Biochemical Toxicology of Fluorosulfate, an Intermediary Metabolite of the Fumigant Sulfuryl Fluoride

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ABSTRACT

Sulfuryl fluoride is a fumigant used for extermination of wood-borne insects. Current hypotheses involving sulfuryl fluoride's toxicity implicate hydrolysis of fluoride ions, which are innately toxic to living organisms. However, in this thesis I hypothesize that novel secondary biochemical pathways may contribute to sulfuryl fluoride's toxicity. To investigate this, I performed enzymatic inhibition assays and a thin layer chromatography screen using fluorosulfate – a relevant metabolic intermediate - to identify novel reactivity involved with conjugation to endogenous molecules or derivatization of essential enzymes. I found that both fluorosulfate and hydrolyzed fluoride ion inhibited multiple enzymes (acetylcholinesterase, butyrylcholinesterase and sulfatase) but only fluorosulfate inhibited glutathione S-transferase (GST). To identify intermediate steps by which this occurred, I performed nuclear magnetic resonance (NMR) spectroscopy on incubated fluorosulfate and glutathione mixtures and compared it to glutathione and synthesized glutathione S-sulfonate (a known inhibitor), with inconclusive results. Nonetheless, the inhibition of glutathione S-transferase by fluorosulfate remains relevant for understanding potential secondary toxicity of the fumigant sulfuryl fluoride and for future evaluations of its safety.

KEYWORDS

Glutathione S-transferase (GST), glutathione (GSH), cholinesterase, nuclear magnetic resonance (NMR)

INTRODUCTION

Sulfuryl fluoride (SO_2F_2) is a fumigant with a long history of use for the extermination of wood-borne insects in both residential and agricultural applications. Developed in 1957 (Kollman 2006), sulfuryl fluoride's importance increased in the 1990s as it replaced the fumigant ozone depletor methyl bromide (EPA 2009). Like most fumigant pesticides, sulfuryl fluoride is non-selective and broadly toxic – meaning it is lethal to any exposed organisms. Fatal human exposures to the fumigant have been reported despite heavy regulation (New York Times 1988). However, with respect to risk, concern for sulfuryl fluoride has primarily focused on low level exposure to fumigant applicators and residues left in homes post-fumigation. Although a phase out for sulfuryl fluoride has been proposed (EPA 2011), understanding the toxicity will remain relevant to assessing its safety and possibility of continued use.

Multiple studies have investigated the toxicity of sulfuryl fluoride, but none have definitively determined its mechanism. The most commonly hypothesized mechanism of action occurs through the hydrolysis to fluoride ions - which inhibit enzymes involved in metabolism (Meikle et al 1963) and cause kidney damage (Eisenbrandt and Nitschke 1989). However, rats exposed to an acutely toxic dose of sulfuryl fluoride were rescued by both fluoride antidotes (calcium gluconate) and anticonvulsants which otherwise should not have mitigated fluoride toxicity (e.g., diazepam) (Nitschke et al 1986). Additionally, some symptoms in rats and rabbits exposed to subchronic doses (300 and 600 ppm) were not fluoride specific; including neurotoxicity and respiratory inflammation (Eisenbrandt and Nitscke 1989). Lastly, human exposures in fumigant applicators have been reported to have minor but significant changes in cognitive and olfactory functions (Calvert et al 1998). Together, these findings suggest a fluoride-independent mechanism through which sulfuryl fluoride can be either acutely or subchronically toxic.

The conjugation of fluorinated sulfate compounds to specific proteins, like those seen in fluorinated phosphates, is an alternative explanation of sulfuryl fluoride's pathology. Fluorinated phosphates are a class of compounds with a comparable structure to sulfuryl fluoride which have been shown to derivatize key proteins making them

potent inhibitors and extremely toxic. For example, diisopropyl fluorophosphate binds to the serine residue on acetylcholinesterase as well as a tyrosine residue on human serum albumin (Means and Wu 1979), making it a potent inhibitor of both (Lanks and Seleznick 1981). Sarin, a chemical weapon, is another fluorinated phosphate which binds with a serine residue of acetylcholinesterase, making it a deadly poison (Abu-Qare and Abou-Donia 2002). Given the high potential for protein derivatization and the unexplained symptoms of toxicity, experiments targeting the reactivity of sulfuryl fluoride may provide an alternative explanation for toxicity.

The primary objective of this study is to explore uncharacterized reactivity of sulfuryl fluoride. Because sulfuryl fluoride is a volatile poison, potassium fluorosulfate ($KFSO_3$) will serve as an intermediary metabolite to study interactions and reactions with proteins of interest. Using fluorosulfate, the goal of this study is to discover (a) derivatized forms of key metabolic enzymes and (b) conjugation to key endogenous compounds (Figure 1). Given the tendency for sulfuryl fluoride to react with reactive electron-rich amino groups (Meikle 1964), I hypothesized that other electron-rich residues including tyrosine and serine will be likely sites of conjugation. This study allows me to postulate the role of single protein interactions in whole organism toxicity, further elucidating the specific mechanisms of toxicity for this fumigant.

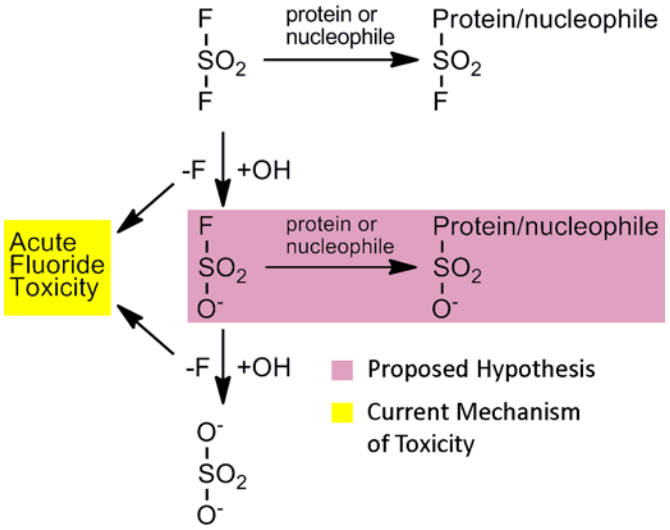

Figure 1. Schematic representation of proposed hypothesis versus current understanding of sulfuryl fluoride toxicity. Conjugation of proteins or nucleophilic compounds by fluorosulfate is represented in purple. Acute fluoride toxicity is represented in yellow.

METHODS

I employed a variety of methods to recognize and understand the reactivity of fluorosulfate. First, I explored inhibition of key mammalian enzymes using colorimetric assays. Second, I used thin layer chromatography to investigate conjugation with common biological compounds. Lastly, I characterized reactions with enzymes using nuclear magnetic resonance spectroscopy (NMR).

Chemical Reagents and General Methods

My primary compound of interest was a potassium salt form of fluorosulfate ($KFSO_3$) (Sigma-Aldrich) due to structural similarity to and role in intermediate hydrolysis of sulfuryl fluoride. I also investigated sodium fluoride (NaF) (Sigma-Aldrich) in parallel for comparative reactivity. Enzymes (acetylcholinesterase, butyrylcholinesterase, trypsin, chymotrypsin, albumin, sulfatase and glutathione *S*-transferase) were acquired from Sigma Aldrich. Peak absorbances were quantified using a Versamax (Molecular Devices, Sunnyvale, CA) plate reader. Proton NMR spectra were obtained using either a Bruker AVB-400 spectrometer with a Z-gradient 5 mm QN probe or a Bruker AVQ-400 spectrometer with a 5 mm Z-gradient broad band probe.

Enzyme Inhibition Assays

To measure the effect of FSO_3^- on acetylcholinesterase (AChE) activity, I incubated approximately 0.005 units of AChE (sonicated with 2.5% Triton X-100) in a solution containing either $KFSO_3$ or NaF and dithionitrobenzoic acid (DTNB) in 100 mM phosphate buffer (pH 7.4). After 30 minutes, I introduced acetylthiocholine (ATCh) to the solution and read absorbance for 5 minutes at 412 nm. Final concentrations were 0.3 mM DTNB, 0.3-30 mM $KFSO_3$ or NaF and 4 mM ATCh.

To measure the effect of FSO_3^- on butyrylcholinesterase (BuChE) activity, I incubated approximately 0.005 units of BuChE in a solution containing either $KFSO_3$ or NaF in addition to DTNB in 100 mM phosphate buffer (pH 7.4). After 30 minutes, I

added butyrylthiocholine (BuTCh) and read absorbance for 5 minutes at 412 nm. Final concentrations were 0.3 mM DTNB, 0.3-30 mM $KFSO_3$ or NaF and 2 mM BuTCh.

Albumin (Alb), trypsin and chymotrypsin (Xtr) activity was determined while in the presence of FSO_3^- by incubating 70 μM, 210 μM and 24 units/mL respectively, in a solution containing either $KFSO_3$ or NaF in 10 mM phosphate buffer (pH 7.4). After 30 minutes, 4-nitrophenyl acetate (*p*-NPA) was added and after another 15 minutes absorbance was read at 412 nm. Final concentrations were 0.3-30 mM $KFSO_3$ or NaF and 1.1 mM *p*-NPA.

Sulfatase activity was determined while in the presence of FSO_3^- by incubating 0.0125 units/mL respectively, in a solution containing either $KFSO_3$ or NaF in 100 mM TRIS buffer. After 30 minutes, 4-nitrophenyl sulfate (*p*-NPS) was added. 15 minutes after the addition of *p*-NPS absorbance was read at 412 nm. Final concentrations were 0.3-30 mM $KFSO_3$ or NaF and 4 mM *p*-NPS.

To measure changes in activity of glutathione *S*-transferase (GST) after addition of FSO_3^-, I incubated 0.08-0.155 units of GST in a solution containing either $KFSO_3$ or NaF and glutathione (GSH) in 100 mM phosphate buffer with 0.01% EDTA (pH 6.5). After 30 minutes, 1-chloro-2,4-dinitrobenzene (CDNB) in 95% ethanol was added to the solution. Absorbance was measured at 340 nm 15 minutes after the addition of CDNB. Final concentrations were 2.5 mM GSH, 0.3-30 mM $KFSO_3$ or NaF and 1 mM CDNB.

Thin Layer Chromatography

To investigate potential reactions with FSO_3^- and a variety of amino acids or their derivatives, I mixed solutions containing 30 mM of a compound with 30 mM of either $KFSO_3$, NaF or water. After at least 24 hours, I spotted each solution onto a silica plate and ran with a solution containing 2 mL methanol with a drop of water. Spots were visualized with potassium permanganate and mild heating.

Chemical Synthesis of Glutathione *S*-Sulfonate Standard

I prepared a glutathione *S*-sulfonate ($GSSO_3$) standard using a modification of the

method described in Robinson and Pasternak (1964). I mixed 0.06 mM oxidized glutathione (GSSG) with 1 M sodium sulfite and 0.01 M cupric sulfate overnight in a shaking bath at 37 °C and filtered the product on Whatman 1 filter paper to remove precipitate. The structure was confirmed using ^{1}H NMR with D_2O solvent.

Analysis of Potential Glutathione Fluorosulfate Conjugate

A solution of 100 mM GSH and 100 mM $KFSO_3$ was incubated in water overnight at 37 °C and the ^{1}H spectra was recorded with D_2O as the solvent.

Analysis

Colorimetric Enzyme Assays.

I normalized the absorbance for treatment groups (NaF, fluorophosphate and fluorosulfate) to the positive and negative controls to determine percent activity of enzyme. Then, I used the dose-response curve to determine the concentration of inhibitor where enzyme activity was 50% of normal conditions (IC_{50}). Dose-response curves and their IC_{50}'s were generated using Sigma Plot's (SPSS Science) Four Parameter Logistic Curve algorithm (see equation below).

$$y = m_{} + \frac{i_{} n_{} m_{} - m_{} a_{} x_{} i_{}}{1 + 1_{} 0_{}} \qquad \text{(Equation 1)}$$

Nuclear Magnetic Resonance

Spectra generated for GSH, GSH and FSO_3^- and glutathione s-sulfonate ($GSSO_3$) were analyzed using MestraNova (Mestralab Research).

RESULTS

Colorimetric Enzyme Assays

Butyrylcholinesterase.

Using BuChE-catalyzed hydrolysis of BuTCh with the color reagent DTNB, I found that $KFSO_3$ inhibited butyrylcholinesterase with an IC_{50} of 1.04 ± 0.10 mM and NaF inhibited at an IC_{50} of 0.27 ± 0.04 mM (Fig. 2) (Table 1).

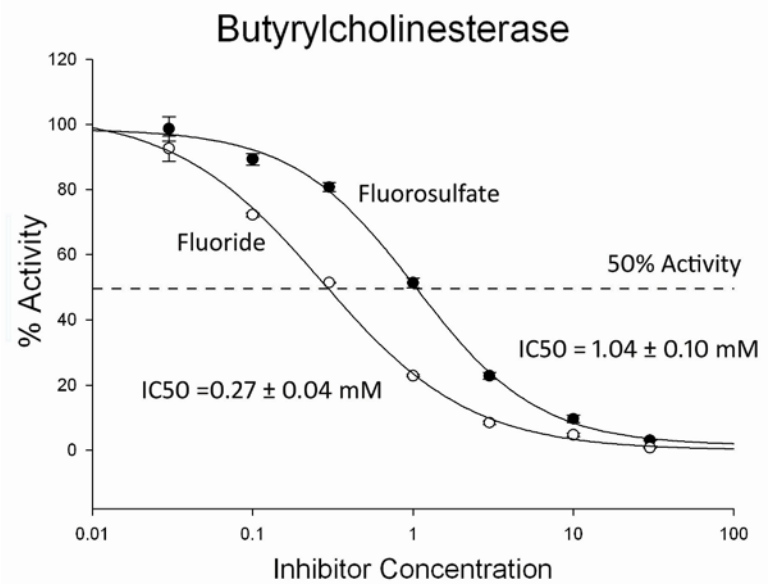

Figure 2. Inhibition curve of butyrylcholinesterase representing derivation of IC_{50} value. Percent activity (100% is normal activity) is plotted against inhibitor concentration (in mM).

Acetylcholinesterase.

Using AChE-catalyzed hydrolysis of ATCh with the color reagent DTNB, I found that $KFSO_3$ inhibits AChE with an IC_{50} of 1.67 ± 0.19 mM and NaF inhibits at a lower IC_{50} of 0.58 ± 0.05 mM (Table 1).

Chymotrypsin, Albumin and Trypsin.

Using chymotrypsin-catalyzed hydrolysis of *p*-NPA, I found that neither $KFSO_3$ nor NaF inhibited chymotrypsin, albumin or trypsin, even at concentrations as high as 30 mM.

Sulfatase.

Using sulfatase-catalyzed hydrolysis of *p*-NPS, I found that sulfatase was inhibited by both $KFSO_3$ (IC_{50} = 6.04 ± 1.54 mM) and NaF (IC_{50} = 1.58 ± 1.22 mM) (Table 1).

Glutathione S-Transferase.

Using a GST-catalyzed conjugation of GSH to CDNB, I found that $KFSO_3$ did inhibit GST (IC_{50} of 11.23 ± 1.92 mM) but NaF did not inhibit (Table 1).

Table 1. Enzymatic Inhibition. Doses of either $KFSO_3$ or NaF where application to the given enzyme provides 50% inhibition.

	IC_{50} (mM)	
Enzyme	**Fluorosulfate (FSO_3^-)**	**Fluoride (F^-)**
Acetylcholinesterase (AChE)	1.67 ± 0.19	0.58 ± 0.05
Butyrylcholinesterase (BuChE)	1.04 ± 0.10	0.27 ± 0.04
Trypsin	>30*	>30*
Chymotrypsin	>30*	>30*
Albumin	>30*	>30*
Sulfatase	6.04 ± 1.54	1.58 ± 1.22
Glutathione *S*-Transferase (GST)	11.23 ± 1.92	>30*

* >30 denotes no inhibition at concentrations tested

Glutathione Conjugate

NMR Characterization.

Incubated solutions containing $KFSO_3$ and GSH resulted in peaks at 2.12, 2.51, 2.91, 3.80, 4.52, 8.44 and 8.49 which were common to the GSH (Fig. 3). An additional peak was located at 3.93 which remains unaccounted for.

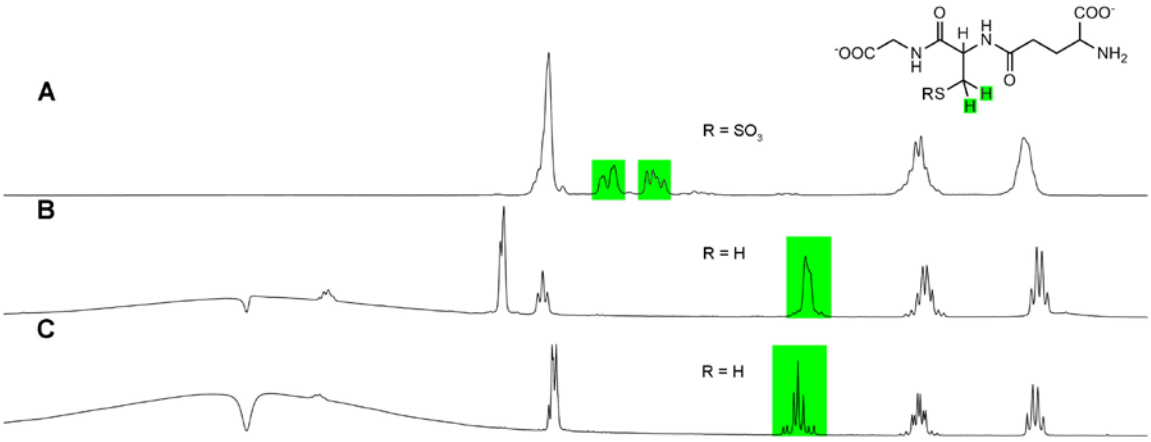

Figure 3. NMR Spectra of glutathione, glutathione and fluorosulfate and glutathione s-sulfonate. A is a synthesized glutathione S-sulfonate standard; B is a mixture containing glutathione and fluorosulfate and C is a mixture containing only glutathione. Relevant hydrogens are labeled in green.

Thin Layer Chromatography

I observed no migration change after NaF or $KFSO_3$ incubation (Table 2).

Table 2. Thin Layer Chromatography Migration. Migration values (R_f) of various peptides spotted on a TLC plate. Columns to the right denote whether incubation with $KFSO_3$ or NaF changed migration value.

Compound	R_f	$KFSO_3$	NaF
Proline	0.13-0.31	-	-
Hydroxy-Proline	0.05-0.31	-	-
Cysteine	0-0.20	-	-
Methionine	0.25-0.56	-	-
Tyrosine	0	-	-
Tryptophan	0.41-0.63	-	-
Histidine	0-0.13	-	-
Glutatmate	0.30-0.65	-	-
Glutamine	0.01-0.23	-	-

Asparagine	0.01-0.19	-	-
Cystine	0	-	-
Lysine	0	-	-
Threonine	0.11-0.33	-	-
Alanine	0.24-0.43	-	-
Dopamine	0-0.43	-	-
DOPA	0.14-0.57	-	-

DISCUSSION

The purpose of my study was to investigate potential biochemical targets for FSO_3^-, an intermediate of the fumigant SO_2F_2, using a series of enzyme specific assays and a non-specific chemical assay. Although many mechanisms of SO_2F_2 toxicity have been previously suggested (Meikle 1963, Nitschke et al 1986, Eisenbrandt and Nitschke 1989), they have all involved toxicity caused by hydrolysis to F^-. Additionally, these studies have only elucidated pathways involving acute or sub-chronic doses of the fumigant and the long term potential effects are poorly understood. In this project, I demonstrated multiple enzymes not previous identified as inhibited by the SO_2F_2 hydrolyzed intermediate, FSO_3^-. GST was the only enzyme inhibited by FSO_3^- but not F^-. Additionally, I found that FSO_3^- does not react with any of the relevant small molecules tested.

Enzymatic Targets

Of the enzymes I investigated using colorimetric assays, three were inhibited by both $KFSO_3$ and NaF: AChE, BuChE and sulfatase. The first, AChE, is an esterase responsible for termination of neurochemical signals by hydrolyzing ACh. Given the essential physiological role of neuronal signals, compounds which inhibit this target (such as organophosphate pesticides and chemical warfare agents) can be particularly potent poisons (Casida and Quistad 2004). The second, BuChE, is an esterase that has no known distinct physiological function but has been demonstrated to compensate for

deficient cholinesterase activity (Casida and Quistad 2004). For that reason, specific BuChE inhibitors, such as ethephon, increase the potency of other cholinesterase inhibitors (Haux et al 2002). Lastly, sulfatase from *Aerobacter aerogenes* is homologous to sulfatase enzymes responsible for sulfate metabolism (Rammler et al 1964).

Inhibition by F^- has been previously reported in both AChE (Krupka 1966) and BuChE (Page et al 1985), although this study is the first where inhibition has been demonstrated in either by $KFSO_3$. The inhibition by either F^- or FSO_3^- has not been previously demonstrated for sulfatase. Given that the inhibition by NaF in these enzymes were at a much lower concentration than $KFSO_3$, FSO_3^- inhibition likely occurs by hydrolysis to free F^-. Whether the F^- hydrolysis was mediated by any of these enzymes was not examined.

In addition to those enzymes inhibited by both F^- and FSO_3^-, three enzymes were not inhibited by either compound: trypsin, chymotrypsin and albumin. Trypsin and chymotrypsin are proteases in the intestine which are responsible for digestion (Vercruysse 2005). Albumin is an essential protein as a storage site for a variety of compounds, particularly for non-endogenous compounds in the process of removal and excretion. Compounds expected to competitively bind to albumin would increase the bioavailability of toxic compounds and therefore make normally unharmed individuals susceptible to toxicity (Kragh-Hansen et al 2002). However, I found none of the enzymes to be affected by either F^- or FSO_3^-.

GSTs are part of phase II metabolism of xenobiotics, a process which removes toxic compounds from the human body. To make compounds more hydrophilic, it conjugates GSH to electrophilic compounds and improves their excretability (Hayes et al 2005). Here, I have demonstrated that FSO_3^- but not F^- inhibits GST at fairly high concentrations.

Glutathione Conjugate

Previous studies on an analogous compound, sulfite, have demonstrated comparable inhibition by a mechanism involving conjugation to GSH, forming the active inhibitor $GSSO_3$ (Leung 1985). To determine whether FSO_3^- would form a similar

conjugate, I compared FSO_3^- incubated with GSH overnight to a $GSSO_3$ standard with NMR spectroscopy. The peaks predicted for the FSO_3^- and GSH product were surprisingly absent.

If the conjugation of FSO_3^- to GSH did occur in a similar manner to the conjugation of sulfite to GSH, there would be a possible link to cancer with chronic exposure to SO_2F_2. Sulfite has been previously shown to be involved with increased benzo[a]pyrene adducts to DNA, leading to increased mutations (Leung et al 1989, Green et al 1994); however it is still unclear whether this is $GSSO_3$ mediated. Exposure to sulfite increases the levels of $GSSO_3$ (Keller and Menzel 1989) which then inhibits GST (Leung et al 1985, Sun and Morgenstern 1994). However, Green et al (1994) argues that conjugation by GST is not a significant pathway for benzo[a]pyrene detoxification, but instead sulfite modifies benzo[a]pyrene to a species that is more capable of binding DNA. If it were true that intermediate $GSSO_3$ inhibits GST, increased cellular damage caused by reactive epoxide metabolites of benzo[a]pyrene could explain the inflamed respiratory system demonstrated in subchronic exposure studies for sulfuryl fluoride (Eisenbrandt and Nitschke 1989).

Amino Acids and Related Compounds

In addition to enzymatic studies, I investigated multiple amino acids and their related compounds for their ability to interact with $KFSO_3$ using a general screen with thin layer chromatography (TLC). However, using this technique I could not detect any changes in these compounds. Out of those tested, it is not surprising that the functional groups of non-reactive hydrophobic amino acids such as valine and isoleucine were not altered by incubation with FSO_3^-. However, it is interesting that other, more reactive amino acids such as serine, tyrosine or cysteine did not form any conjugated products. These amino acids are at the catalytic sites of many of the enzymes tested in this study: serine is at the active site of AChE, BuChE, trypsin and chymotrypsin (Casida and Quistad 2005); tyrosine is at the active site of albumin (Kragh-Hansen et al 2002); and cysteine is at the active site of sulfatase (Bond et al 1997). The lack of conjugation by the amino acid provides further evidence that these enzymes are not inhibited by a derived

active site residue.

Limitations

An important limitation of this study has been the inability to test the compound SO_2F_2 due to toxicity and limited access. Although I used a product of its hydrolysis, FSO_3^- is not completely comparable to reactivity of the fumigant itself. Most likely, the extra fluoride on the sulfur core would confer increased reactivity, heightening its toxicity. However, without the pure compound, there remains considerable uncertainty.

Additionally, using TLC to investigate reactivity of amino acids and related compounds was limited in utility. Originally, I planned to screen for structural changes by measuring an absorbance spectrum in the ultraviolet range for a number of amino acids. Had the compounds reacted, their signature spectrum would have changed. However, I discovered that standard 96-well plates were either made of polypropylene or polysterene and thus unsuitable for this assay. The issue could be resolved using a glass cuvette, however this would require more reagent and is time-consuming. Using TLC instead of UV spectrophotometry has advantages and disadvantages. TLC is fairly low resolution and requires more experimental trial and error with varying solvents and spotting reagents. Most importantly, TLC is not accurately quantitative and requires other methods to further validate its findings. However, TLC is cheap, quick, easy and requires fairly little reagent to perform. Additionally, given it does not rely on UV absorption, it is not limited to conjugated compounds like UV spectrophotometry.

Future Directions

Given the findings presented here, the next steps in characterizing the mechanisms of sulfuryl fluoride toxicity would be to further investigate the role of FSO_3^- on GST. With additional NMR or even LC/MS experiments, it is reasonable that the GSH conjugate which inhibits GST could be identified. Additional experiments could also be performed based on those designed for sulfite (Leung et al 1989, Green et al 1994) to determine if exposure may cause DNA damage within a cell model. Lastly, the same

assays performed with SO_2F_2 would measure the extent of inhibition of SO_2F_2, not just the intermediate.

Conclusions

In this study, I have demonstrated that $KFSO_3$ can inhibit a number of enzymes relevant to mammalian toxicity. Of these, GST is the most interesting because of structural similarity to sulfite, a known carcinogen. Further investigation may implicate the inhibition of this detoxifying enzyme as a mechanism of cancer initiation. However, this hypothesis remains to be tested. If true, it would provide better occupational risk assessment and cancer intervention for fumigant applicators.

In the next several years, the relevancy of SO_2F_2's toxicity will diminish as it becomes phased out of an American market (EPA 2011). However, use may continue across the world and therefore better understanding its chronic toxicity will be important to evaluate its use.

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