic conditions of 0.1% trifluoroacetic acid in water with 6% ACN (v/v), a flow rate of 50μL/min, and detection with a photodiode array detector (absorbance at 202 and 280nm). The peak area was then converted to concentration units through comparison to a standard curve achieved from metabolite standards. The percent activity of ALDH for each sample was determined by comparing the linear slopes (0–60min) for the time-dependent formation of DOPAC from treated synaptosomes to controls.

Amino Acid Reactivity

Thiol reactivity of molinate, molinate sulfoxide, and molinate sulfone was determined using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)) and a procedure adapted from the literature (39). *N*-acetyl Cys (50μM) was incubated with each compound (0–100μM) for 15min at 37°C. DTNB (0.5mM) was added, and absorbance monitored at 412nm using a SpectraMax plate reader to determine free thiol concentration ($\epsilon = 13,600\text{M}^{-1}\text{cm}^{-1}$). In order to determine amine reactivity of each compound, TNBSA was used according to manufacturer specifications (Pierce) and literature (40). An amine-containing model peptide (20μM), RKRSRAE, was incubated with each compound (0–100μM) for 20min at 37°C in sodium bicarbonate buffer, pH 8.0 in a quartz 96-well plate. TNBSA (100μM, final concentration) in sodium bicarbonate buffer pH 8.0 was then added and absorbance readings were taken every minute for 2.5h at 335 and 420nm. The absorbance values were compared to those obtained from a standard curve produced using *N*-acetyl lysine (0–20μM) and TNBSA (0.0031% w/v), in order to determine the primary amine concentration in each sample.

Mass Spectrometry of Modified Peptide

A Cys-containing model peptide, rat ANP(1–11) (SLRRSSCFGGR, 50μM), from American Peptide, was incubated with either molinate, molinate sulfoxide, or molinate sulfone (500μM) for 1h at 37°C in 50mM sodium phosphate buffer, pH 7.4. These samples (2.5–5 ng/μL) were then analyzed by LC/MS using a Waters Acquity UPLC interfaced with a Q-TOF Premier equipped with an Acquity BEH C18 column (2.1 × 100mm). The mobile phase consisted of (A) 5% ACN in water with 0.1% formic acid and (B) 0.1% formic acid in ACN with a flow rate of 200μL/min. The gradient conditions used were 0% B at 1min to 45% B at 15min, and held for 10min. Mass spectrometric detection was conducted using positive ion mode, scanning 100–1500 *m/z*, and analysis was completed using MassLynx 4.0 software. MS/MS data was collected on the samples (2–3 ng/μL) by direct injection using a Harvard Apparatus syringe pump running at 10μL/min interfaced with a Waters Premier Q-TOF. The collision energy was set to 20V, scanning 100–1500 *m/z*. Analysis was conducted using MassLynx 4.0 and MaxEnt deconvolution software.

Protein Mass Spectrometry

Wild-type hALDH2 and the C302S mutant of hALDH2 were incubated with molinate and molinate sulfone at molar ratios of 1:2 for 13 hours in 10 mM Tris-HCl, pH 7.5, and then analyzed for covalent adducts by whole protein mass spectrometry. Samples were introduced into the mass spectrometer using an Agilent 1200 series capillary HPLC with a flow rate of 10 μ L/min consisting of 70% H₂O and 30% acetonitrile with 0.1% formic acid. An Agilent 6520 quadrupole-time of flight (Q-TOF) mass spectrometer, operating in positive ion ESI-TOF mode, was interfaced with the HPLC, and the data was processed and deconvoluted using Mass Hunter/Bioconfirm software suite.

Statistics

All statistics and linear regressions were calculated using GraphPad Prism version 5.0c (GraphPad Software, San Diego, CA). SEM for the comparison of ALDH activity in mouse striatal synaptosomes was calculated with the standard rules of error propagation for quotients using the SE from linear regression calculations. All other values are reported as the mean $\pm$ SEM ($n \geq 3$). One-way ANOVA with Newman-Keuls Multiple Comparison test was conducted to determine significance between all samples for the bimolecular rate constants and percent activity of ALDH (Tables 1 and 2). All values are significantly different from control and other treated samples, $p < 0.01$, unless otherwise noted.

Results

Kinetics of Aldehyde Dehydrogenase Inhibition

To compare the inhibitory potency of molinate and its metabolites towards ALDH, the activity of hALDH2 was monitored in the presence of these compounds. The specific activity of this recombinant protein was determined to be 0.375 μ mol/min/mg protein using propionaldehyde as the substrate. This value is comparable to the specific activity previously determined for this recombinant protein (41). Figure 1A is a representative primary plot, demonstrating the natural log transformation of percent activity of hALDH2 treated with molinate sulfone (1–25 μ M). This experiment was also conducted with molinate and molinate sulfoxide (data not shown). The slope (k') representing each concentration of molinate sulfone is graphed in Figure 1B. The linear slope of this secondary plot represents the bimolecular rate constant (k_i (μ M⁻¹s⁻¹)) of molinate sulfone. The bimolecular rate constants for all three compounds are shown in Table 1. Relative inhibitory potency was determined to be molinate sulfone > molinate > molinate sulfoxide. The inhibition of hALDH2 was found to be irreversible, because upon dilution, no return of activity was observed (data not shown).

Disruption of Dopamine Catabolism

ALDH activity was also monitored in a more complex model system, mouse striatal synaptosomes (containing multiple carbonyl metabolism enzymes). Synaptosomes were used as a model of dopamine metabolism because they retain most of the structural and functional characteristics of neurons, including active MAO (18,42,43) and multiple ALDHs (44,45). They have also proven to be a good model for looking at the effects of drugs on synaptic processes (46,47). The synaptosomes were pre-incubated with dopamine, to allow for sufficient levels of DOPAL to be produced, and then treated with either molinate, molinate sulfoxide, or molinate sulfone. To assess ALDH activity, the conversion of DOPAL to DOPAC was monitored at various time points using HPLC. A time-dependent increase in DOPAC concentration was observed for the control, as illustrated in Figure 2. However, treatment of the synaptosomes with molinate sulfoxide and molinate sulfone yielded significantly decreased DOPAC production, compared to controls, indicating

inhibition of ALDH activity in this model system. The percent activity, calculated by comparing the initial linear slopes of the time-dependent DOPAC formation to control, is shown in Table 2. Molinate sulfone was found to be the most potent inhibitor of aldehyde metabolism in the synaptosomes, followed by molinate sulfoxide, then molinate.

Amino Acid Reactivity

The reactivity of each compound with two protein nucleophiles, Cys and Lys, was determined in order to characterize possible mechanisms of protein modification. To determine Cys reactivity, various concentrations of molinate, molinate sulfoxide, or molinate sulfone were incubated with *N*-acetyl Cys and free thiol concentration was monitored using DTNB. The results are shown in Figure 3 and demonstrate that under these conditions, only molinate sulfone is reactive with *N*-acetyl Cys, indicated by a decrease in free thiol concentration. The amine-reactivity of these compounds was determined by incubating an amine-containing peptide (RKRSRAE) with each compound and monitoring the primary amine concentration using TNBSA. No reactivity towards this peptide was observed with any of the compounds (data not shown).

Mass Spectrometry of Modified Peptide

In order to further investigate thiol reactivity as well as Ser reactivity, a model peptide containing Cys and Ser was used. This peptide (ANP(1–11), SLRRSSCFGGR) was incubated with each compound and then analyzed using LC-MS. The HPLC chromatogram for the unmodified peptide (retention time (RT) 4.57min) is shown in Figure 4A. The chromatogram for ANP(1–11) incubated with molinate sulfone is shown in Figure 4D, indicating the presence of modified peptide (RT 7.37min) and a small amount of unmodified peptide (RT 5.12min). Chromatograms were also obtained for ANP(1–11) incubated with molinate and molinate sulfoxide, however, only a peak corresponding to the unmodified peptide was observed (data not shown). In the chromatogram for the unmodified peptide, peaks at 613.4 *m/z* (theoretical, 613.2 *m/z*) and 409.2 *m/z* (theoretical, 409.1 *m/z*), corresponding to the doubly and triply charged unmodified peptide, were observed, Figure 4B. Treatment of the peptide with molinate sulfone resulted in peaks at *m/z* 676.4, 451.3, and 338.7 (theoretical, 676.3, 451.2, and 338.6 *m/z*), representing the doubly, triply, and quadruply charged ANP(1–11) peptide with a 126 Da thiocarbamate adduct, respectively (Figure 4E). In this chromatogram there is also a peak at 126.1 *m/z*, corresponding to the charged loss of the azepine ring.

Fragmentation of the triply charged ion corresponding to the unmodified peptide (408.95 *m/z*) was obtained via MS/MS analysis (Figure 4C). The following major ions were found: *b*₂ (201.1), *y*₂ (232.1), *y*₃ (289.1), *b*₃ (357.2), *y*₄ (436.2), *b*₄ (513.3), *y*₅ (539.2), *b*₅ (600.3), *y*₆ (626.2), *b*₆ (687.4), *y*₇ (713.3), *b*₇ (790.3), *y*₈ (869.4), *b*₉ (994.4), and *b*₁₀ (1051.5). Fragmentation of the triply charged ion corresponding to the modified peptide (450.8 *m/z*) was also obtained via MS/MS analysis (Figure 4F), allowing determination of the target residue (Cys) for molinate sulfone adduction. The following major ions were found: charged loss of adduct (126.1), *y*₁ (175.1), *b*₂ (201.1), *y*₂ (232.1), *y*₃ (289.2), *b*₃ (357.2), *y*₄ (436.2), *b*₄ (513.3), *b*₅ (600.3), *y*₅ (664.3), *b*₆ (687.4), *y*₆ (751.3), *y*₇ (838.3), *y*₈ (994.4). The *y*₄, *y*₅, *y*₆, *y*₇, and *b*₆ ions, along with the charge loss (126.1) corresponding to the carbamate adduct, conclusively map the 126.1 Da, thiocarbamate adduct to the Cys residue of the ANP(1–11) peptide, Figure 5. No evidence of primary amine modification, i.e. amine terminus of the peptide, nor Ser modification was observed. Results further confirm the aforementioned findings conducted using *N*-acetyl Cys and the RKRSRAE peptide, indicating reactivity towards thiols but not primary amines by molinate sulfone.

Protein Mass Spectrometry

Based on the ability of molinate sulfone to inhibit ALDH and to modify Cys residues, the reactivity of molinate and molinate sulfone with hALDH2 was investigated using mass spectrometry. Results are outlined in Table 3. When hALDH2 was treated with molinate, no mass shift was observed, indicating no covalent modification occurred. However, when hALDH2 was incubated with molinate sulfone, a complete mass shift of 125 m/z was observed (m/z 54445 to m/z 54570), indicating the presence of a single thiocarbamate adduct on this protein. Such a finding supports the results obtained using the ANP(1–11) model peptide, demonstrating Cys reactivity with molinate sulfone, but not with molinate. In order to verify that molinate sulfone was reacting with catalytic Cys302 of hALDH2, a C302S mutant of hALDH2 was also treated with molinate and molinate sulfone. No reactivity was observed for either molinate or molinate sulfone with the C302S mutant, confirming covalent modification of Cys302 of hALDH2 by molinate sulfone.

Discussion

Exposure to molinate can lead to disruptions in motor activity and other signs of neurotoxicity (2,7). The neurotoxic effects observed may be a consequence of inhibition of ALDH in the brain, resulting in an accumulation of endogenous neurotoxins, such as DOPAL or 4HNE, oxidative stress, or mitochondrial dysfunction (15–21). The current study investigates the relative reactivity of molinate and both of its sulfoxidation metabolites, molinate sulfoxide and molinate sulfone, towards hALDH2, as well as establishes their protein reactivity profiles and identifies the mechanism of ALDH2 inhibition.

Previous work with molinate and its metabolites has indicated that in rat liver mitochondria and microsomes, molinate and molinate sulfoxide both inhibit ALDH (molinate sulfone was not evaluated) (12). In rat brain mitochondria the parent molinate was not found to inhibit ALDH, but neither sulfoxidation metabolite was investigated (22). There have also been a couple *in vivo* studies assessing ALDH activity. One study found liver ALDH inhibition in mice dosed ip with molinate (13), and the other found inhibition of ALDHs from the brain, liver, and testicle in rats dosed with molinate (10). These studies all indicate molinate or a metabolite is capable of inhibiting ALDHs, but the rates of this inhibition and the metabolite responsible for it are not clear. In the current study, molinate and molinate sulfone were both found to inhibit hALDH2, with molinate sulfone ($245 \text{ M}^{-1}\text{s}^{-1}$) being three times more potent than the parent molinate ($76 \text{ M}^{-1}\text{s}^{-1}$). Molinate sulfoxide was not found to be a potent hALDH2 inhibitor ($11.6 \text{ M}^{-1}\text{s}^{-1}$).

In both of the model systems used, hALDH2 and mouse striatal synaptosomes, molinate sulfone was determined to be the most potent ALDH inhibitor. Molinate sulfoxide was found to only inhibit ALDH activity in the mouse striatal synaptosomes, which contain multiple ALDHs (14,44,45,48) and also have oxygen sources that may be able to oxidize the sulfoxide to the more potent sulfone. However, when the sulfoxide was tested against human ALDH2, it was determined not to be a good inhibitor of the enzyme, indicating that in the synaptosome it may be targeting other isoforms of ALDH, or there are species differences affecting its potency (14,48). The parent pesticide, molinate, was found to inhibit hALDH2, but showed no potency towards inhibiting ALDH activity in the synaptosomes. It may be concluded that molinate is a potent inhibitor of ALDH2, particularly in humans, but is not capable of inhibiting the ALDHs found in the striatal region of the mouse brain, or is less capable than the sulfoxidation metabolites to access these enzymes (24).

Previous studies with molinate have also demonstrated an inconsistency regarding the relative reactivity of molinate vs molinate sulfoxide dependent upon the model system employed (3,23). Extensive work has been previously conducted investigating the inhibition

of esterases by molinate and its sulfoxidation metabolites. These studies involved the use of rat liver and testicular microsomes and illustrated molinate sulfoxide was the most potent esterase inhibitor, followed by molinate sulfone, then by molinate (3). Another recent report using Leydig cells isolated from rats, found molinate sulfone was the most potent esterase inhibitor followed by molinate sulfoxide and molinate (23). Even though both studies addressed rat testicular carboxylesterase activity, differences in the relative esterase inhibition potency were observed.

The reactivity profile of each of the molinate metabolites with protein nucleophiles (i.e. Cys and Lys) was also established. It was determined that molinate and molinate sulfoxide were not reactive towards *N*-acetyl Cys, ANP(1–11) (containing Cys and Ser residues), or a Lys containing peptide (RKRSRAE). Molinate sulfone was found to modify *N*-acetyl Cys and the Cys residue in the ANP(1–11) peptide, resulting in a mass shift of 126 *m/z*. This confirms the proposed mechanism of action in Scheme 2, involving a nucleophilic substitution reaction between Cys and the carbonyl of the thiocarbamate moiety of molinate sulfone. The reactivity of molinate sulfone towards thiols has been utilized in previous studies to form a standard for selected reaction monitoring to compare the total number of carbamate adducts on Cys residues between microsome and mitochondrial fractions from various organs (10,49). From these studies it was concluded that molinate or one of its metabolites was capable of modifying proteins, such as hemoglobin, although specific sites of adduction were not identified, nor were other target proteins. The authors noted that the degree of Cys modification did not necessarily correlate with ALDH inhibition (10), but specific modifications on ALDH enzymes were not assessed.

Molinate and molinate sulfoxide were not observed to react with Cys, Lys, or Ser residues in either peptide used. It is important to note that *in vivo* molinate or a metabolite has been reported to be able to inhibit carboxylesterases via carbamylation of the active-site Ser, confirmed via MALDI-TOF (3,23). The Ser residues in the active-site of carboxylesterases are highly polarized and activated, rendering them much more reactive than the Ser residues found in other proteins and in ANP(1–11) (50,51).

The last part of this study investigated the covalent modification of hALDH2 by molinate sulfone. Literature reports have indicated that exposure to molinate can cause inhibition of ALDH activity (10,12,13), but the mechanism of this inhibition, including identifying which metabolite is responsible and the site of covalent modification, has not been previously determined. Using mass spectrometry and site-directed mutagenesis, molinate sulfone was found to be highly reactive towards the active-site Cys302 of hALDH2, resulting in a single thiocarbamate adduct. While it was determined that Cys302 is the primary target of molinate sulfone, other residues (e.g., Cys) are predicted to be modified by the inhibitor at higher concentrations. It was found that treatment of the Ser302 mutant of hALDH2 with a ten-fold excess of molinate sulfone yielded partial adduction of the enzyme, i.e. peaks at *m/z* 54445 and *m/z* 54570 were observed.

In the present study, molinate sulfone was the only compound observed to inhibit hALDH2 activity and to covalently modify this enzyme. Based upon these results it is concluded that this metabolite is most likely responsible for the inhibition of ALDH, and not molinate or molinate sulfoxide. The production of molinate sulfone is very difficult to monitor *in vivo*, and has not been detected in blood from animals dosed with molinate (12,52,53). This is most likely due to its high reactivity, but many previous studies have ruled out the role of this metabolite in enzyme inhibition. The results of the current study indicate this metabolite is the most likely contributor to ALDH inhibition observed *in vivo*. Based on its bimolecular rate constant, only a small amount of molinate sulfone needs to be produced in order for enzyme inhibition to occur.

In summary, molinate sulfone was found to be the most potent inhibitor of ALDH activity, using hALDH2 and mouse striatal synaptosomes. This indicates the sulfoxidation of molinate and then of molinate sulfoxide leads to increased inhibitory potency of this pesticide. The reactivity profile of all three compounds was established against various protein nucleophiles, i.e. Cys and Lys. The only reaction that was observed under the conditions used (pH 7.4 and 37°C) was between Cys and molinate sulfone. It was determined that this reaction involved nucleophilic attack of the thiol to the carbonyl of molinate sulfone, resulting in the formation of a 126 Da carbamate adduct, based upon results from LC/MS and MS/MS. A single 125 Da adduct was also found on Cys302 of hALDH2 upon treatment with molinate sulfone, using mass spectrometry and site-directed mutagenesis. From these results, it may be inferred that the sulfone metabolite of molinate is capable of covalently modifying the active-site Cys residue of ALDH2, resulting in the inhibition of this critical enzyme.

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Figure 1.

Inhibition kinetics of hALDH2 by molinate sulfone. (A) Primary plot, representing the natural log transformation of percent activity calculated from treatment of hALDH2 with molinate sulfone (1–25 μM). The slope of each curve gives $k' \text{ (s}^{-1}\text{)}$. (B) Secondary plot, representing the linear correlation between $k' \text{ (s}^{-1}\text{)}$ and $[\text{molinate sulfone}]$, with the slope of this curve equal to $k_i \text{ (}\mu\text{M}^{-1} \text{ s}^{-1}\text{)}$.

Figure 2.

Inhibition of aldehyde metabolism (i.e. DOPAL), represented by a decreased production of DOPAC, in mouse striatal synaptosomes (0.5mg/mL protein) pre-incubated with 0.1mM dopamine and treated with 50 μM molinate ($\square$), molinate sulfoxide (Δ), molinate sulfone ($\circ$), or control ($\diamond$). The values shown represent the mean $\pm\text{SEM}$ ($n = 3$, except for controls where $n = 6$), where peak area was converted to concentration using a standard curve.

Figure 3. Reactivity of molinate (□), molinate sulfoxide (Δ), and molinate sulfone (○), (0–100 μM) with *N*-acetyl Cys (50 μM), monitored using DTNB (500 μM) to quantify free thiol concentration. The values shown represent the mean ± SEM ($n = 3$).

Figure 4.

LC/MS and MS/MS analysis of the model peptide, ANP(1–11) (MH^+ at m/z 1225.4, SLRSSCFGGR) (50 μ M), incubated with molinate sulfone (0 μ M (panels A–C) or 500 μ M (panels D–F)). (A) ANP(1–11) peptide HPLC chromatogram, RT 4.57min. (B) Mass spectrum at 4.57min with peaks 613.4 and 409.2 m/z corresponding to the doubly and triply charged, unmodified peptide. (C) Deconvoluted MS/MS of unmodified peptide (408.95 m/z). (D) HPLC chromatogram of ANP(1–11) peptide modified by molinate sulfone, RT 7.37min. (E) Mass spectrum at 7.37min with peaks at 676.4, 451.3, and 338.7 m/z corresponding to the doubly, triply, and quadruply charged peptide modified by molinate sulfone. (F) Deconvoluted MS/MS of modified peptide (450.8 m/z). *, charged loss of adduct (126.1 m/z).

Figure 5.

Sequence of the thiocarbamate-modified ANP(1-11) peptide with y and b fragment ions identified from MS/MS analysis. C*, modified Cys residue.

Scheme 1.
Metabolism of Molinate

Scheme 2.

Reactivity of Molinate Sulfone with Cys Containing Peptide, ANP(1–11)

Table 1

Bimolecular rate constants, k_i ($M^{-1}s^{-1}$), for the inhibition of hALDH2 by molinate, molinate sulfoxide, and molinate sulfone.

Compound	k_i ($M^{-1}s^{-1}$)
Molinate	76.0 ± 6.94
Molinate Sulfoxide	11.6 ± 15.3^a
Molinate Sulfone	245 ± 7.38

^aSlope not significantly different from zero

Table 2

Percent activity of ALDH in striatal synaptosomes following treatment with 50 μ M compound, determined through comparison of the initial linear slopes (0–60min) of time-dependent DOPAC formation to control.

Compound	% Activity
Molinate	117 $\pm$ 3.67
Molinate Sulfoxide	62.6 $\pm$ 2.87
Molinate Sulfone	27.6 $\pm$ 2.98

Table 3

Deconvoluted whole protein mass spectrometric analysis of wild-type hALDH2 or C302S hALDH2 treated with molinate or molinate sulfone.

	Wild-type hALDH2		C302S hALDH2	
	MH ⁺ (<i>m/z</i>)	$\Delta m/z^a$	MH ⁺ (<i>m/z</i>)	$\Delta m/z^a$
Control	54445	0	54429	0
Molinate	54445	0 ^b	54429	0 ^b
Molinate Sulfone	54570	125.00	54429	0 ^b

^a Denotes complete change in mass from control

^b No shift in mass from control observed