CHARACTERIZATION OF LUNG INFLAMMATION INDUCED BY EXPOSURE TO
FIPRONIL

A Thesis Submitted to the College of Graduate Studies and Research in Partial
Fulfilment of the Requirements for the Degree of Master of Science in the
Department of Veterinary Biomedical Sciences

University of Saskatchewan
Saskatoon

By
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ABSTRACT

Fipronil is an insecticide that acts at the gamma-aminobutyric acid receptor and glutamate-gated chloride channels in the central nervous systems of target organisms. The use of fipronil is increasing across the globe. Presently, very little data exist on the potential impact of exposure to fipronil on the lungs. We studied the same by exposing mice to fipronil intranasally (N=8) or orally (N=7) for 7 days followed by collection of blood, broncho-alveolar lavage (BAL) fluid and lung tissues. Control mice were given corn oil (N=15). The oral and intranasal exposure routes were chosen because these are the most common routes of exposure for humans and animals. Hematoxylin-eosin stained lung sections showed normal histology in the control lungs compared to the thickened alveolar septa, disruption of the airways epithelium and damage to vascular endothelium in the intranasal and the oral groups. Lung sections stained for von Willebrand factor showed that mice exposed to fipronil either orally or intranasally had increased staining in the endothelium and septal capillaries. Compared to the control mice, TLR4 expression in lungs from animals treated orally with fipronil was reduced while animals exposed intranasally had increased TLR4 staining in the airway epithelium. Similarly, TLR9 stained lungs showed that orally treated animals had reduced TLR9 reaction in the airway epithelial cells but intranasally exposed animals had intense TLR9 staining in the alveolar septa and airway epithelium. The slides were also scored blindly to gain a quantitative understanding of the staining; there were a significantly higher number of TLR4 positive stained cells in the intranasal fipronil
group (P=0.010) but no significant differences between treatments for TLR9 positive stained cells (P=0.226).

The U937 cell line was employed to compliment the in vivo work. Cells were exposed to fipronil in DMSO at concentrations of 0.29 μm to 5.72 μm per 1 ml for various times from 3, 9 and 24 hours. Viability was assessed and western blots on Toll-like receptors 4 and 9 were completed in addition to immunofluorescence. Cell death was determined with trypan blue method. A significant increase in cell death was observed when the cell line was exposed to higher concentrations of fipronil (P<0.0001). Western blots on TLR4 and 9 revealed no significant differences (TLR4 {P=0.49}, TLR9 {P = 0.94}) between cells exposed to fipronil and those exposed to the control (DMSO). The data taken together show that fipronil causes cell death in vitro, and induces lung inflammation following oral or intranasal exposure but has different effects on the expression of TLR4 and TLR9 in vivo. Because of the central roles of TLR4 and TLR9 in lung inflammation, fipronil-induced changes in the expression of these receptors would alter the pulmonary response to bacterial infections in the host exposed to fipronil. Further studies are needed to examine the mechanisms through which fipronil regulates expression of immune receptors and also the pulmonary response of fipronil-exposed animals to subsequent microbial infections.
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<th>Description</th>
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<tbody>
<tr>
<td>BALT</td>
<td>Bronchial associated lymphoid tissues</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>H&amp;E Staining</td>
<td>Hematoxylin and eosin staining</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INL</td>
<td>Intranasal</td>
</tr>
<tr>
<td>LD50</td>
<td>Lethal dose, 50%</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen activated proteins</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with Tween 20</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>SMD</td>
<td>Sodium methyldithiocarbamate</td>
</tr>
<tr>
<td>TFN-α</td>
<td>Tumor-necrosis-factor-α</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
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</table>
Chapter 1: Review of Literature

1.1 Overview of lung inflammation

1.1.1 Inflammation

Five recognizable signs traditionally describe acute inflammation: Redness, heat, swelling, pain, and loss of function (Rankin, 2004). It is generally accepted that two types of inflammation, acute and chronic, exist and are distinguished by their duration and the type of cells present (Rankin, 2004). Chronic inflammation generally persists for longer than a few weeks and is characterized by an increase in lymphocytes and monocytes while in acute inflammation a greater number of neutrophils and macrophages are recruited (Rankin, 2004).

1.1.2 Components of the Innate Immune System

The activation of the innate immune system is linked with PAMPs (Pathogen associated molecular patterns) and DAMPs (Damage associated molecular patterns) and is a quick and nonspecific response (Medzhitov, 1997). PAMPs are recognized by receptors known as pattern recognition receptors (PRRs) and include Toll-like receptors (TLRs) (Medzhitov, 2001).

In addition to PRRs, specific immune cells are recruited once PAMPs have been ligated and activated. The activated cells secrete cytokines and chemokines that are instrumental in the recruitment of inflammatory cells. One of the first cells to respond are neutrophils, granulocytes characterized by their multilobulated nucleus (Kolaczkawska, 2013). Once they have migrated to the site of inflammation, neutrophils act through various mechanisms to clear the pathogen. Neutrophils are
considered to be short-lived cells and typically undergo apoptosis or necrosis in order to facilitate the prevention of tissue damage once inflammation is resolved (Kolacxkawska, 2013). The presence of von Willebrand Factor, a large glycoprotein synthesized in endothelial cells (Pendu, 2006), has been traditionally used as a positive control in immunohistochemistry protocols. However, recent evidence has shown that vWF may play a role in inflammation, binding leukocytes and facilitating the transport of inflammatory cells from the blood to the injured tissue (Pendu, 2006). Due to this interaction between vWF and inflammatory cells, I will also examine the expression of vWF between our different treatment groups.

Although many other cells are part of the innate immune response, the other cell of especial interest in this project will be the macrophage. Macrophages are derived from monocytes once they have migrated into the bloodstream (Rankin, 2004) where they are recruited to the injured area and act to clear it in order to facilitate healing (Rankin, 2004). Macrophages mostly reside in extravascular tissues. However, there are intravascular macrophages in the liver and lungs. Traditionally, macrophages clear pathogens by phagocytosis (Aderem, 2001), which can then lead to activation of another arm of the immune system, adaptive immunity. Additionally, macrophages express one of the most well characterized PRRs, TLR4 (Medzhitov, 2001).

1.1.3 Toll-like receptors

The toll-like receptors (TLRs) are a component of the innate immune system that function to protect our mucosal surfaces against pathogens through the
regulation of inflammation (Schneberger, 2013). For example, TLR4 interacts with lipopolysaccharide (LPS), which is present in the cell walls of Gram-negative bacteria. Following exposure to LPS, it has been previously reported that TLR4 expression is increased in lungs for up to 36 hours (Janardhan, 2006). TLR4 ligates LPS followed by induction of cell signals that leads to translocation of NF-κB into the nucleus and initiation of transcription of pro-inflammatory genes such as TNF-α and IL-1β. The expression of inflammatory mediators induces adhesion molecules such as P-selectin and integrins on endothelium and neutrophils leading to the migration of neutrophils into inflamed organs. The activated neutrophils extend their lifespan for a few hours and produce reactive oxygen species that kill pathogens and also cause significant tissue damage and delay repair. The newly arriving monocytes/macrophages act to remove cellular debris and secrete cytokines such as TGF-β and IL-10 that resolve inflammation and restore homeostasis (Tizard, 2012).

Another member of the TLR family, which plays an important role in innate immunity, is TLR9. Schneberger et al. were the first to describe the expression of TLR9 in intact lungs of mice, human, horse, cattle, pig, and dogs (Schneberger, 2013). TLR9 was localized in vascular endothelium, bronchiolar epithelium, alveolar septal cells, and alveolar macrophages in mice and human lungs (Schneberger, 2013). Other studies have shown that TLR9 activation leads to expression of proinflammatory cytokines (Li, 2004). As TLR9 expression on epithelial cells and alveolar macrophages may be considered part of the initial defence in the lungs, the alternation of TLR9 expression will be of especial interest in this study.
1.2 Introduction to pesticides

When our early human ancestors encountered insects and vermin their pest control was limited to scratching, swatting and squashing. However, with the advent of agriculture and our nomadic ancestors adopting a more sedentary lifestyle along with food stores and domestic animal stocks, more effective measures were required. Some of the earliest documented use of pesticides included by the Sumerians in 2500 BC, who used elemental sulphur against insects and mites, while ancient Chinese cultures treated body lice with arsenic and mercury. While the ingenuity of these ancient cultures can be appreciated, it is unlikely arsenic would be embraced today as an effective pesticide due to its deleterious effects on human health. Nevertheless, in the field of pest control, how far has science really advanced in the last thousand years? Hundreds of different chemicals are still being used to “protect” humans but the effects on non-target organisms are still a widely debated field of interest and research.

The most prominent example of the struggle between insect control and public health concerns is dichlorodiphenyltrichloroethane (DDT). Following World War II chemical intervention of pests rose drastically in popularity, an increase that corresponded to tropical outbreaks of Malaria. DDT is unparalleled in effectiveness, for example 78, 000 cases in 1942 in the Island of Sardinia dropped to just 9 cases in 1951 following DDT intervention. DDT belongs to the organochlorine group of pesticides, which are notable for their persistence in the environment and their lipophilicity (Kannan, 1997), making them highly toxic to both insects and mammals (DDT LD50 is 200 mg/kg for rats). Nowadays organochlorines are banned in the
western world but are still sometimes employed in some developing countries (Kannan, 1997).

1.2.1 Categories of pesticides

A pesticide is defined as anything that works to kill a pest, be it insect, weed, or vermin. There are several categories of chemical compounds that act through various mechanisms to eradicate insects.

A group of the most notorious insecticides are the organochlorines that can cause hyperexcitation, tremors and paralysis. However, as mentioned above, most insecticides of this nature have now been banned in most of the developed world. Another group is the organophosphates, which include the insecticides chlorpyrifos, malathion and parathion. This group inhibits acetylcholinesterase, meaning that in addition to insects these chemicals are also toxic to mammals at low doses (LD50 of parathion is 10 mg/kg). Another group of insecticides are the phenylpyrazoles, which are moving into the niche formerly occupied by the organophosphates (Lassiter, 2009). This group of insecticides was developed as an attempt to overcome problems encountered with similar compounds such as insect resistance and potential public health concerns (New, 1996). A prominent insecticide that belongs to the phenylpyrazole family is fipronil. Fipronil is the active component in products such as Frontline, an anti-flea and tick spray used on common household pets (Jennings, 2002). Products containing fipronil are also used in the agriculture industry on crops such as corn and potatoes to control for pests. The IUPAC name
for fipronil is (±)-5-amino-1- (2,6-dichloro-α,α,α-trifluoro-p-tolyl) - 4-
trifluoromethylsulfinylpyrazole-3-carbonitrile (New, 1996).

1.2.2 Fipronil

Previous research has determined fipronil and other related compounds
belonging to the phenylpyrazole family to act at GABA-gated chloride channels
where they are considered GABA antagonists and block the passage of chloride ions
through the channels (Cole, 1993; Ratra, 2001). Fipronil has a higher affinity for
these channels in insects when compared to non-target organisms such as humans,
making it a seemingly safer product in these regards (Ratra, 2001). However, it has
been demonstrated the primary metabolite of fipronil, fipronil sulfone, actually has
a much greater affinity for these channels in mammals than those in insects (Zhao,
2005), indicating potential detrimental effects of the break-down products to non-
target organisms. One study, which explored the disposition of fipronil in various
tissues of rats following a single oral exposure, found fipronil sulfone to persist for much longer than fipronil in high fat containing tissues especially adipose tissue, adrenals and the liver (Cravedi, 2013).

1.2.3 Usage of fipronil

Fipronil is an insecticide that belongs to the phenylpyrazole family. Since its introduction into the market in 1996, it has become widely used in both agricultural settings, for crops, and domestic settings, for pets. Currently, minimal information exists on the effects of fipronil on non-target organisms and virtually no information is available on the relationship between fipronil and pulmonary health. Currently, little data are available on persistence of fipronil residues in food sources. However, it has been found that fipronil may be used on feed crops such as potatoes, which are then fed to animals used for meat (i.e. cattle). The lipophilic properties of fipronil result in the accumulation of fipronil in fat tissues (New, 1996), an accumulation that increases along the food chain in process known as bioamplification. The dissolution of fat stores during starvation has the potential to release stored pesticides into the circulation leading to systemic effects (Cecchini, 2006).

One study comparing the efficacy of fipronil and indoxacarb found that only 15.6% of the pets in the fipronil treated group were rid of fleas after a two-month study period (Dryden, 2013). The same study design had been used years prior with a much higher efficacy and, while the reason for decreased efficacy is currently
unknown, it is speculated that fleas may be developing resistance or may be part of a innately tolerant strain (Dryden, 2013).

The use of fipronil is escalating in the Indian province of Punjab. Often farmers spraying pesticides on crops will do so without taking necessary safety precautions such as wearing adequate personal protective equipment. Practices such as this lead to an increase in incidence of pulmonary exposure. Another route of exposure is through the accumulation of fipronil in food derived from animals and plants. One study conducted in Ludhiana, examined the biochemical alterations induced by the use of fipronil in conjunction with fluoride following subchronic oral exposure on buffalo calves (Gill, 2013). Unfortunately there are currently no published data on inhalation exposure of fipronil on animals beyond initial studies conducted by the Environmental Protection Agency. These studies determined inhalation exposure to fipronil to be of low to moderate toxicity in rats (New, 1996). Furthermore, the effect of fipronil exposure on the lung’s abilities to generate an inflammatory response may also be compromised/enhanced, which has implications for host response to bacteria or bacterial products such as endotoxin.

1.2.4 Pesticides and non-target organisms

Although some of the health effects of chronic and acute exposures are established, the pulmonary effects of chronic exposure to insecticides are still unclear. Most studies that are focused on pesticide exposure and pulmonary health are limited to anecdotal accounts and cross sectional surveys; models directly exposing organisms to pesticides are few and far between. However, there are
several studies that point to deleterious effects of insecticides on lungs following repeated exposure at sub-lethal doses. One cross-sectional study in Spain indicated farmers displayed reduced pulmonary function in correlation to chronic exposure to pesticides such as neonicotinoids (Hernandez, 2008). Another study investigating the use of paraquat found ingestion of the substance was likely to play a role in multi-organ failure, lung carcinomas and other respiratory symptoms (Wesseling, 2001). Another set of data from women working on plantain fields in Costa Rica showed that exposure to chlorpyrifos and terbufos increased likelihood of having wheeze (Fleten, 2007). It appears that pulmonary effects of oral or inhalational exposure to pesticides have been documented but such data on fipronil are highly limited.

1.3 Cell lines and Pesticides

It is common to use specific cell lines to study the effects of chemicals on cells. This approach though highly reductionist does reduce the number of animals used in research and also provides more specific data and more freedom to manipulate the experimental conditions.

U937 is a cell line derived from a male 37-year-old human patient. U937 cells are a popular cell line to choose when investigating the effects on human immune cells, as they are one of the few cell lines that display monocyte characteristics. Following stimulation with various reagents (in this study Phorbol 12-myristate 13-acetate (PMA), was used) the monocytic cells will differentiate into macrophages. Macrophages are cells present in the early stages of inflammation and secrete
various cytokines (Tizard, 2012). U937 cells have provided a useful tool to investigate the biology of monocytes and macrophages in vitro.

U937 cells have been utilized to solve a number of research questions but to our knowledge, no studies have yet examined the relationship between fipronil and its affect on U937 cells. However, one study did explore the similarly related compound, chlorpyrifos, and if exposure to it induced apoptosis in the U937 cells (Nakadi, 2006). This study found that chlorpyrifos and one of its metabolites, chlorpyrifos-oxon induced apoptosis in a dose and time dependent manner as determined through flow cytometry and other various staining (Nakadi, 2006).

Due to the mode of action of various insecticides (blocking acetylcholinesterase, GABA antagonists, etc.) other studies have used cell lines to investigate the neurological effects of pesticides. For example, a study conducted in 2009 compared the neurotoxic effects of fipronil vs. chlorpyrifos on the PC12 cell line, which is derived from the rat adrenal medulla (Lassiter, 2009). Interestingly, this study found that fipronil was more detrimental to the cells in a fourfold response. Differentiated cell number was reduced, oxidative stress was increased, a more deleterious phenotype was predominant and cell death was delayed (Lassiter, 2009).
Chapter 2: Hypothesis and Objectives

2.1 Hypotheses

1) Exposure to low levels of fipronil will induce lung inflammation characterized by increased expression of inflammatory molecules.

2) Exposure to low levels of fipronil will induce cell death in a concentration and time-dependent manner.

2.2 Objectives

A. To study whether fipronil induces lung inflammation in mice.

B. To study whether fipronil induces cell death in U937 cell line.

2.3 Rationale:

Numerous previous studies have determined that exposure to pesticides in non-target organism may have the potential to result in deleterious effects to a varying extent. Fipronil is a relatively new insecticide and its long-term effects in the environment and how it may affect non-target organisms, including humans, is still not well understood. Especially, there are no data on the effects of fipronil on the respiratory system. In order to create a well rounded story of the relationship between the insecticide fipronil and lung inflammation, I decided that an in vitro study using the U937 cell line would be conducted to compliment the in vivo experiments with mice. To determine if fipronil leads to lung inflammation, various inflammatory markers would be measured in both in vivo and in vitro experiments.
Chapter 3: Materials and Methods

3.1 In Vivo Experiments

3.1.1. Animals

Experiment was conducted following approval from the Institutional Animal Ethics Committee (IAEC), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Swiss albino mice, ages 8-10 weeks, were housed in laboratory animal cages at 18-22 °C and 12:12 light-dark cycles. Mice had access to feed (Ashirwad Industries, Chandigarh, Punjab, India) and water ad libitum.

3.1.2 Experiment Design

An initial experiment was first conducted to determine an appropriate dose of fipronil (Table A). It was decided 8 mg of fipronil per kg of body weight was to be the dose used for both intranasal and oral exposure routes. This dose was determined as it was 10% of the oral LD_{50} for mice (New, 1996). For a negative control, mice received ethanol (intranasal) and groundnut oil (oral) for 7 days (Table B). Unfortunately the intranasal groups of mice experienced high mortality following anaesthesia and the ketamine/xylazine dose was adjusted daily.

A ketamine/xylazine mixture was prepared by mixing 0.5 ml xylazine, 2ml ketamine and 7.5 ml Pyrogen free solution. After 7 days mice were euthanized with a lethal dose of ketamine/xylazine (0.1 μl/10 g of body weight) and cardiac puncture was done to collect blood. The trachea was isolated and a small cannula was inserted to perform lung lavage. Lungs were lavaged 3 times with 0.5 ml of phosphate buffered saline to collect broncho-alveolar lavage (BAL) fluid. The left
lung was fixed in 10% formalin overnight (24h). Lungs were placed in filter capsules and washed in increasing concentrations of ethanol (70, 80, 90, 100%) for 1 hour and then washed in acetone and benzene (15 minutes each) and then embedded in paraffin blocks.

The paraffin blocks were then transported back to Canada and H&E staining and immunohistochemistry was performed according to standard protocols.

### 3.1.3 Hematoxylin and eosin staining

Tissue sections were washed with xylene to de-paraffinize the tissues and then washed with decreasing concentrations of ethanol to rehydrate the tissues (100, 95, 70 and 50%, 2 minutes each). After a rinse with tap, then distilled water, slides were stained with hematoxylin dye for 5 minutes and, dipped in acid alcohol (1 ml HCl in 400 ml 70% ethanol) to stop the reaction then quickly washed (20 seconds) with tap and distilled water. Next, the slides were stained with eosin dye for 3 minutes, rinsed (30 seconds) with tap water, and then dipped in increasing concentrations of ethanol (70, 95, 100%, less than 10 seconds each time). The slides were washed with xylene (2 minutes) and cover slips were applied.

### 3.1.4 Immunohistochemistry

Tissue sections were washed with xylene to de-paraffinize the tissues (2 x 15 minutes) and then washed with decreasing concentrations of ethanol to rehydrate the tissues (100, 95, 70 and 50%, 15 minutes each). To remove peroxidases, tissues were incubated for 20 minutes with 0.5% H₂O₂ in methanol. Pepsin (2 mg/ml in
0.01N HCl) was used to unmask antigen-binding sites (60 minutes) and then 1% bovine serum albumin (BSA) in PBS was used to prevent non-specific binding (30 minutes). Next the tissues were incubated overnight (16 hrs) at 4°C with the following antibodies: von Willebrand Factor (1:500, DAKO A0082), Toll-Like Receptor 4 (1:25, IMG-578A, IMGENEX) and Toll-Like Receptor 9 (1:50, IMG-3051, IMGENEX). Slides were washed 3 times in 1x PBS for 5 minutes for excess antibody removal. The slides were then incubated with secondary antibody (vWF at 1:300 and TLR at 1:100, all from DAKO) for 30 minutes at room temperature and a colour-developing step followed (VECTOR VIP Peroxidase Substrate Kit; Vector laboratories, Burlingame, CA). Finally, methyl green (Vector laboratories) was used for counter staining and then the tissues were dehydrated with increasing concentrations of ethanol (50, 70, 90, 100%, 1 minute each), washed with xylene (5 minutes) and mounted with cover slips.

3.1.5 Grading for Immunohistochemistry

One individual who was blinded to what treatments of each animal performed the scoring. For each tissue section five random fields of vision was assessed and a score from 1-4 (1 being least intense, 4 being most intense) was assigned for each of the criteria depending on the intensity of the staining and whether the epithelium was interrupted or intact. The criteria for grading (Table B) included the intensity of vWF in the large blood vessels, or TLR4 or TLR9 staining in the bronchial epithelium (Figure 5) as well the number of TLR4 or TLR9 positive cells that were present in the alveolar septa (Figures 6 and 7).
### Table 1: Pilot Study

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of animals treated</th>
<th>Number of animals used for tissue collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 mg/mouse fipronil in ethanol Intransasal</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2 mg/mouse fipronil in ethanol Intransasal</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Absolute Ethanol Intransasal</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>8 mg/mouse fipronil in Groundnut oil Oral</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Groundnut oil Oral</td>
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</table>

### Table 2: Mice receiving fipronil or control treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Number of animals treated</th>
<th>Number of animals used for tissue collection</th>
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<tr>
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<td>8 mg/mouse fipronil in ethanol Intransasal</td>
<td>8</td>
<td>6</td>
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<tr>
<td>2</td>
<td>Absolute Ethanol Intransasal</td>
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<tr>
<td>3</td>
<td>8 mg/mouse fipronil in Groundnut oil Oral</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>Groundnut oil Oral</td>
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</table>
**Table 3: Mouse lung histology grading scores**

<table>
<thead>
<tr>
<th>Score</th>
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<tbody>
<tr>
<td>4</td>
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<tr>
<td>3</td>
<td>Intense Staining</td>
</tr>
<tr>
<td>2</td>
<td>Moderate Staining</td>
</tr>
<tr>
<td>1</td>
<td>Low Staining</td>
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</tbody>
</table>
3.2 In vitro Experiments

3.2.1 Thawing the Cells

U937 cell line was obtained from ATCC®. RPMI Complete Media +10% Fetal Bovine Serum (FBS) (Gibco) was warmed to 37 °C. Aliquots of cells were thawed in a hot water bath (37°C) and combined with 10 ml warm media in 15 ml clinical centrifuge tubes. The tubes were spun at 258 g for 3 minutes, the supernatant was discarded and the pellet then resuspended in 10 ml media in a closed flask and incubated at 37°C, 5% CO₂.

3.2.2 Passaging of Cells

The cell suspension was removed from the flask and transferred to a 15 ml centrifuge tube and spun at 258 g for 3 minutes. The supernatant was discarded and cells were resuspended in 5 ml media, split and transferred into 2 new flasks and topped with media (~15 ml). Incubation continued at 37°C, 5% CO₂ and this procedure was repeated every 2-3 days depending on the concentration of cells (split when cells reach ~ 5 x 10⁶ cells/ml of media).

3.2.3 Fipronil Exposure and Viability Assessment

Once the cells reached a concentration of 5 x 10⁵ cells/ml, the cells were transferred to a 12 well plate and differentiated into macrophages after being incubated with 12 ml media + Phorbol-12-myristate-13-acetate (PMA) for 48 hours. Following this incubation, various concentrations of fipronil (the concentrations of fipronil used in these experiments ranged from 0.29 μm to 5.72 μm per 1 ml)
dissolved in DMSO were added to each well and incubated for 3 hours. A 50 µl of 0.25% Trypsin in Ethylenediaminetetraacetic acid (EDTA) was added to each well in order to facilitate removal of the differentiated macrophages. A sample of 150 µl of the cells in media were removed and put in 1.5 ml centrifuge tubes and combined with 50 µl Trypan blue. Viability of cells was then assessed via trypan blue exclusion method.

### 3.2.4 Protein Extraction

Following a 3-hour exposure to fipronil, 50 µl of 0.25% Trypsin in EDTA was added to each well for 5 minutes. Each sample was collected in 1.5 ml centrifuge tubes and spun for 10 minutes at 258 g. The supernatant was aspirated off and the pellet was washed with 1 ml Hanks Balanced Salt Solution (HBSS). This step was repeated twice. The supernatant was again aspirated off and one tablet of Protease inhibitor (Roche) was added to 7 ml Radioimmunoprecipitation assay (RIPA) (Sigma-Aldrich) buffer. A 300 µl of this RIPA buffer mix was added to each centrifuge tube and vortexed thoroughly. The samples were kept in a 4°C refrigerator for 15 minutes and vortexed twice during this incubation. The samples were put in the centrifuge at 10,000 g for 5 minutes. The supernatant was then carefully removed without disturbing the pellet and stored at -45 °C until needed for future assays for up to 1 month.
3.2.5 Western Blot

25 µl of protein samples plus indicator were boiled for 5 minutes and then loaded into a 12% SDS-PAGE gel. The proteins were separated via gel electrophoresis at 160V for 45-60 minutes. The gel was collected and placed between 2 sponges, 4 filter papers and Immobilon – FL membrane in a western blot sandwich all previously soaked in the transfer buffer. The protein transfer was performed at 100V for 70 minutes. Following transfer, the membrane was washed in 15 ml PBS and then incubated in ~15 ml blocking buffer (5% BSA in PBS) for 1 hour. Primary antibodies were mixed with the above blocking buffer with the addition of 0.1% Tween-20. Primary antibodies (Anti-TLR4, AF1478 R&D at 1:200 and Anti-TLR9, IMG 305A at 1:200, IMGENEX) were incubated with membranes overnight at 4°C. Following overnight incubation membranes were washed with PBS and PBST and then incubated with secondary antibody (Goat, anti-mouse Cy5.5 or Donkey Anti-goat Cy3, both at 1:1000, AbCam) in PBS for 30 minutes. Washing was repeated after this step and then membranes were allowed to dry before visualization using the Typhoon 3 laser fluorescence scanner.

3.2.6 Immunofluorescence

Cells adhered to confocal cover slips in a 24 well plate while being differentiated with PMA for 48 hours. They were incubated with either DMSO or 5.72 µm fipronil in DMSO for 3 hours. Following the incubation, cells were washed with 0.5 ml PBS and then fixed with 0.5 ml of 4% PFA in PBS and incubated for 15 minutes. Cells were washed again 3 times with 0.5 PBS for 5 minutes each time.
Cells were incubated with 0.5 ml of 1% BSA for 30 minutes. Cells were washed again with PBS and then incubated with the primary antibody (Rabbit anti-TLR4, G2808, Santa Cruz) in 0.1% BSA (1:250 concentration). After a 1 hour incubation cells were washed with PBS and incubated with the secondary antibody (Anti-rabbit Alexa 555, A21428, Invitrogen) in 0.1% BSA (1:500 concentration) for a 30 minute incubation. Following a last PBS wash, the cover slips were fixed to slides with Mounting Media containing (4’6-diamidino-2-phenylindole) DAPI.

3.3 Statistical Analyses

Statistical analysis was performed using statistical software (SPSS, IBM version 21.1 for Windows). For in vivo work a one-way analysis of variance (ANOVA) was run to determine if the number of TLR4/9 positive cells present in the alveolar septa was significantly different between treatments groups. If there was a significant difference, Tukey’s Multiple Comparison test was performed to see which treatments differed.

For in vitro work, one-way or two-way ANOVA was run to see if there were significant difference between average cell viability. Dunnet’s or Tukey’s Multiple Comparison Test was ran to see the differences. Since only 2 treatments were used for western blots, Student’s Independent T-test was used to determine significant differences.
Chapter 4: Results

4.1 In vivo Results

4.1.1 Hematoxylin and eosin staining

Representative images of H&E staining depict normal lung morphology for all animals in the oral and intranasal control groups though some black spots are present indicating some evidence of dust particles that the animals could have easily been exposed to throughout the experiment (Figure 1a and 1b). Animals in the oral fipronil group display an accumulation of inflammatory cells around the terminal bronchioles. There was a dilatation of perivascular spaces in lung sections from all the animals. An increase in activated epithelial cells was consistently visible from the enlarged and domed appearance of the cells (Figure 1c and d). In comparison to the oral group, the intranasal fipronil group displayed overall normal lung architecture and the epithelium did not appear to be activated. Additionally, there was an increase in accumulation of inflammatory cells in the alveolar septa and the alveoli (Figure 1e, f and g). Many blood cells were attached to the vascular endothelium (Figure 1f).
Figure 1. H&E staining of mice lungs. Lung sections from control mice (1a-b) have normal lung histology of alveolar septa (arrows) and bronchiolar (B) epithelium (Ep). The oral treatment with fipronil caused lung inflammation and lung sections (1c-d) show inflammation (asterisks) around bronchioles (B) and blood vessels (BV). Lung sections from mice treated intranasally with fipronil (1e-g) show septal congestion in septa (arrows; 1e)), cells adhering (arrows) to endothelium (En; 1f) and swollen epithelium (Ep) of bronchioles (B; 1g). Bar: 100 micron.
4.1.2 von Willebrand Factor

Lung sections from control animals showed staining in vascular endothelium that was more prominent in larger blood vessels compared to the alveolar septal capillaries (Figure 2a and b). As expected there was no staining of the bronchiolar epithelium in lung sections from any of the treatment groups. The mice of the oral fipronil group did not have an altered expression of vWF though there was an indication of inflammation in the lung (Figure 2c and d). An increase in vWF staining was displayed in representative images from the intranasal fipronil group in areas such as septal capillaries and in the cells accumulated in septal areas (Figure 2e, f and g). There was specially increased focal vWF staining in endothelial cells and the adhering blood cells (Figure 2f).
**Figure 2. von Willebrand Factor expression in mice lungs.** Lungs from control groups (2a-b) show normal vWF staining (arrows) in endothelium of blood vessels (BV) but not in bronchiolar (B) epithelium. The oral treatment with fipronil (2c-d) caused inflammation but the expression of vWF (arrows) in blood vessels (BV) remained unchanged. The intranasal fipronil (2e-g) showed increased expression (arrows) in alveolar septum (2e), endothelium of blood vessels (2f-g) as well as the vascular cells. Note vascular cells (lightening arrow) attaching to the endothelium (2f). Bar: 100 micron.
4.1.3 Toll-like Receptor 4

Representative images from oral and intranasal control groups showed TLR4 staining in the bronchiolar epithelium, which is common in normal animals (Figure 3a and b). The high magnification images showed TLR4 staining in the cytoplasm of bronchiolar epithelium (Figure 3b inset). Bronchial associated lymphoid tissues (BALT) were present indicating the animals were kept in a dusty environment, as BALTs are not normally present in animals kept in sterile environments. The BALTs were not expressing TLR4. Animals from the oral fipronil group displayed evidence of reduced staining for TLR4 on their lungs (Figure 3c, d). The staining in bronchiolar epithelium was clearly reduced to minimal levels. The blood vessel endothelium gave a folded appearance; a possible indication of the underlying smooth muscles contraction and changes in basement membrane. There was an apparent increase in smooth muscle mass. The intranasal fipronil group showed TLR4 staining in alveolar septa, bronchiolar epithelium and vascular endothelium (Figure 3e, f, g). The airway epithelium showed TLR4 reaction on the surface (Figure 3f). The interface of blood cells and vascular endothelium had TLR4 staining (Figure 3g). Additionally, alveolar macrophages were positive for TLR4.
Figure 3. Toll-like receptor 4 expression in mice lungs. Lungs from control groups (3a-b and) show strong staining (arrows) in bronchiolar epithelium (B) and alveolar septum. Note rich cytoplasmic staining in bronchiolar epithelial cells (3b and inset). The lungs sections (3c-d) from mice treated orally with fipronil show barely minimal staining (arrows) in alveolar septum (3c) while it is nearly absent in bronchiolar (B) epithelium. Lung sections from mice exposed to fipronil (3e-g) show TLR4 staining similar (arrows) to the control lungs in alveolar septum (3e) and epithelium (arrows) of bronchioles (B, 3f). Note TLR4 staining (3g) in endothelium (arrow) and adhering cells (lightening arrows). Bar=100 micron
4.1.4 Toll-like Receptor 9

Representative images of animals from the oral and intranasal control groups showed TLR9 staining in septa, airway epithelium and blood vessels (Figure 4a,b). In animals of the oral fipronil group, TLR9 staining assumed focal appearance in the septa but the airway epithelial staining was considerably reduced (Figure 4c,d). Vascular endothelium showed very minimal TLR9 reaction (Figure 4d). TLR9 expression in lungs of mice exposed intranasally to fipronil was intense and clearly more prominent in the areas of alveolar septa where larger cells were localized (Figure 4e). The airway epithelium in the lungs of intranasal fipronil mice showed intense surface staining for TLR9 while the cytoplasmic reaction was reduced compared to the lungs from normal mice (Figure 4f)
Figure 4. Toll-like receptor 9 expression in mice lungs. Lungs sections from control groups (4a-b) have TLR9 staining (arrows) in alveolar septum (4a) and bronchiolar epithelium (4a inset). The staining is also seen in the septal cells (arrows) in lung sections from control mice (4b). The oral treatment reduced TLR9 staining in lung sections (4c-d) and the staining (arrows) was observed in occasional septal cells (4c). Bronchiolar epithelium (B; 4d and insets) showed much reduced TLR9 staining compared to the controls. Lung sections from mice treated with intranasal fipronil (4e-f) showed intense staining (arrows) in alveolar septum (4e) and bronchiolar epithelium (4f). Bar: 100 micron
4.1.5 Grading for Immunochemistry

To gain a more quantitative understanding of the immunochemistry staining, slides were assigned a histology grading score. For the number of TLR4 positive cells present in the alveolar septa (Figure 6), there was a significantly higher number present in the intranasal fipronil group than were present in the intranasal control group (P=0.050) and in the oral fipronil group (P=0.010). There was no difference between the intranasal fipronil group and the oral control (P=0.145) or between any of the other treatments. For the number of TLR9 positive cells present in the alveolar septa (Figure 7) there was not a significant difference between any of the treatment groups with one-way ANOVA.
Figure 5. Histology grading scores for mice lungs. (A): Scoring for intensity of vWF staining in large blood vessels. (B): Scoring for intensity of TLR4 staining in bronchial epithelium. (C): Scoring intensity of TLR9 staining in bronchial epithelium.
Figure 6. A graph of the means for the number of TLR4 positive cells present in the alveolar septa. Standard error of the means is presented. A 1-way analysis of variance (ANOVA) revealed there was a significant difference between at least two of the treatment groups at p < 0.05 (F_{2,12} = 8.696, P = 0.005). Tukey’s Multiple comparison test determined there was a significant difference between the intranasal treatment and the intranasal control (n=5, n=7) (P = 0.050). There was also a significant difference between the intranasal and oral treatments at (n=5, n=8) (P = 0.010). There was no significant difference between the intranasal treatment and the oral control (n=5, n=7) (P = 0.145) or any of the other treatments. * denotes significant results.
Figure 7. A graph of the means for the number of TLR9 positive cells present in the alveolar septa. Standard error of the means is presented. A 1-way ANOVA revealed there was not a significant difference between the means at p<0.05. (F_{313} = 1.651, P = 0.226). There was no significant effect of either oral or intranasal fipronil on TLR9 positive cells present in the alveolar septa of mice lungs. Oral control (n=5), INL control (n=5), oral fipronil (n=7), INL fipronil (n=5).
4.2 In Vitro Results

4.2.1 Cell Viability

Trypan blue exclusion method was used to determine the percentage of U937 cells that were still alive after incubation with increasing concentrations of fipronil dissolved in DMSO. Following incubation for 3 hours (Figure 8) Dunnet’s Multiple Comparison Test determined that each treatment was significantly different from the control (DMSO alone). In addition to 3 hour incubations with fipronil, a 24 hour incubation was performed, where cell viability was determined at 3, 9, and 24 hours. A low (0.29 μm) and high (5.72 μm) concentration of fipronil was used as well as a control (DMSO). For all time points there was a significant difference in percentage of living cells between the control and both fipronil concentrations. At 3 and 9 hours the low fipronil concentration also had a significantly higher percentage of living cells than the high concentration. At 24 hours there was not a significant difference in the % of living cells between the low and high fipronil concentrations (Figure 9). There was not a significant result for the interaction between time vs. concentration (\( F_{4,120} = 11.01, P = 0.115 \)).
Figure 8. Average U937 cell viability (%) is presented as a function of concentration (µM) of fipronil. Data is presented as means with error bars representing standard error of the mean. Significant results were obtained with a 1-way analysis of variance (ANOVA) and Dunnet's Multiple comparison Test ($F_{3,13} = 1.651$, $P = 0.226$). * depicts the result is significantly different from the control (DMSO). $F_{6,67} = 14.03$, $P < 0.0001$.
Figure 9. Average cell viability (%) as a function of concentration (μM). Data is presented as means with error bars representing standard error of the mean. Significant results were obtained via a 2-way ANOVA and Tukey’s Multiple Comparison Test. No significant result was found for an interaction between time and concentration (F_{4,120} = 11.01, P = 0.115). There was a significant result for time (F_{2,120} = 29.6, P < 0.0001) and for concentration (F_{2,120} = 94.82, P < 0.001). * represents significant results.
4.2.2 Western Blots

After the U937 cells were incubated with a high (5.72 μm) concentration of fipronil for 3 hours, protein extraction was done and western blots were performed for TLR4 and TLR9 followed by densitometric quantification. Figures 10 and 11 depict the average relative density of western blots that were performed three times. There were no differences shown between the group treated with fipronil vs. the group treated only with DMSO. TLR4 (P=0.49), TLR9 (P = 0.94).

4.2.3 Immuno-fluorescence staining of U937 cells with TLR4

The U937 cells were stained with TLR antibodies. The negative control groups didn't include the primary antibody step and resulted in negative reactions with the secondary antibody (Figure 14a). The TLR4 expression was observed in the cytoplasm and plasma membrane of DMSO-treated (Figure 14b) as well as fipronil-treated (Figure 14c) U937 cells. There was an appearance of reduced fluorescence intensity in fipronil-treated cells; however, it was not quantified.
Figure 10. Western blot of TLR4: Western blots show TLR4 expression in control cells (lanes 1 and 2) as well as those treated with fipronil and DMSO (5.72 μm) (lanes 3 and 4). The experiment was repeated three times.
Figure 11. Western blot of TLR9: Western blots show TLR9 expression in control cells (lane 1) as well as those treated with fipronil and DMSO (5.72 μm) (lanes 2 and 3). The experiment was repeated three times.
Figure 12. A graph of the means of the relative densities for a Western Blot of Toll-like Receptor 4. A t-test was performed between the 2 treatments and the results were not significant (P=0.49). DMSO (n=5), fipronil (n=4).
Figure 13. A graph of the means of the relative densities for a western blot of Toll-like Receptor 9. A t-test was performed between the 2 treatments and the results were not significant (P = 0.94). DMSO (n=5), fipronil (n=4).
Figure 13. Staining of U937 cells with TLR4 antibody: Cells stained with only secondary antibody show no labeling except nuclear stain (blue) with DAPI. Both DMSO-exposed control cells (4b and inset) and fipronil-treated (4c and inset) cells showed TLR4 staining (red) in the cytoplasm as well as plasma membrane.
Chapter 5: Discussion

5.1 Discussion of in vivo Results

The experiments conducted as part of this project investigated the effects of fipronil on lung physiology and on a macrophage cell line. The data collectively show that intranasal, but not oral, administration at the indicated dose affects the expression of vWF, TLR4 and TLR9, and the histopathology. Additionally, the intranasal route increased the number of TLR4 positive cells in the lung. Interestingly, the in vitro treatment with fipronil caused a concentration dependent reduction in the number of viable U937 macrophage cells but had no effect on the TLR4 or TLR9 expression.

Looking at the results for histology grading scores of mice lungs alone, there does not seem to be any prevalence of any of the groups exposed to fipronil having a higher score for any of the markers (vWF, TLR4, TLR9), (Figure 5). However, there is a significant difference between treatments in the number of TLR4 positive cells present in the alveolar septa (Figure 6). The intranasal fipronil group had a significantly higher number of positive cells compared to the intranasal control and the group exposed to fipronil via oral gavage (Figure 6). Interestingly, the intranasal fipronil group did not have a significantly higher number of positive cells compared to the oral control group. Based on these results, there is preliminary evidence that exposure to fipronil via the intranasal route has an effect on expression of TLR4 positive cells in the alveolar septa. It could be speculated that this effect is causative because of the anaesthesia the intranasal group received, but this does not seem to be the case since an increase in the expression of TLR4 positive cells was not
observed in the intranasal control group. My experiments do not address the reasons for a significant increase in TLR4 positive cells after intranasal but not oral treatment with fipronil. One of the reasons may be the fipronil in the oral group was metabolized and a significantly lesser amount of the original chemical or the metabolite reached the lungs thus activating a lesser number of TLR4 positive cells. Possibly the passage of the chemical or its metabolite(s) through the liver may have attenuated its toxicity for the lung. Therefore, if this study was to be repeated it would be beneficial to collect and analyze the liver in addition to the lung. Nevertheless, to the best of our knowledge, this is the first study to explore a relationship between fipronil exposure and TLR4 expression.

The data from previous studies have shown that pesticide exposure may be linked with altered TLR4 expression. For example, one study investigating ingestion of the herbicide paraquat and myocardial damage found that there was increased TLR4 mRNA following intraperitoneal injection to paraquat (Dong, 2013). Another study examined the relationship between sodium methyldithiocarbamate (SMD), a commonly used pesticide in the U.S., and altered expression of innate immunity, including TLR4 and macrophages expression (Pruett, 2005). In this study SMD was given via oral gavage, followed by administration of lipopolysaccharide (LPS), and finally a challenge by *Escherichia coli* (*E. coli*) in order to measure the response of the innate immune system. It was found that the mice exposed to the pesticide had a significantly suppressed immune response following *E. coli* challenge, compared with the group that was not exposed (Pruett, 2005). The TLR4 activation was altered via the inhibition of the MAP kinases leading to alteration in the production
of pro-inflammatory cytokines (Pruett, 2005). The increased susceptibility of exposed animals and human workers to secondary challenge with microbes or their products such as TLR4 ligands is a serious occupational health hazard. Therefore, we need to undertake additional studies where experimental animals exposed to fipronil are challenged with LPS. Also, we could make use of TLR4 knockout mice to further understand the role of TLR4 in lung inflammation in animals exposed to fipronil and microbial LPS.

Unlike TLR4, there were no significant differences between any of the treatment groups for TLR9 positive cells present in the alveolar septa. The groups to display the highest number of positive TLR9 cells were the intranasal control and intranasal fipronil groups. Interestingly, the group with the lowest number of positive cells was the oral fipronil group. It would be worthwhile to investigate further to determine if TLR9 expression is altered following exposure to pesticides or if the result may stem due to the anaesthesia with xylazine/ketamine, that was employed in both of the intranasal groups. Currently, extremely limited data is available on TLR9 expression and pesticide exposure. To my knowledge there are very limited data on the effects of two organochlorine insecticides on the leukocytes of gilthead seabream, which found that exposure led to an upregulation of TLR9 gene expression (Cuesta, 2008). Clearly much more work is needed before it can be established if there is any link or not between TLR9 and pesticide exposure.

The changes in alveolar septa of animals are always of importance because of the interface with blood. Therefore, an increase in TLR4 may be associated with an increase of monocyte/macrophages, which can be recruited into lungs of animals
such as rats and mice, which may not normally have such cells (Aharonson-Raz, 2012). The recruitment of such cells, especially pulmonary intravascular macrophages, has been linked to increased lung injury and mortality in response to secondary challenge with E. coli LPS (Gill, 2008). Therefore, the alveolar septum as a unique morphological and physiological area is worthy of special focus in studies on the effects of pesticide exposures.

5.2 Discussion of in vitro Results

Trypan blue exclusion method may be employed when one is interested in seeing a percentage of living cells following a specific cell exposure. In this study, this method was used following exposure to specific concentrations of fipronil over various time points. To begin, U937 cells were exposed to decreasing concentrations of fipronil dissolved in DMSO (Figure 8). This experiment was repeated 3 times and the average of these 3 experiments is presented in this figure. This figure depicts the percentage of living cells significantly decreases as the concentration of fipronil increases. In addition to the 3-hour exposure, a 24-hour exposure with 2 concentrations (low and high) was performed 3 times (Figure 9). Percentage of living cells was significantly decreased as the concentration of fipronil was increased at 3 and 9 hours. There was a significant difference between the control (DMSO) and the two concentrations at 24 hours, but not a significant difference between the low and high concentrations of fipronil, however the percentage of living cells for both of these concentrations was very low (~12% of living cells
remained). These data show that fipronil induces cell death in a dose and time dependent manner.

To our knowledge, no other studies have yet looked at a link between U937 cell death and fipronil exposure, but one study did investigate the effect of the pesticide chlorpyrifos (Nakadai, 2006). Chlorpyrifos occupies a similar niche in the market to fipronil in that it is used in both agricultural settings for crop pests and in domestic settings to treat termites; it has also been linked to abnormal immune responses in humans (Nakadai, 2006). This study treated the U937 cells in a similar fashion to ours, with various time points and concentrations but analysis was then performed using propidium iodide (PI) staining and flow cytometry. Similar to my observations, it was found that chlorpyrifos induced U937 cell death in both a dose and time dependent manner (Nakadai, 2006). These findings were repeated with other assays evaluating cell death: MTT assay, measurement of LDH, FITC-Annexin V staining and DNA fragmentation to determine that chlorpyrifos induced apoptosis in U937 cells through caspase-3 activation (Nakadai, 2006). While the cell death was increased in vitro we actually found that the number of cells expressing TLR4 was increased in the lung in vivo following intranasal fipronil exposure. The reasons for the differences between the two experiments are not clear. We speculate that the cells of single type are more directly exposed in vitro compared to the uptake of the fipronil by many cell types and this potentially leads to a more diffused metabolism of the pesticide. Taken together with the results of our study, it might be worthwhile to further investigate these findings and determine if it is apoptosis that is the cause of cell death or the cells are dying due to necrosis.
To gain a better understanding about how fipronil impacts U937 cells, a surrogate for macrophages, western blot analyses for TLR4 and TLR9 were performed. Following a 3-hour exposure using the 5.72 μm of fipronil or DMSO (control), protein was extracted and western blot was performed, the resulting membrane was then incubated either with TLR4 or TLR9 antibody and visualized using secondary antibodies linked to fluorescent markers. This was performed 3 times with each TLR antibody and the results were analyzed using densitometry, however, neither marker showed a significant difference in protein expression between the control and the fipronil exposed group (Figures 10 and 11). The lack of a significant difference between the two treatments is difficult to conclude without conducting further experiments. Perhaps fipronil is inducing cell death through a mechanism similar to that of chlorpyrifos, which would mean that the amount of TLR4 and TLR9 detected with western blots is actually derived from a fewer number of cells than those in DMSO groups. This would in turn suggest that the expression of TLR4 and TLR9 on live cells may actually be increased as was the case \textit{in vivo} where the number of cells expressing TLR4 was increased and thus there is no increased expression off the Toll-like receptors. As previously discussed, as of yet there is very limited literature on the interactions between pesticide exposure and the Toll-like receptors so it is difficult to make robust conclusions about our findings at this time.
5.3 General Discussion and Ideas for Future Directions

A large obstacle encountered during the *in vivo* study was problems stemming from anaesthesia with xylazine/ketamine. The mice were weighed every second day in order to receive an appropriate dose of anaesthesia, related to body weight. However, sometimes it seemed as if they were not given an adequate amount, as some animals would not succumb to anaesthesia. Other times it seemed as if too much was administered, as the mice would die while under anesthesia. This anomaly was most pronounced in the intranasal control group, where only 2 of the mice lived until day 7 of the study. Other studies have debated the efficacy of this method of anaesthesia vs. ones such as isoflurane. However, isoflurane is traditionally administered through inhalation and would likely not be an appropriate anaesthesia for groups where the lung is the organ of interest.

The standards were drastically different in the animal care units of GADVASU vs. at the University of Saskatchewan. The lack of insulation in the building made it difficult to regulate the temperature. To combat this, a space heater was employed at night times; despite this the mice appeared to be cold, as they would be found huddled together every morning, sometimes shivering. In addition to the trouble with maintaining temperature, the environment where the mice were housed was not entirely free of contaminants, as indicated by the black spots believed to be dust particles which showed up in some of the H&E and IHC slides. As further evidence of the dusty environment, the presence of bronchial associated lymphoid tissues (BALT) was evident in some of the immunohistochemistry slides. Exposure to dust particles and subsequent inflammation has been linked to TLR2 (Poole, 2011); a
TLR that was not investigated throughout this project. However, due to this link between the up-regulation of the TLR2 expression following dust exposure, it would be of benefit to look at this marker if an experiment such as this was to be repeated, especially to see if there was association between dust and pesticide exposure and lung inflammation since oftentimes those working in an agricultural setting are exposed to the two contaminants. TLR2 is expressed on macrophages (Poole, 2011), which has previously been established to be an important component of the innate immune response. Unlike TLR4, commonly regarded as the receptor for cell wall components of gram-negative bacteria, TLR2 recognizes components of gram-positive bacteria (Takeuchi, 1999).
5.4 Conclusions

In vivo results

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<tr>
<td>Von Willebrand Factor Staining</td>
<td>vWF expression</td>
<td>No change</td>
</tr>
<tr>
<td>Toll-like Receptor 4 Staining</td>
<td>TLR4 Expression</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td># of TLR4 positive cells</td>
<td>No change</td>
</tr>
<tr>
<td>Toll-like Receptor 9 Staining</td>
<td>TLR9 Expression</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td># of TLR9 positive cells</td>
<td>No change</td>
</tr>
</tbody>
</table>

In vitro results

<table>
<thead>
<tr>
<th>Expression</th>
<th>Western Blot</th>
<th>Immunofluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4 Expression</td>
<td>No change</td>
<td>Decrease</td>
</tr>
<tr>
<td>TLR9 Expression</td>
<td>No change</td>
<td>Not evaluated</td>
</tr>
</tbody>
</table>

Figure 14. Summary of experiments.
A synopsis of the significant increases or decreases that occurred in each experiment performed when compared with the corresponding control group.
The primary hypothesis of this study was that mice exposed to low levels of fipronil would have lung inflammation characterized by increased expression of inflammatory molecules. As stated above, there was an increase in some of the inflammatory molecules (inflammatory cells, TLR4 expression, etc.), especially in the group that was exposed to fipronil via the intranasal route (Figure 14). Contrarily, the same increased expression was not replicated in the mouse group exposed to fipronil via the oral route, and in fact there was sometimes a decrease in expression of inflammatory markers when compared to the control groups.

The second hypothesis was to study whether exposure to fipronil induces cell death in the U937 cell line. This was demonstrated via the cell viability results, which showed that fipronil induced cell death in both a time and concentration dependent manner (Figure 14). However, exposure to fipronil did not seem to lead to an increase in inflammatory molecules in U937 cells as no changes were observed between controls and fipronil exposed cells in either western blots or immunofluorescence assays. However, this was not an objective of the second hypothesis but merely additional work done to complement the in vivo work.

Ultimately, the primary objective was realized in part as intranasal exposure to fipronil did indeed increase some inflammatory molecules, furthermore, fipronil did induce cell death in the U937 cell line. These results will provide insight into the further exploration of pesticides and how they affect non-target organisms.
LIST OF REFERENCES


Singh, A. K.; Jiang, Y. Lipopolysaccharide (LPS) induced activation of the immune system in control rats and rats chronically exposed to a low level of the organothiophosphate insecticide, acephate. *Toxicology and Industrial Health*, 2003, 19, 93–108


