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**Induction of amyloid- $\beta$ 42 production by fipronil and other pyrazole insecticides**

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**Running title:** A $\beta$ <sub>42</sub> inducers in the human chemical exposome

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## ABSTRACT

Generation of amyloid  $\beta$  peptides (A $\beta$ s) by proteolytic cleavage of the amyloid precursor protein (A $\beta$ PP), especially increased production of A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> over A $\beta$ <sub>40</sub>, and their aggregation as oligomers and plaques, represent a characteristic feature of Alzheimer's disease (AD). In familial AD (FAD), altered A $\beta$  production originates from specific mutations of A $\beta$ PP or presenilins 1/2 (PS1/PS2), the catalytic subunits of  $\gamma$ -secretase. In sporadic AD, the origin of altered production of A $\beta$ s remains unknown. We hypothesize that the 'human chemical exposome' contains products able to favor the production of A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> over A $\beta$ <sub>40</sub> and shorter A $\beta$ s. To detect such products we screened a library of 3500+ compounds in a cell-based assay for enhanced A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> production. Nine pyrazole insecticides were found to induce a  $\beta$ - and  $\gamma$ -secretase-dependent, 3-10 fold increase in the production of extracellular A $\beta$ <sub>42</sub> in various cell lines and neurons differentiated from induced pluripotent stem cells derived from healthy and FAD patients. Immunoprecipitation/mass spectrometry analyses showed increased production of A $\beta$ s cleaved at positions 42/43, and reduced production of peptides cleaved at positions 38 and shorter. Strongly supporting a direct effect on  $\gamma$ -secretase activity, pyrazoles shifted the cleavage pattern of another  $\gamma$ -secretase substrate, alcadein $\alpha$ , and shifted the cleavage of A $\beta$ PP by highly purified  $\gamma$ -secretase towards A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub>. Focusing on fipronil, we showed that some of its metabolites, in particular the persistent fipronil sulfone, also favor the production of A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> in both cell-based and cell-free systems. Fipronil administered orally to mice and rats is known to be metabolized rapidly, mostly to fipronil sulfone, which stably accumulates in adipose tissue and brain. In conclusion, several widely used pyrazole insecticides enhance the production of toxic, aggregation prone A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> peptides, suggesting the possible existence of environmental "Alzheimerogens" which may contribute to the initiation and propagation of the amyloidogenic process in sporadic AD.

## INTRODUCTION

Alzheimer's disease (AD) is a major disease in countries with aging populations. Despite unresolved questions on the initial causes of AD and numerous clinical trial failures in recent years, the lack of satisfactory treatments and the extremely high prevalence of AD calls for fundamental research to determine its underlying molecular and cellular causes and mechanisms and for applied research to identify options for prevention, therapeutic targets and disease-modifying drug candidates [reviews in 1-4].

Although the aggregation of specific forms of amyloid  $\beta$  peptides (A $\beta$ s) into soluble oligomers is undoubtedly associated with the onset of AD, the upstream etiological events underlying this pathological process remain unclear, including whether there is an absolute, or relative, increase in the production of longer aggregation-prone A $\beta$  species. A $\beta$ s are derived from the successive action of two proteases,  $\beta$ -secretase and  $\gamma$ -secretase on one of their numerous substrates, the transmembrane Amyloid precursor protein (A $\beta$ PP). According to its cleavage site  $\gamma$ -secretase liberates A $\beta$ s of different sizes. While A $\beta$ <sub>40</sub> is considered as the main physiological product, the appearance of A $\beta$ <sub>42</sub> and A $\beta$ <sub>43</sub>, or increase of the A $\beta$ <sub>42</sub>-A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratio is clearly associated with the onset of AD [5-7]. These longer A $\beta$ s show an increased propensity to form oligomers, which can assemble into large extracellular deposits, the amyloid plaques, a characteristic feature of AD. A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> oligomers are now considered as the toxic, AD initiating elements rather than the more prominent plaques initially discovered by Alois Alzheimer. This 'amyloid cascade theory' is further supported by the identification of the genetic causes of early onset familial AD (FAD). These rare forms of AD (less than 1% of all AD cases) are indeed all associated with specific mutations of either *A $\beta$ PP* or *PS1* or *PS2* genes (the latter encodes for the catalytic subunits of the  $\gamma$ -secretase complex) [review in 8, 9]. In addition, a specific, protective mutation of A $\beta$ PP was recently found to be associated with reduced risk for developing AD [10]. Furthermore, animal models that express mutated forms of A $\beta$ PP and/or PS1, or human A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub>, develop molecular, cellular and cognitive deficits reminiscent of AD [6, review in 11, 12]. Yet, the level of the different forms of A $\beta$ s is not well correlated with cognitive deficits in mice, suggesting that A $\beta$ s may represent markers rather than inducers of AD in these models [13]. An altered processing of  $\gamma$ -secretase substrates other than A $\beta$ PP might also be important in the pathogenesis of AD. Although FAD is clearly a consequence of genetic mutations of genes encoding the proteins responsible for the production of A $\beta$ s, the initial trigger(s) of late-onset, sporadic AD (>99% of AD cases) remain unknown,

despite extensive genome-wide association studies (GWAS) and the identification of various risk factors [review in 14].

Environmental neurotoxic agents including pesticides, organic solvents, metals and some natural toxins (cyanobacteria) are likely sources of AD-inducing factors [reviews in 15-25]. According to the US Environmental Protection Agency (EPA) Toxic Substances Control Act, over 84,000 chemicals are manufactured or imported at levels >10 tons per year, not including pesticides, cosmetics, food stuffs and food additives which are covered by other legislations ([www.epa.gov](http://www.epa.gov)). It is estimated that we are exposed to over 85,000 substances which, along with all natural substances to which we are exposed from conception to death, constitute the ‘human chemical exposome’ (HCE) [26-34]. In search for such potential “Alzheimerogens” (named by analogy with carcinogens), and encouraged by the discovery of Aftins (**A**myloid  $\beta$  **F**orty-**T**wo **I**nducers) [35-37], a class of synthetic compounds which specifically induce the production of A $\beta$ <sub>42</sub>, we have started to assemble and screen a library of compounds belonging to the HCE for A $\beta$ <sub>42</sub>-inducing products. Our first study identified some triazine herbicides as products stimulating the production of A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> in numerous cell lines [38].

In this new study, we show that several members of the pyrazole class of insecticides, exemplified by fipronil, also increase the  $\gamma$ -secretase-dependent production of A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> peptides in various cell lines and in a cell-free system with highly purified  $\gamma$ -secretase. Interestingly fipronil is metabolized to fipronil sulfone, a very persistent product that accumulates in adipose tissue and brain. These results show that the HCE contains various brain permeable products able to induce the production of pathogenic A $\beta$ s. If long exposures occur in real life and if accumulation of A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> is sufficient to trigger neurodegeneration/neuroinflammation, such products may contribute to the initiation, development and acceleration of sporadic AD and might thus be collectively qualified as potential “Alzheimerogens”. Some of them might also be used to develop useful pesticide-induced animal models of AD.

## MATERIAL AND METHODS

Material and methods are described in full in the Supplementary Information (SI) section. They include: 1. Pyrazoles and other reagents. 2. Cell cultures: cell lines, iPSCs-derived neurons & HEK293-alcadein $\alpha$  cells. 3. Purified  $\gamma$ -secretase preparation. 4. A $\beta$  cell-based and cell-free assays. 5. Cell viability. 6. Pharmacokinetics studies. 7. Proteomics study.

## RESULTS

### Screening HCE compounds reveals pyrazole insecticides as A $\beta$ <sub>42</sub> inducers

We first screened a library of over 3500 low molecular weight compounds representative of the HCE for their ability to induce the production and secretion of A $\beta$ <sub>42</sub> by N2a cells stably expressing A $\beta$ PP695 (N2a-A $\beta$ PP695). An MTS-based cell viability assay was run in parallel to assess cell survival. Although the vast majority of compounds were unable to induce A $\beta$ <sub>42</sub> production, a few active products including several triazine herbicides [38] and pyrazole insecticides (this article) were identified. We next assembled a small library of 18 pyrazoles (**1-18**, SI, Tables S1, S2) that we further tested, along with aftin-5 (**19**) as a positive control, for their ability to trigger A $\beta$ <sub>42</sub> production at 5, 10, 25 or 50  $\mu$ M, in both N2a and CHO cells stably expressing A $\beta$ PP695 and A $\beta$ PP751, respectively (SI, Table S2) (Fig. 1). Nine pyrazoles were found to induce more than a 3-fold change in A $\beta$ <sub>42</sub> levels, showing that A $\beta$ <sub>42</sub> induction is an intrinsic property of some, but not all, pyrazoles: fenpyroximate (**2**), tebufenpyrad (**3**), bixafen (**4**), fluxapyroxad (**5**), isopyrazam (**6**), penthiopyrad (**8**), chlorantraniliprole (**14**), tolfenpyrad (**16**) and fipronil (**18**) (Fig. 1, 2). We next focused on fipronil (**18**)<sup>1</sup>, a widely produced insecticide used to treat crops and pet parasites [reviews in 39-44], and we assembled a small library of fipronil analogues and metabolites (Fig. 3) which were tested for their A $\beta$ <sub>42</sub> production ability in N2a-A $\beta$ PP695 cells (SI, Table S3). The two fipronil enantiomers (**18a**, **18b**) were separated by SFC chiral chromatography (Regis Whelk O1 SS column) and found to be roughly equipotent. Interestingly the main metabolites of fipronil (**18**) [45], fipronil sulfone (**20**), fipronil desulfinyl (**21**) and fipronil sulfide (**22**) displayed A $\beta$ <sub>42</sub> inducing activity equivalent or close to that of the parent compound. In contrast, a few other environmental metabolites such as fipronil chloramine (**24**) [46] and hydroxy-fipronil (**25**) [47] were found to be inactive (SI, Table S3).

A $\beta$ <sub>42</sub> production triggered by pyrazoles in N2a-APP695 cells requires  $\beta$ - and  $\gamma$ -secretase activity as demonstrated by the fact that it was strongly inhibited by  $\beta$ - (inhibitor IV) and  $\gamma$ -secretases (BMS 299897, DAPT) inhibitors and by a  $\gamma$ -secretase modulator ('Torrey Pines' compound, inducing an increase in A $\beta$ <sub>38</sub> and a decrease in A $\beta$ <sub>42</sub> and A $\beta$ <sub>40</sub>) [48, 49] (Fig. 4). A $\beta$ <sub>38</sub>

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<sup>1</sup> Although fipronil is not the most potent A $\beta$ <sub>42</sub> inducer, it was selected for more detailed studies, among other pyrazoles, for several reasons: (1) its very wide use all over the world (despite its ban in Europe in America for food plant cultures), (2) its wide use against fleas and ticks in domestic animals, (3) its wide presence in urban and rural environment, (4) its chemical stability and environmental persistence (especially that of its metabolite, fipronil sulfone), (5) the biological activity of the main metabolite, (6) the fact fipronil sulfone accumulates in adipose tissue, crosses the blood brain barrier and accumulates in the brain, (8) its availability in large quantities for very cheap prices (in preparation of our current long term exposure experiments the supply issue needed to be solved). Incidentally, fipronil was selected before the public health scandal of summer 2017 in Europe!

production was also strongly reduced following pyrazole treatment, while A $\beta$ <sub>40</sub> levels were only modestly affected, as measured by ELISA (data not shown) and by mass spectrometry (Fig. 5). Increased A $\beta$ <sub>42</sub> production triggered by pyrazoles was further confirmed in HEK293 cells stably expressing A $\beta$ PP<sub>sw</sub> (data not shown) and neurons derived from human iPSCs (see below).

### Mass spectrometric quantification and profile analysis of pyrazole-induced A $\beta$ s

A $\beta$ <sub>38</sub>, A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> were quantified in N2a-A $\beta$ PP695 cell culture supernatants using LC-MS/MS [50, 51]. Like aftins [35, 36] and triazines [38], pyrazoles induced a reduction in A $\beta$ <sub>38</sub> levels, a slight increase in A $\beta$ <sub>40</sub> levels and a strong increase in A $\beta$ <sub>42</sub> levels (Fig. 5).

We next analyzed, by IP-MS [52], the profile of A $\beta$ s produced by N2a-A $\beta$ PP695 exposed to fipronil (**18**), fipronil sulfone (**20**) and fipronil desulfinyl (**21**) (Fig. 6). Cell supernatants were collected and A $\beta$ s were immunoprecipitated and analyzed using MALDI-TOF/TOF [52] (Fig. 6). Exposure to pyrazoles decreased the production of A $\beta$ <sub>1-18</sub>, A $\beta$ <sub>1-19</sub>, A $\beta$ <sub>1-33</sub>, A $\beta$ <sub>1-37</sub> and A $\beta$ <sub>1-38</sub>. A $\beta$ <sub>1-40</sub> levels showed a modest increase. In contrast, the two highly neurotoxic [6, 7, 53-55] amyloid peptides A $\beta$ <sub>1-42</sub> and A $\beta$ <sub>1-43</sub>, which were undetectable in supernatants of control cells, were strongly induced in cells treated with fipronil (**18**) and its two metabolites.

### Neurons differentiated from human iPSCs of AD patient and healthy control

We next tested the effects of the active pyrazoles and aftin-5 on neurons differentiated for 4 weeks from human iPSCs derived from a healthy individual (A $\beta$ PP WT, wild-type) or from a patient with familial AD (A $\beta$ PP K724N mutation) [56-58] (Fig. 7). A $\beta$ PP K724N neurons produced more A $\beta$ <sub>42</sub> versus A $\beta$ <sub>40</sub> compared to A $\beta$ PP WT neurons. As observed with triazines [38], addition of any of the active pyrazoles or aftin-5 resulted in further increase in A $\beta$ <sub>42</sub> production, in both A $\beta$ PP WT and A $\beta$ PP K724N neurons (Fig. 7).

### Pyrazoles affect the specificity of APP-C99 cleavage and A $\beta$ production in a cell-free $\gamma$ -secretase activity assay

In order to assess whether the pyrazole-based compounds modulate A $\beta$  production through a direct effect on the specific processing of APP-C99 by the  $\gamma$ -secretase complex, we tested fipronil (**18**), fipronil sulfone (**20**) and fipronil desulfinyl (**21**) in a previously described cell-free activity assay performed with highly purified  $\gamma$ -secretase protease and APP-C99 substrate (a recombinant APP C-terminal fragment expressed in *Escherichia coli* as a fusion protein consisting of a N-terminal Met for translation initiation, amino acids 597- 695 of the

695-amino acid human isoform of APP, and a C-terminal Flag tag sequence) [59, 60]. More specifically, A $\beta$ s generated by purified  $\gamma$ -secretase in the presence of 100  $\mu$ M of the pyrazole compounds were analyzed by immunoprecipitation and MALDI-TOF (IP/MS), as previously described [60]. We found that all three pyrazole compounds increased the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio when compared to the DMSO control (Fig. 8). Importantly, our analysis further revealed that fipronil sulfone (**20**), but not fipronil (**18**) or fipronil desulfinyl (**21**), drastically increased the A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratio by ~2-fold when compared to the DMSO control (Fig. 8). These results strongly support the increased A $\beta$ <sub>43</sub> production seen exclusively with fipronil sulfone (**20**) in N2a-A $\beta$ PP695 cells (Fig. 6). Altogether, we found that pyrazoles favor the production of the toxic A $\beta$ <sub>42</sub> and/or A $\beta$ <sub>43</sub> peptides, both of which contribute to the formation of amyloid plaques and the development of AD.

### **Pyrazoles shift the cleavage pattern of another $\gamma$ -secretase substrate, alcadein $\alpha$**

Like A $\beta$ PP, alcadeins (Alcs) are sequentially cleaved by secretases, first by  $\alpha$ -secretase, leading to N- and C-terminal fragments, the latter being then cleaved by  $\gamma$ -secretase to an intracellular domain and the p3-Alcs peptide, in a way similar to A $\beta$ PP [61, 62] (see Fig. 6A in Ref. 38). HEK293 cells stably expressing full length alcadein $\alpha$  were used to investigate the effects of pyrazoles on alcadein cleavage. Alcadein $\alpha$  is first cleaved on the N-terminal side (two possible sites) followed by cleavage by  $\gamma$ -secretase leading to p3-Alc $\alpha$ <sub>35</sub> and p3-Alc $\alpha$ <sub>2N+35</sub>, the latter representing the major peptide in cultured cells. HEK293-alcadein $\alpha$  cells were grown till 70% confluence and treated with 100  $\mu$ M pyrazoles for 48 h. The secreted p3-Alc $\alpha$  peptides were recovered by immunoprecipitation and analyzed by MALDI TOF/MS (Fig. 9A). Quantification of p3-Alc $\alpha$  peptides showed that, compared to the p3-Alc $\alpha$  peptide profile in vehicle treated cells, concentrations of the main alcadein $\alpha$  peptide (p3-Alc $\alpha$ <sub>2N+35</sub>) and p3-Alc $\alpha$ <sub>2N+37</sub> peptide remained stable. In contrast, both p3-Alc $\alpha$ <sub>2N+34</sub> and p3-Alc $\alpha$ <sub>2N+36</sub> concentrations dropped significantly while the level of p3-Alc $\alpha$ <sub>2N+38</sub> peptide concentration strongly increased (Fig. 9B). These results show that, like for A $\beta$ PP, pyrazoles induce a shift in the cleavage pattern of Alcadein $\alpha$ , another  $\gamma$ -secretase substrate. Similar effects were seen previously with aftin-5 and triazines [38]. These observations further suggest that pyrazoles are more likely to interact with  $\gamma$ -secretase than with their substrates.

### **Proteomics study of fipronil's effects**

In order to understand the molecular mechanisms of action of pyrazoles, we decided to analyze protein expression of cells exposed to fipronil sulfone (**20**) and dipropetryn, two



structurally unrelated products which, despite their unidentified targets, share the property of inducing A $\beta$ <sub>42</sub> production. N2a-A $\beta$ PP cells were exposed for 18 h to 20  $\mu$ M fipronil sulfone (**20**), 100  $\mu$ M dipropetryn or 0.1% DMSO (control) and processed cells for proteomics analysis. Cell supernatant A $\beta$ <sub>42</sub> levels were increased as expected (data not shown). A total of 8,027 proteins were identified in the multiplex experiment (data not shown). The expression of 1,634 and 1,638 proteins was modified, respectively, in fipronil sulfone (**20**)- and dipropetryn-treated cells compared to control, DMSO-treated cells (Fig. 10A). Among these, 261 proteins were shared by both products-treated cells, out of which 178 were up-regulated or down-regulated in the same direction for both products (SI, Table S4). DAVID analysis of these 178 proteins showed that they gather in mitochondrial pathways, cell cycle control, AD (Fig. 10B)

### **Fipronil is metabolized to fipronil sulfone, a stable metabolite which accumulates in adipose tissue and brain**

We next investigated the pharmacokinetics and biodistribution of fipronil (**18**) and its metabolites in mice (Fig. 11) and rats (SI, Fig. S2). Mice first received a single oral dose of fipronil (**18**) and blood, brain and adipose tissue were collected at various times during 72 h (Fig. 11). Plasma pharmacokinetics showed rapid elimination of fipronil (**18**) and parallel appearance of fipronil sulfone (**20**) which remained stable during the 72 h time-course (SI, Fig. S1). No fipronil desulfinyl (**21**) was detected. Similarly, fipronil (**18**) transiently appeared in brain and adipose tissues, while fipronil sulfone (**20**) accumulated to a stable level in both tissues (Fig. 11A, 11B). Fipronil sulfone (**20**) reached a 5-6 fold higher concentration in adipose tissue compared to brain. Given the metabolic stability of fipronil sulfone (**20**), we next ran a two months duration study following a single oral administration of fipronil (**18**) (Fig. 12A). Fipronil sulfone (**20**) levels were determined at various times up to 56 days after the single administration. The half-life of fipronil sulfone (**20**) was found to be  $14 \pm 3$  days,  $17 \pm 2$  days and  $26 \pm 3$  days in plasma, brain and adipose tissue, respectively (Fig. 11A). We next administered fipronil (**18**) daily, 5 days/week for 3 weeks and measured fipronil sulfone (**20**) in plasma, brain and adipose tissue (Fig. 12B). This repeated oral dosing allowed the maintenance of a stable level of fipronil sulfone (**20**) in plasma, brain and adipose tissue (Fig. 12B). Pharmacokinetics and biodistribution of fipronil (**18**) and fipronil sulfone (**20**) were next studied in rats during a 14 days period, following a single oral administration. Results confirmed rapid metabolism of fipronil (**18**) to fipronil sulfone (**20**) (SI, Fig. S2A). Fipronil sulfone (**20**) was much more stable and showed extensive accumulation in adipose tissue (peak concentration 48

h after oral administration of fipronil (**18**) followed by slow release and/or metabolism (SI, Fig. S2B).

## DISCUSSION

This study reports on the induction of A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> production, and the increased ratio of long vs. short A $\beta$ s, in various cell cultures and by highly purified  $\gamma$ -secretase following exposure to several pyrazole insecticides. These results support previous results obtained with various drugs (fenofibrate, celecoxib, indomethacin, isoprenoids) [63], DAPT under certain conditions [64, 65], steroids [66], ceramide analogs [67], the peroxy nitrite donor SIN-1 [68], Zinc [60], aftins [35-37] and several triazine herbicides [38]. Altogether these results have two main implications:

(1) they support the idea that the HCE contains several compounds able to shift the cleavage of A $\beta$ PP towards the production of long, toxic, aggregation-prone A $\beta$ s, such as A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub>, those which are classically associated with AD in humans and numerous animal models of AD. These compounds, if proven to modify this balance in animals (as suggested by the first preliminary results obtained with aftin-4 [28] and celecoxib or FT-1 [63]) and in human, may contribute to the onset, development and/or acceleration of AD. We suggest that they be collectively named “Alzheimerogens”, by analogy with cancer-inducing carcinogens. Identification of such compounds in our environment appears to be a priority to develop a prevention strategy.

(2) some of these compounds, particularly the metabolically stable, brain-permeable molecules, constitute new pharmacological research tools to investigate the role of  $\gamma$ -secretase, its substrates and products, in the onset of AD, in cell lines and animal models. In this context, one of our objectives is, by analogy with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced Parkinsonism, to develop a pesticide-induced animal model of AD, which would allow the study of AD onset in wild-type (WT) animals. This is exemplified by the orally available fipronil (**18**) which is rapidly metabolized to fipronil sulfone (**20**), a very stable compound which accumulates in adipose tissue, crosses the blood brain barrier and reaches stable levels in the brain (Fig. 11, 12; SI, Fig. S2). We are currently running long-term exposure experiments with fipronil (**18**) administered orally in WT mice and Tg2576 mice (overexpressing isoform 695 of human A $\beta$ PP with the Swedish mutation (KM670/671NL)).

### Mechanism of action of fipronil and other pyrazoles

As also observed with aftins [36] and triazines [38], pyrazoles insecticides, including fipronil derivatives, show a structure/activity relationship (SI, Table S1) in their effects on A $\beta$  production - some are active, others are inactive -, showing that pyrazole insecticides devoid of A $\beta$ <sub>42</sub> induction properties can be developed. The limited number of pyrazoles and their wide structural diversity precludes a clear identification of key structural features interacting with the unknown target leading to A $\beta$ <sub>42</sub> production. Unspecific, detergent, hydrophobic, membrane or protein structure disrupting actions are unlikely to account for the effects of pyrazoles. Several arguments support an effect on  $\gamma$ -secretase and/or its micro-environment:

- (1) induction of A $\beta$  production by pyrazoles is inhibited by  $\gamma$ -secretase inhibitors or modulators (Fig. 4),
- (2) pyrazoles also affect the cleavage of another substrate of  $\gamma$ -secretase, alcadeins (Fig. 10),
- (3) pyrazoles modify the cleavage pattern of A $\beta$ PP by highly purified  $\gamma$ -secretase.

Our cell-free  $\gamma$ -secretase activity assays performed with highly purified enzyme and substrate (Fig. 8) show that fipronil (**18**), Fipronil sulfone (**20**) and fipronil desulfinyl (**21**) clearly increased the A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio. Remarkably, Fipronil sulfone (**20**) additionally increased by ~2-fold the A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratio. At the molecular level, the  $\gamma$ -secretase complex cleaves the APP-C99 substrate within the transmembrane domain (TMD), first at the  $\epsilon$ -site located at the intracellular interface, to generate two different C-terminal APP intracellular domains (AICDs) of 50 or 51 aa length [69]. To generate A $\beta$ s of different lengths,  $\gamma$ -secretase next cleaves the N-terminus of the TMD every  $\alpha$ -helical turn (corresponding to 3-4 amino acids), starting from  $\epsilon$ -sites, following two production lines: the AICD51/A $\beta$ <sub>48</sub> pathway (A $\beta$ <sub>48</sub>  $\Rightarrow$  A $\beta$ <sub>45</sub>  $\Rightarrow$  A $\beta$ <sub>42</sub>  $\Rightarrow$  A $\beta$ <sub>38</sub>) and the AICD50/A $\beta$ <sub>49</sub> pathway (A $\beta$ <sub>49</sub>  $\Rightarrow$  A $\beta$ <sub>46</sub>  $\Rightarrow$  A $\beta$ <sub>43</sub>  $\Rightarrow$  A $\beta$ <sub>40</sub>) [69]. Consistent with a direct effect of pyrazoles on APP-C99 processing, our results support an altered A $\beta$ <sub>48</sub> pathway leading to an increased A $\beta$ <sub>42</sub> production. Surprisingly, our study further revealed that fipronil sulfone (**20**) unambiguously and drastically increases the A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratio (Fig. 8). This observation is important since A $\beta$ <sub>43</sub> is known to be highly amyloidogenic and a main species involved in the formation of senile plaques [6, 7, 70]. As of today, A $\beta$ <sub>43</sub> is known to be a direct precursor of A $\beta$ <sub>40</sub> [69]. Therefore, the increased A $\beta$ <sub>43</sub> production in the cell-free assay suggests that fipronil sulfone (**20**) blocks the processing of APP-C99 at position 43 in the A $\beta$ <sub>49</sub> pathway, an event which is likely associated with reduced A $\beta$ <sub>40</sub> production.

Further quantitative experiments are required to explain the increased A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> or A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratios, caused either by increased A $\beta$ <sub>42</sub> or A $\beta$ <sub>43</sub> production, a reduced A $\beta$ <sub>40</sub> production or both. In any case, an altered A $\beta$  profile in the presence of pyrazole insecticides strongly suggests that the compounds directly affect the positioning of the A $\beta$ PP substrate in

the lipid-bilayer, as previously observed for compounds known as inverse  $\gamma$ -secretase modulators (iGSM) [64]. Similarly to what we observed with fipronil (**18**) and fipronil desulfinyl (**21**), the iGSM nonsteroidal anti-inflammatory drugs (NSAIDs) Fenofibrate and Celecoxib have indeed been reported to be A $\beta$ <sub>42</sub>-raising and A $\beta$ <sub>38</sub>-lowering compounds [63]. Interestingly, Celecoxib is a pyrazole. The pyrazole scaffold has in fact been used extensively in therapeutic drug development [review in 71]. In contrast to iGSMs, some NSAID-based  $\gamma$ -secretase modulators (GSM), including sulindac-sulfide, lower A $\beta$ <sub>42</sub> and increase A $\beta$ <sub>38</sub> productions [63]. GSMs are of great pharmaceutical interest and are currently under clinical evaluation for their ability to reduce the production of the amyloidogenic A $\beta$ <sub>42</sub> and A $\beta$ <sub>43</sub> peptides. At the same time the potential risk from A $\beta$ <sub>42</sub> and A $\beta$ <sub>43</sub> producing iGSMs needs careful evaluation since some of these compounds are FDA-approved and widely used as NSAIDs. At the molecular level, it has further been shown that residues 29–36 (GAIIGLMV) of the A $\beta$  motif in APP-C99 located at the TMD N-terminus represent the binding site of both A $\beta$ <sub>42</sub>-lowering GSMs and A $\beta$ <sub>42</sub>-raising iGSMs [63]. This raises the question whether the same binding site could be involved in the pyrazole-induced A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> and A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratio increase. Interestingly, we recently found that zinc can drastically raise the A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratio by influencing the positioning of the APP-C99 TMD through binding to Lysine 28, which is located at the surface of the membrane and is involved in anchoring APP-C99 in the lipid bilayer [69]. This observation further supports the possibility that Lys28 and more globally residues 29–36 in APP-C99 are involved into the pyrazole-induced A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> increase. Finally, novel GSMs have recently been reported that directly bind on PS1 [72]. Binding of these compounds to PS1 induced a conformational change in the enzyme-substrate interaction that modulated the protease activity and consequently the A $\beta$  profile. Thus, one cannot exclude that fipronil (**18**), fipronil sulfone (**20**) or fipronil desulfinyl (**21**) also modulate APP-processing by directly binding to the  $\gamma$ -secretase complex. Further experiments are needed to decipher the mode of action of these compounds (see SI, Fig. S3 for working model).

### **Fipronil exposure and consequences**

Fipronil has been developed a selective inhibitor of insect GABA-gated chloride channels [review in 44]. It is one of the most widely used pyrazole insecticides in agriculture (seed, culture & crop treatment), in urban pest control (coackroaches, termites, wasps, flies & ants management in & around buildings) and in veterinary applications (fleas & ticks control for pets and cattle). Fipronil and its metabolites are therefore widely found [73] in urban soil, dust, wastewater and residential runoff [74], in rural soil, rivers and atmosphere [46, 73, 75,

76], but also in various tissues of pet, farmland and wild-life animals [75, 77, 78] (only a very small number of references are cited). Although fipronil (**18**) is relatively resistant to degradation, it is mostly oxidized to fipronil sulfone (**20**), reduced to fipronil sulfide (**22**), photodegraded to fipronil desulfinyl (**21**), hydrolyzed to fipronil amide (**23**, **28**). Many other metabolites have been described [45-47, 73, review in 39]. Although fipronil shows low affinity for mammalian GABA-gated chloride channels compared to those of insects, toxicity studies carried out with fipronil and its metabolites in various vertebrates [39, 40, 43, 44, 77] as well as studies performed with mammalian cells studies [79-83] cast some doubts about the safety of fipronil to humans (see SI, Table S5). This concern is amplified by (1) the existence of numerous metabolites which are poorly characterized in terms of toxicity, (2) their long persistence in the environment (soil, water, sediment and atmosphere), (3) their accumulation in tissues, especially adipose tissue (Fig. 11, 12), (4) the potency of fipronil sulfone on mammalian GABA-receptor [84], (5) the fact that fipronil and its metabolites essentially meet all drugability criteria of Lipinski's rule of Five (SI, Table S6). In addition, fipronil (along with neonicotinoids) is a major worldwide concern for the survival of pollinators [85-89]. In mammals, fipronil shows oxidative stress, neurotoxic, thyroid, endocrine, lung inflammation, blood pressure and reproductive effects [review in 44] at high doses. In humans, fipronil triggers mild nervous troubles as seen in acute intoxication cases [90, 91] and apparently impacted thyroid functions of workers following occupational exposure [92].

As shown in Fig. 11 & 12, fipronil (**18**) is rapidly metabolized to a very stable product, fipronil sulfone (**20**), which readily accumulates in fat tissue. Table 1 reviews plasma and tissue levels of fipronil sulfone (**20**) reached experimentally in mice and rat or accidentally in humans (our data + literature data). The highest plasma levels reached experimentally during these relatively short exposures (3.7  $\mu\text{g/mL}$ , i.e. 8.2  $\mu\text{M}$ ) are not very far from those which induce A $\beta$ <sub>42</sub> and A $\beta$ <sub>43</sub> production. Adipose tissue and brain levels reached experimentally are probably higher. 72 h after a single oral administration of <sup>14</sup>C-fipronil to rats, about 50% and 2% of the radioactivity (essentially fipronil sulfone) were found in adipose tissue and brain, respectively [93]. Fipronil sulfone is also the primary metabolite produced in human via hepatocyte cytochrome P-450 oxydation [90, 92-95]. Fipronil sulfone was detected in about 25% of 96 human serum samples (0.1-39 ng/mL which corresponds to 0.22-86 nM) [45]. These samples were obtained from individuals with no known fipronil exposure. However fipronil applied to the fur of pets is easily and rapidly transferred to humans [96, 97]. Fipronil and fipronil sulfone levels were measured in urine and blood samples of 159 workers in a factory manufacturing fipronil-containing veterinary products [92]: 33 and 155 had detectable serum fipronil and

fipronil sulfone (0.37-42.45 ng/mL, i.e. 0.82-9.32 nM), respectively. Incidentally, fipronil has recently been shown to promote adipogenesis [98] and adiposity/obesity has been suggested as a risk factor for AD [99, 100]. Adipose tissue may thus act as a trap and storage tissue for lipophilic, A $\beta$ <sub>42</sub>/A $\beta$ <sub>43</sub> inducers like fipronil, other pyrazoles, other pesticides. The persistence of fipronil sulfone, its accumulation in adipose tissue, and its ability to enter the brain raises concerns on its possible long term effects on the central nervous system, in particular on its possible effects on the production of toxic, aggregation-prone amyloidogenic A $\beta$ <sub>42</sub> and A $\beta$ <sub>43</sub>, as shown in this article.

In conclusion, we have added some pyrazole insecticides, especially the main metabolite of fipronil, in the growing list of HCE products which are able to alter the specificity of A $\beta$  production. Our hypothesis is that these products may, on a long-term, cumulative and additive basis, possibly in parallel with microorganisms [101] and microorganisms-derived products, alter the A $\beta$  production pattern sufficiently to lead to AD pathogenesis, and thus qualify as “Alzheimerogens”. Gradual accumulation in and chronic release from adipose tissue and transfer to the brain may contribute to the onset, development and acceleration of sporadic AD.

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**SUPPLEMENTARY INFORMATION** contains a supplementary material & methods, 3 supplementary tables, 3 supplementary figures and supplementary references.

**CONFLICT OF INTEREST STATEMENT.** L. Meijer is one of the co-founders of ManRos Therapeutics. The authors declare that they have no competing interests. B.D. Hammock is a co-founder of Eicosis Human Health.

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## TABLE

**Table 1. Concentrations of fipronil sulfone reached in mammals following fipronil administration (oral, ip, iv, general exposure).** Fipronil sulfone (**20**) levels are expressed either in µg/mL or in µg/g. A 4.53 µg/mL concentration is equivalent to a concentration of 10 µM. bw, body weight.

Species/tissue	Exposure conditions	Fipronil sulfone ( <b>20</b> ) levels reached	Refs.
<b>Mouse</b>			
plasma	10 mg/kg bw, oral, single administration	1 - 1.1 µg/mL	Fig. 11, 12A
plasma	10 mg/kg bw, oral, 5 days/week/3 weeks	3.7 µg/mL	Fig. 12B
plasma	2.5 or 10 mg/kg bw, oral, 1 day/week/2 weeks	0.029 or 0.210 µg/mL	SK, unpubl.
plasma	2.5 or 10 mg/kg bw, oral, 3 days/week/2 weeks	0.211 or 0.662 µg/mL	SK, unpubl.
adipose tissue	2 mg/kg bw, ip, daily/6 days	22-24 ppm	[102]
adipose tissue	10 mg/kg bw, oral, single administration	4.3 - 4.9 µg/epididymal adipose pad	Fig. 11, 12A
adipose tissue	10 mg/kg bw, oral, 5 days/ week/3 weeks	13.5 µg/epididymal adipose pad	Fig. 12B
adipose tissue	2.5 or 10 mg/kg bw, oral, 1 day/week/2 weeks	0.730 or 5.276 µg/g	SK, unpubl.
adipose tissue	2.5 or 10 mg/kg bw, oral, 3 days/week/2 weeks	6.098 or 12.48 µg/g	SK, unpubl.
brain	40 mg/kg bw, ip, 6 or 20 min, single administration	19 or 32 ppm (6 and 20 min, respectively)	[103]
brain	10 mg/kg bw, oral, single administration	0.71 - 0.75 µg/brain	Fig. 11, 12A
brain	10 mg/kg bw, oral, 5 days/week/3 weeks	2.7 µg/brain	Fig. 12B
brain	2.5 or 10 mg/kg bw, oral, 1 day/week/2 weeks	0.049 or 0.405 µg/g	SK, unpubl.
brain	2.5 or 10 mg/kg bw, oral, 3 days/week/2 weeks	0.585 or 1.746 µg/g	SK, unpubl.
<b>Rat</b>			
plasma	10 mg/kg bw, oral, single administration	1.4 µg/mL	Fig. S1
plasma	5 or 10 mg/kg bw, oral, daily/2 weeks	2.4 or 3.6 µg/mL	[104]
plasma	30 mg Regent ®/kg bw, oral, daily/15 days	0.46 µg/mL	[105]
plasma	4 µg/kg bw, oral, 3X/ week/90 days	0.008 - 0.39 ng/mL	[106]
plasma	3.4 (po) or 6.9 (iv) µmole/kg bw, single administration	0.2 (po) or 0.25 (iv) µg/mL	[107]
plasma	3.4 µmole/kg bw, oral, daily/14 days	1.5 µg/mL	[107]
plasma	3 mg/kg bw, oral, daily/13 days	0.043 - 2.8 µg/mL	[108]
adipose tissue	10 mg/kg bw, oral, single administration	152.3 µg/g	Fig. S3
adipose tissue	10 mg/kg bw, oral, 72 h, administration	47.87 µg fipronil equivalent (>90% fipronil sulfone)/g wet weight	[93]
brain	10 mg/kg bw, oral, single dose	3.74 µg/g	Fig. S2
urine	5 or 10 mg/kg bw, oral, daily/2 weeks or single dose	0.023-0.026 µg/mL	[47]
urine	5 or 10 mg/kg bw, oral, daily/2 weeks	0.024 or 0.032 µg/mL	[104]
urine	4 µg/kg bw, oral, 3X/week/90 days	0.009-4.27 ng/mL	[106]
hair	4 µg/kg bw, oral, 3X/week/90 days	4.58-306 pg/mg	[106]
<b>Human</b>			
plasma	no known fipronil exposure (96 plasma samples)	0.1-3.9 ng/mL; detected in 25% of the samples.	[104]
plasma	conducted on 159 workers from a factory manufacturing fipronil-containing veterinary products in France	mean: 7.79 ng/mL (range: 0.37-42.45 ng/mL) detected in 155 out of 159 workers.	[92]
plasma	6 cases of self-poisoning with Regent ®	maximum fipronil + fipronil sulfone level measured was 3.74 µg/mL	[109]
urine	no known fipronil exposure (84 urine samples)	undetectable	[104]

## FIGURE LEGENDS

**Figure 1. Some pyrazoles trigger the production of extracellular amyloid A $\beta$ -42.** Effect of 18 pyrazoles on extracellular amyloid A $\beta$ <sub>42</sub> production by N2a-APP695 and CHO-7PA2-APPsw cells. Cells were treated with 100  $\mu$ M of each compound for 18 h and cell supernatants were collected for extracellular A $\beta$ <sub>42</sub> levels measurement by an ELISA assay. Aftin-5 was used as a positive control and the corresponding volume of vehicle (DMSO) was used as a negative control. Levels are expressed as fold change,  $\pm$  standard error (SE), of A $\beta$ <sub>42</sub> levels over the A $\beta$ <sub>42</sub> level of control, vehicle-treated cells. Average of five experiments performed in triplicate. Horizontal dotted lines indicate levels for 1- and 3- fold increases in A $\beta$ <sub>42</sub> concentration.

**Figure 2.** Molecular structure of the nine active pyrazoles.

**Figure 3.** Molecular structure of fipronil and some of its metabolites and derivatives.

**Figure 4. Extracellular A $\beta$ <sub>42</sub> production induced by pyrazoles is inhibited by  $\beta$ -secretase inhibitor IV,  $\gamma$ -secretase inhibitors DAPT & BMS 299897, and  $\gamma$ -secretase modulator 'Torrey Pines'.** N2a-APP695 cells were exposed to 10  $\mu$ M  $\beta$ -secretase inhibitor IV, 2  $\mu$ M BMS 299897, 2  $\mu$ M DAPT or 10  $\mu$ M 'Torrey Pines' compound. 1.5 h later cells were exposed to 50  $\mu$ M (2, 5, 8, 14, 16, 18) or 25  $\mu$ M (3, 4, 6) pyrazoles or 50  $\mu$ M aftin-5. Extracellular A $\beta$ <sub>42</sub> levels were measured after 18 h and are expressed as fold change,  $\pm$  standard error, of A $\beta$ <sub>42</sub> level in pyrazole-treated cells over the A $\beta$ <sub>42</sub> level of control, vehicle-treated cells. Representative of two independent experiments performed in triplicates. Errors bars represent SE of all six values.

**Figure 5. Absolute quantification of A $\beta$ <sub>38</sub>, A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> using LC-MS/MS.** Levels of the three A $\beta$ s were determined by mass spectrometry in supernatants of N2a-APP695 cells following 18 h treatment with DMSO, 100  $\mu$ M of pyrazoles 18, 20 or 21. Amyloid levels are expressed as percentage of levels in vehicle-treated cells (average  $\pm$  SE of quadruplicate values; absolute values in control cell supernatants are indicated) and A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratios are indicated in parentheses. Horizontal dotted line indicates basal A $\beta$  levels vs. the values in DMSO-treated cells.

**Figure 6. Pattern of A $\beta$ s produced by N2a-APP695 cells exposed to pyrazoles 18, 20 or 21.** Cells were treated for 18 h with DMSO or 20  $\mu$ M of each pyrazole. Cell supernatants were

collected and analyzed as described. Quantification of all A $\beta$ s in N2a-APP695 cells supernatants are presented as percentage of total amyloids. Note the decrease in peptides A $\beta$ <sub>1-37</sub>, A $\beta$ <sub>1-38</sub>, the increase in A $\beta$ <sub>1-40</sub> and the appearance of A $\beta$ <sub>1-42</sub> and A $\beta$ <sub>1-43</sub> in the supernatants of pyrazole-treated cells (these two peptides are undetectable in the supernatant of DMSO-treated cells).

**Figure 7. Pyrazoles trigger enhanced production of A $\beta$ <sub>42</sub> versus A $\beta$ <sub>40</sub> in neurons differentiated from human induced pluripotent stem cells.** Neurons were derived from iPSCs obtained from healthy donor (APP WT, white bars) or from an AD patient with APP K724N mutation (grey bars). They were exposed for 24 h to DMSO (control), 100  $\mu$ M aftin-5 or the nine pyrazoles. Cell supernatants were collected for extracellular A $\beta$ <sub>40</sub> and A $\beta$ <sub>42</sub> levels measurement by an ELISA assay. Levels are expressed as A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratios  $\pm$  SE of triplicate values. Horizontal dotted line indicates level for 1-fold increase in A $\beta$ <sub>42</sub> concentration

**Figure 8. Mass spectrometric analysis of the A $\beta$ s generated in cell-free  $\gamma$ -secretase assays, in the presence of DMSO or fipronils 18, 20 or 21.** **A.** The A $\beta$ s generated by highly purified  $\gamma$ -secretase in the presence of 100  $\mu$ M of fipronil (**18**), fipronil sulfone (**20**) or fipronil desulfinyl (**21**) or DMSO (vehicle) were pooled from triplicates of the activity assays, immunoprecipitated overnight with the anti-A $\beta$  antibody 4G8 and protein G and analyzed by MALDI-TOF in a reflectron mode. The A $\beta$ s generated from the recombinant APP-C99 contain an N-terminal methionine, which results in a mass shift of +149m/z when compared to endogenous A $\beta$  peptides. **B.** Note the increased A $\beta$ <sub>42</sub>/A $\beta$ <sub>40</sub> ratio for fipronils **18**, **20** and **21**, and the increased A $\beta$ <sub>43</sub>/A $\beta$ <sub>40</sub> ratio for fipronil sulfone (**20**).

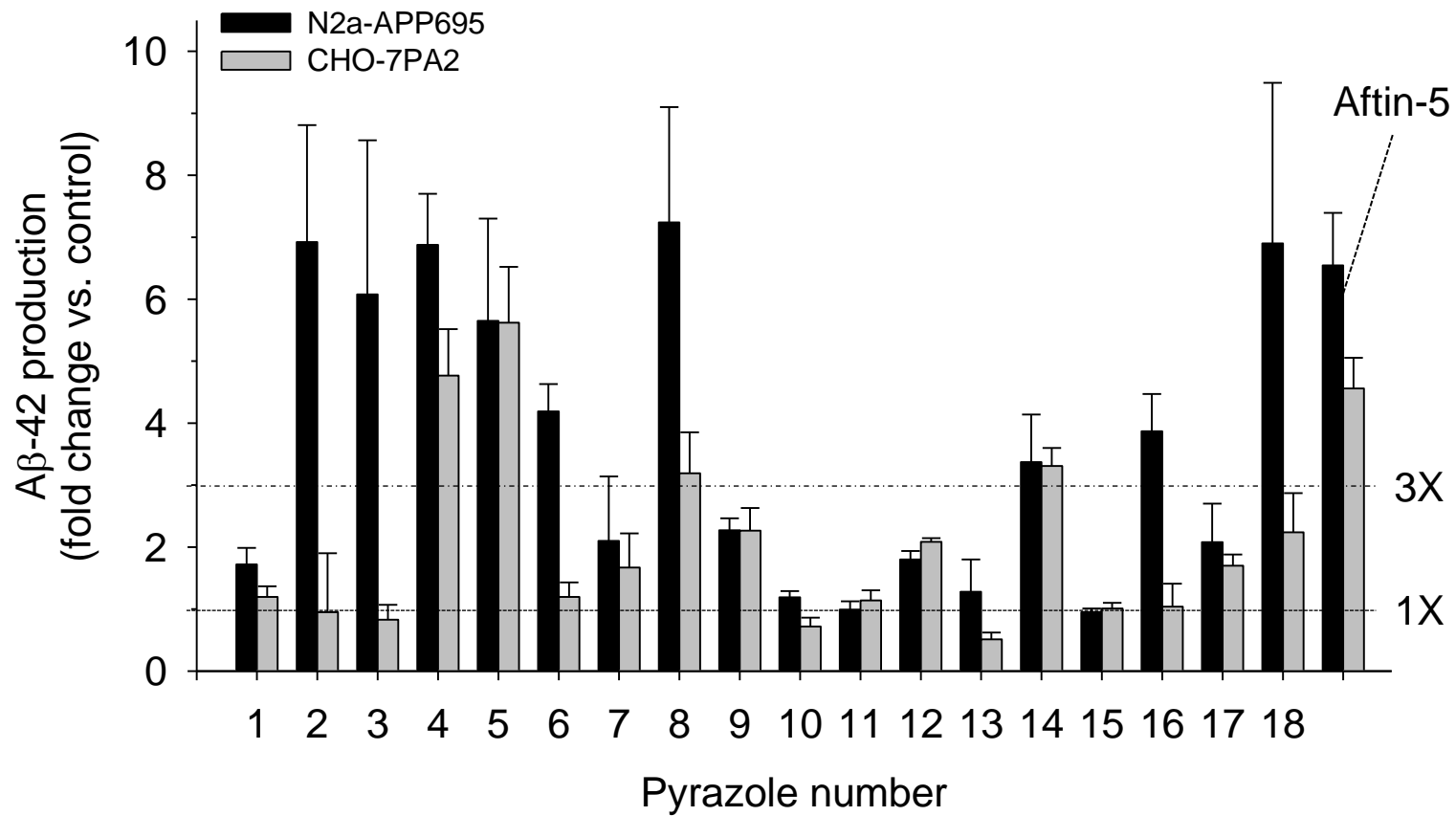
**Figure 9. Pyrazoles alter the cleavage pattern of Alcadein $\alpha$ , leading to increased p3-Alc $\alpha$ 38 production.** **A.** Immunoprecipitation/mass spectrometry spectra of p3-Alc $\alpha$  peptides produced by HEK cells expressing full length Alcadein $\alpha$  exposed to various pyrazoles or DMSO (control). Cells were treated for 48 h with 100  $\mu$ M of each reagent and p3-Alc peptides were analyzed by MALDI-TOF/MS. **A.** Representative profile for each product showing the the p3-Alc $\alpha$ 2N+34, p3-Alc $\alpha$ 2N+35, p3-Alc $\alpha$ 2N+36, p3-Alc $\alpha$ 2N+37 and p3-Alc $\alpha$ 2N+38 peaks. **B.** Relative quantification of p3-Alc $\alpha$  peptides produced by cells exposed to DMSO (control), fipronil (**18**), fipronil sulfone (**20**) or fipronil desulfinyl (**21**). Levels of each peptide are

presented as relative ratios of p3-Alc $\alpha$ 2N+38 versus p3-Alc $\alpha$ 2N+35 (average  $\pm$  SEM of triplicate values).

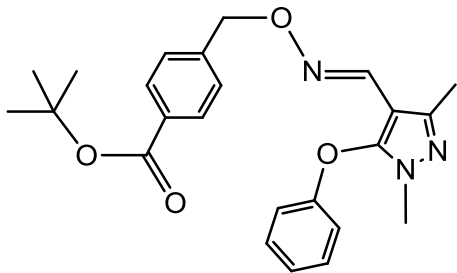
**Figure 10. Proteomics & phosphoproteomics analysis of N2a-APP695 cells exposed to fipronil sulfone (20)- and dipropetryn- treated cells.** **A.** N2a-APP695 cells were exposed for 18 h to 20  $\mu$ M fipronil sulfone (20), 100  $\mu$ M dipropetryn or DMSO. This led to increased extracellular A $\beta$ <sub>42</sub> expression ( $3.58 \pm 0.05$  and  $7.87 \pm 0.81$  fold change for fipronil sulfone (20) and dipropetryn, respectively, compared to DMSO-treated cells). Up/down-regulated proteins in fipronil sulfone- (1634) and dipropetryn- (1638) treated cells vs. DMSO-treated cells were identified and compared. Among the 261 common proteins, 178 proteins (listed in Supplementary Table S1) were either up-regulated by both treatments or down-regulated by both treatments. **B.** DAVID analysis of the 178 proteins common to fipronil sulfone (20) and dipropetryn treatments. Data are selected with p-value <0.01 and FDR <0.05. FDR = False Discovery Rate.

**Figure 11. Short-term time-course of fipronil (18) and fipronil sulfone (20) bioaccumulation in brain (A) and epididymal adipose tissue (B) following a single oral administration of fipronil (18).** Fipronil (18) (10 mg/kg) was administrated by oral gavage at time 0. Animals were sacrificed at various times and plasma, epididymal adipose tissue and brain were collected. Fipronil (18) and fipronil sulfone (20) levels were quantified by LC-MS/MS. Concentrations are expressed as  $\mu$ g/brain or  $\mu$ g/epididymal adipose tissue.

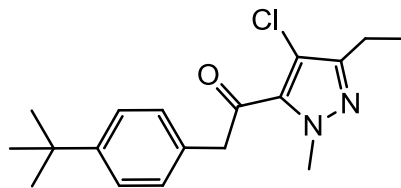
**Figure 12. Long-term time-course of fipronil sulfone (20) production and accumulation following single (A) or repeated (B) oral administrations of fipronil (18).** **A.** Fipronil (18) (10 mg/kg) was administrated by oral gavage on day 0. Animals were sacrificed at various times and plasma, epididymal adipose tissue and brain were collected. Fipronil sulfone (20) levels were quantified by LC-MS/MS. **B.** Fipronil (18) (10 mg/kg) was administrated by oral gavage 5 days /week for 3 weeks (times of administration are indicated by black dots). Animals were sacrificed at various times and plasma, epididymal adipose tissue and brain were collected. Fipronil sulfone (20) levels were quantified by LC-MS/MS. Concentrations are expressed as  $\mu$ g/brain,  $\mu$ g/epididymal adipose tissue or  $\mu$ g/mL plasma.



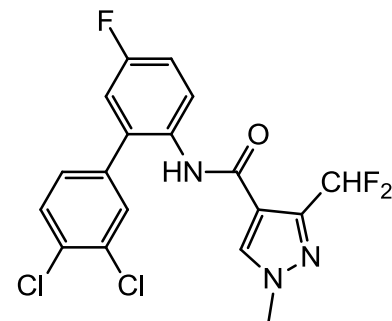
.Figure 1



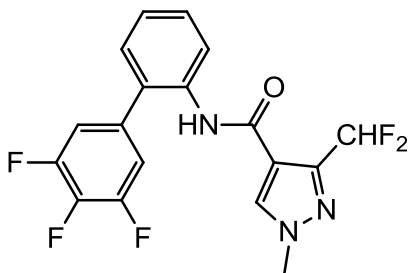
Fenpyroximate (2)



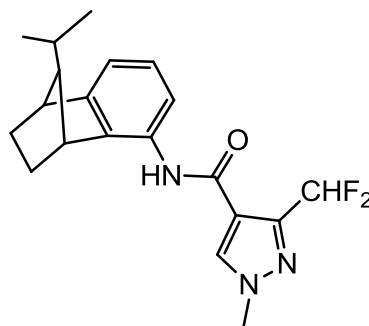
Tebufenpyrad (3)



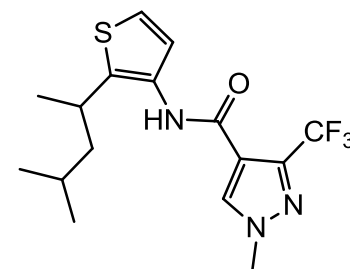
Bixafen (4)



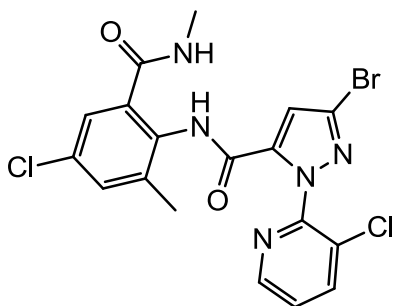
Fluxapyroxad (5)



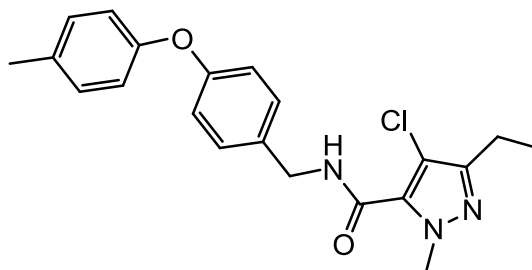
Isopyrazam (6)



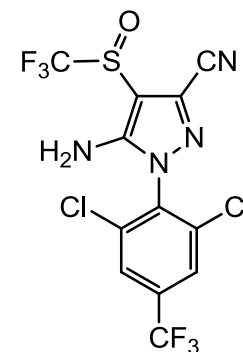
Penthiopyrad (8)



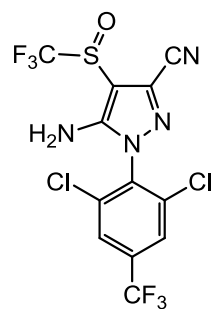
Chlorantraniliprole (14)



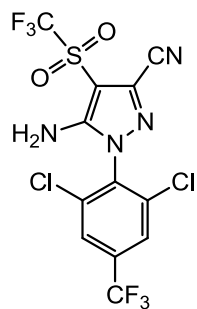
Tolfenpyrad (16)



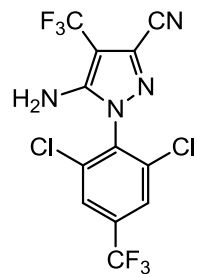
Fipronil (18)



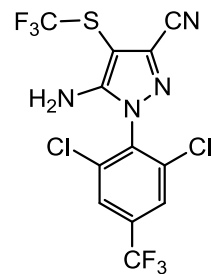
Fipronil (**18**)



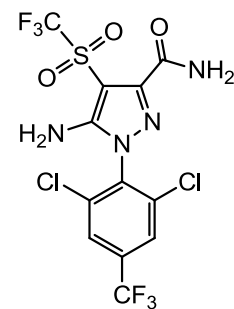
Fipronil sulfone (**20**)



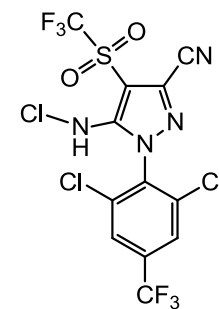
Fipronil desulfinyl (**21**)



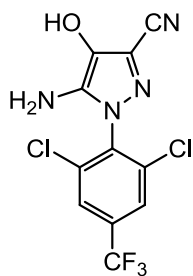
Fipronil sulfide (**22**)



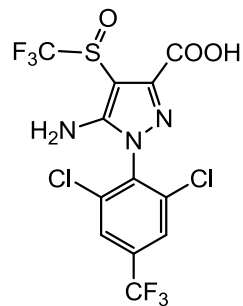
Fipronil sulfone amide (**23**)



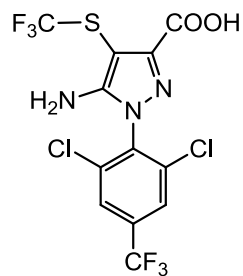
Fipronil sulfone chloramine (**24**)



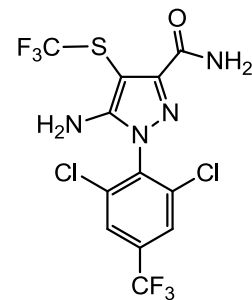
Hydroxy-fipronil (**25**)



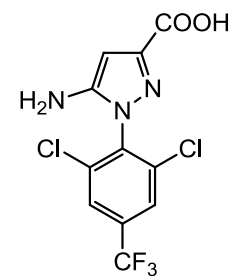
Fipronil acid (**26**)



Fipronil sulfide acid (**27**)



Fipronil sulfide amide (**28**)



Fipronil deSOCF<sub>3</sub> (**29**)



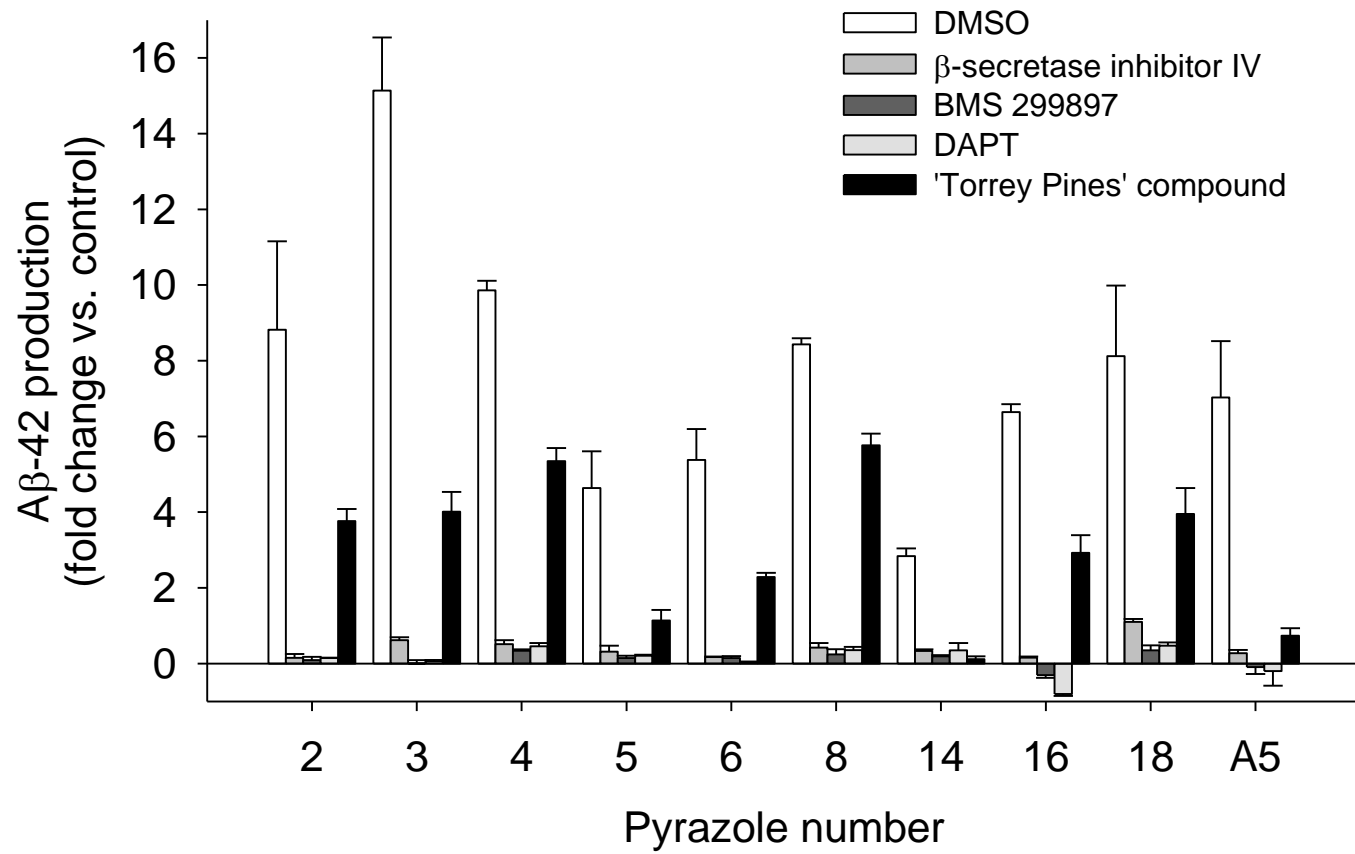


Figure 4

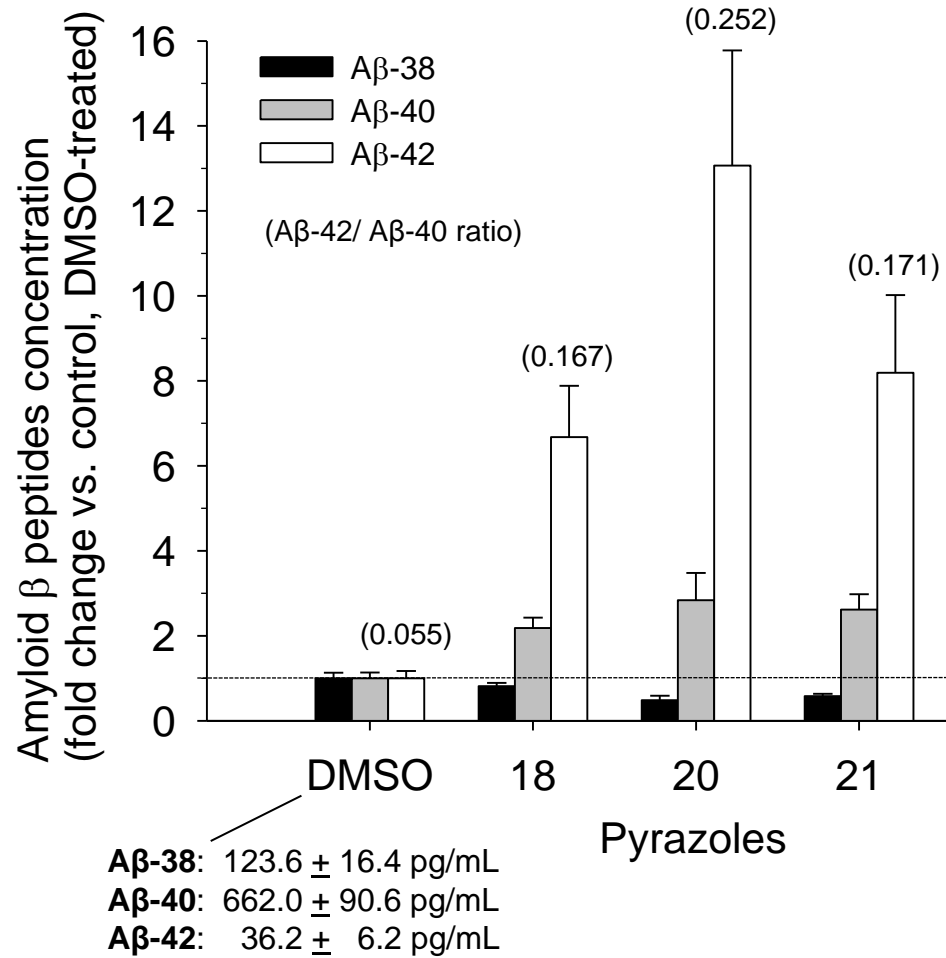


Figure 5

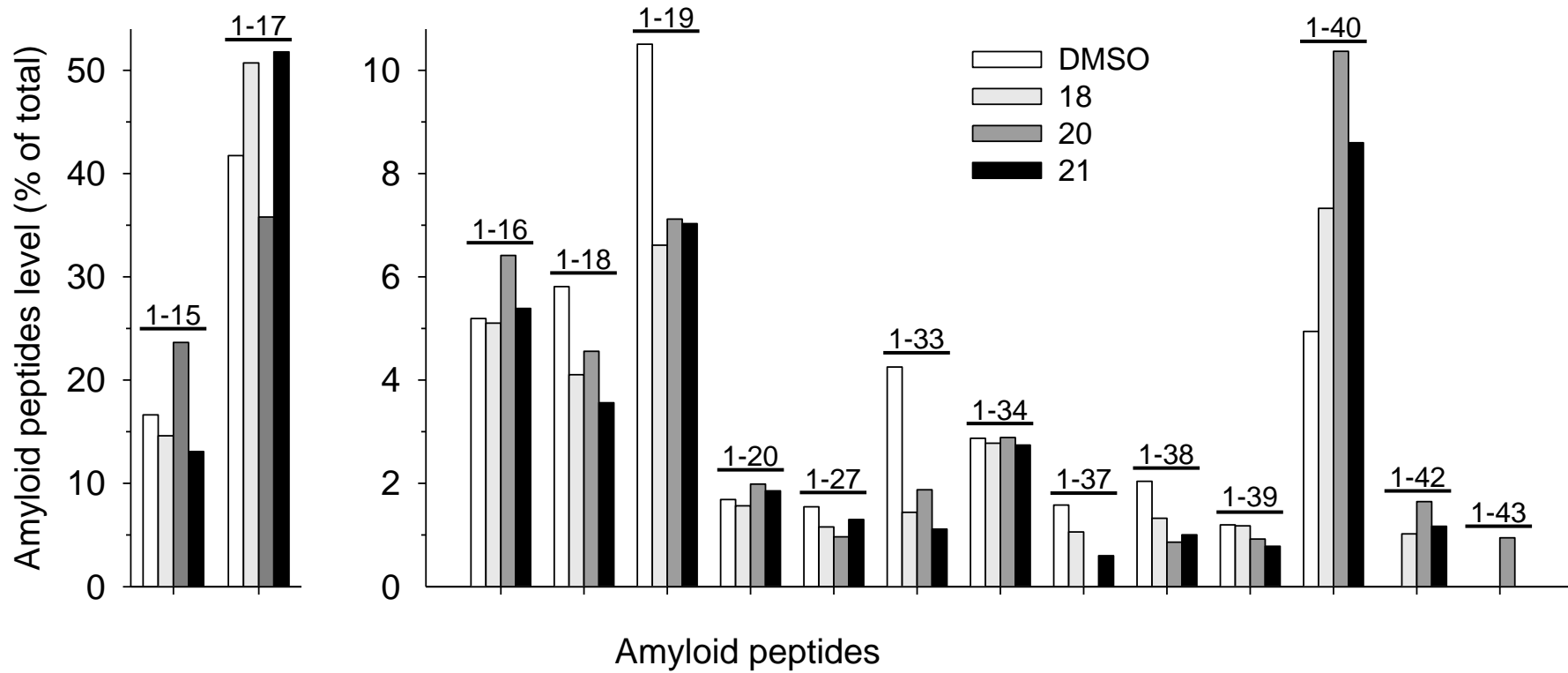


Figure 6

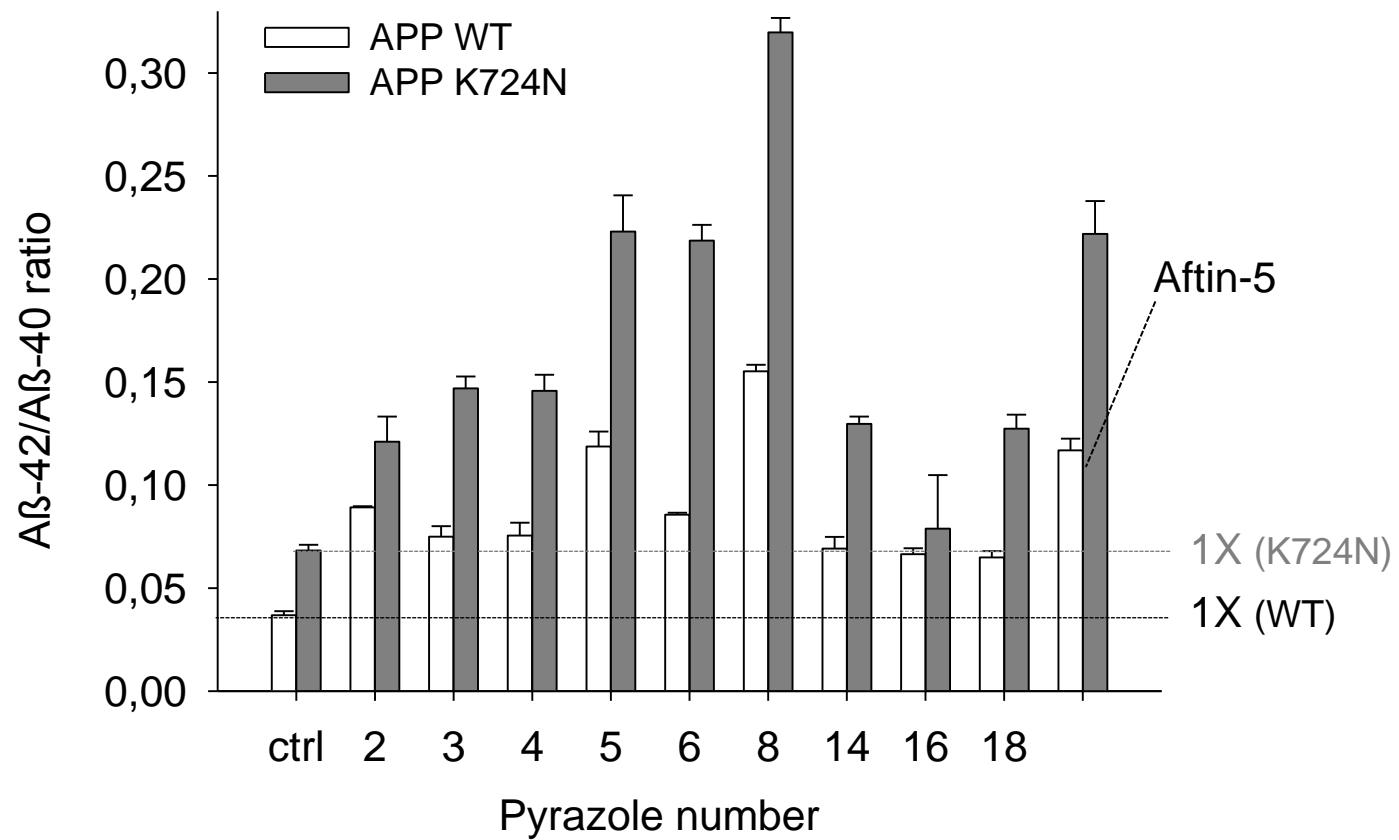
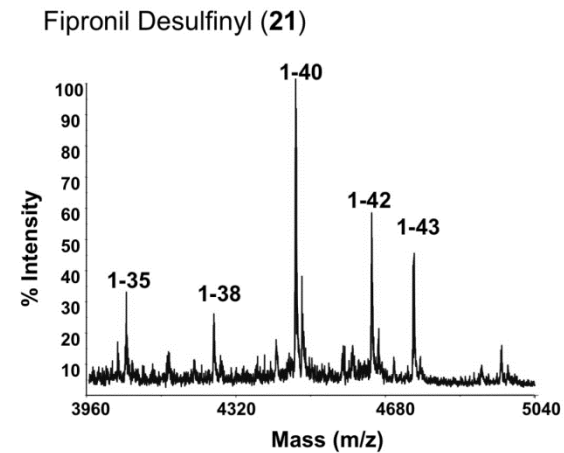
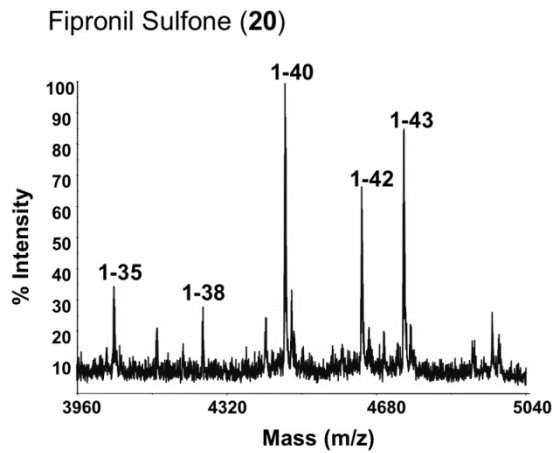
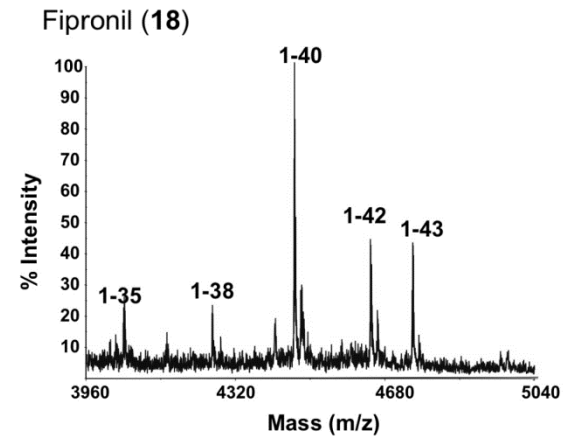
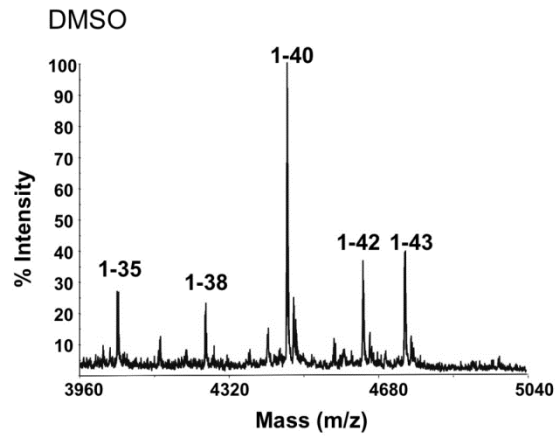


Figure 7

A



B

	A $\beta$ 42 / A $\beta$ 40	A $\beta$ 43 / A $\beta$ 40
DMSO	1.00	1.00
Fipronil (18)	1.23	1.13
Fipronil sulfone (20)	1.76	2.15
Fipronil desulfinyl(21)	1.45	1.10

Figure 8

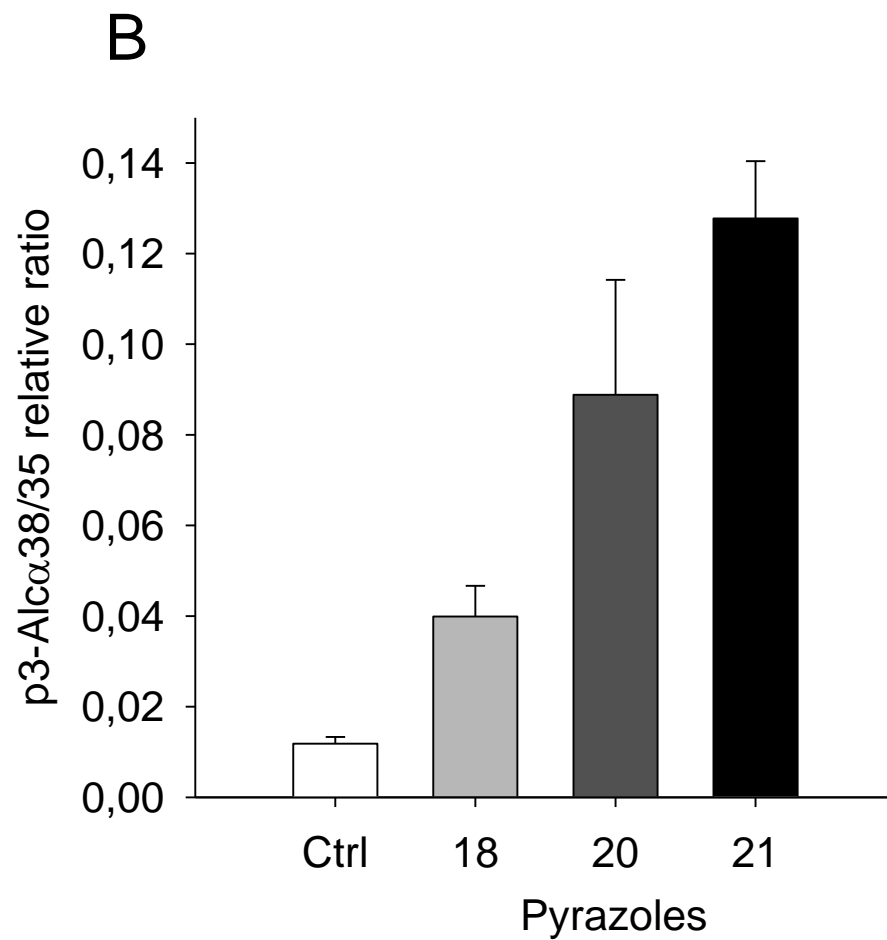
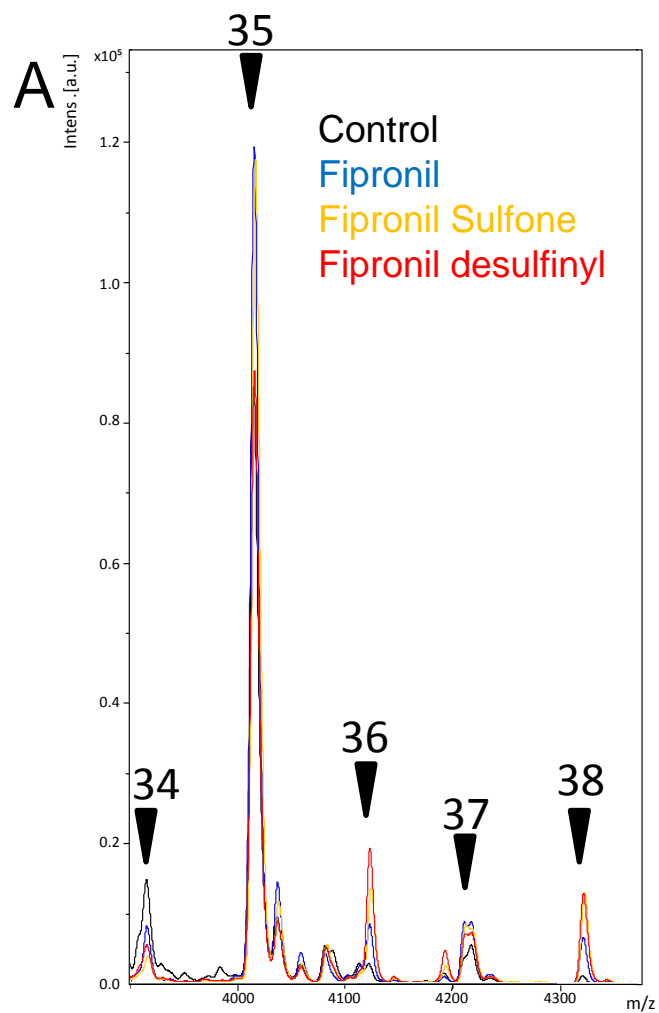
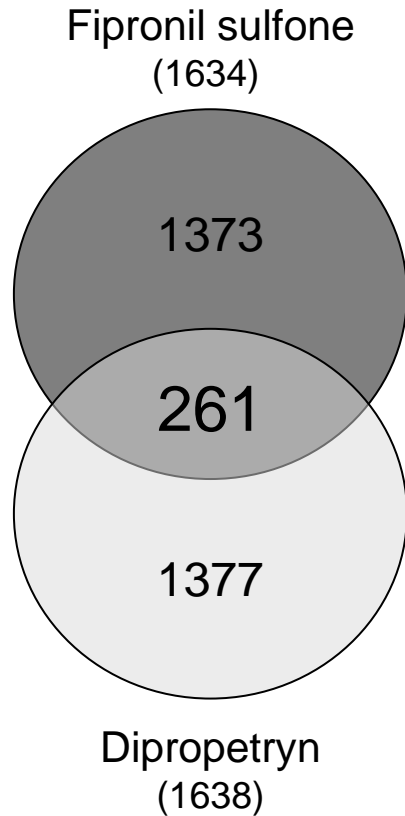


Figure 9

A



B

<b>Pathways (from KEGG)</b>	<b>Genes</b>	<b>%</b>	<b>p-value</b>	<b>FDR</b>
Non-alcoholic fatty liver disease	20	11.3	1.9E-16	2.7E-13
Oxidative phosphorylation	17	9.6	1.4E-13	1.7E-10
Parkinson's disease	17	9.6	4.2E-13	5.1E-10
Huntington's disease	17	9.6	3.5E-11	4.2E-8
Alzheimer's disease	16	9.0	7.9E-11	9.5E-8
Metabolic pathways	31	17.5	7.2E-7	8.7E-4
<b>Biological processes (GOTERM)</b>	<b>Genes</b>	<b>%</b>	<b>p-value</b>	<b>FDR</b>
oxidation-reduction process	25	14.1	3.8E-9	6.0E-6
cell cycle	21	11.9	3.4E-7	5.4E-4
regulation of cell cycle	9	5.1	5.7E-6	8.9E-3
cell division	14	7.9	2.2E-5	3.5E-2
mitotic nuclear division	12	6.8	2.9E-5	4.6E-2
<b>Cellular compartment (GOTERM)</b>	<b>Genes</b>	<b>%</b>	<b>p-value</b>	<b>FDR</b>
mitochondrial respiratory chain complex I	17	9.6	3.1E-22	4.0E-19
respiratory chain	17	9.6	1.5E-20	2.0E-17
mitochondrion	37	20.9	2.0E-7	2.6E-4
mitochondrial inner membrane	16	9.0	9.1E-7	1.2E-3

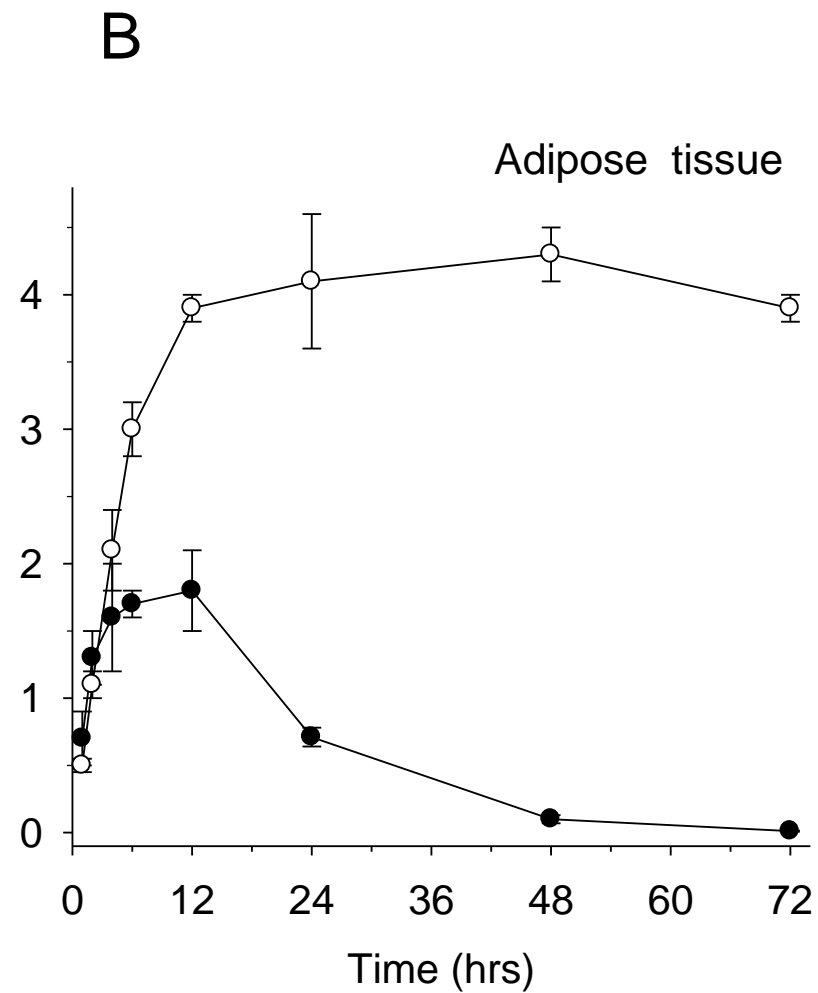
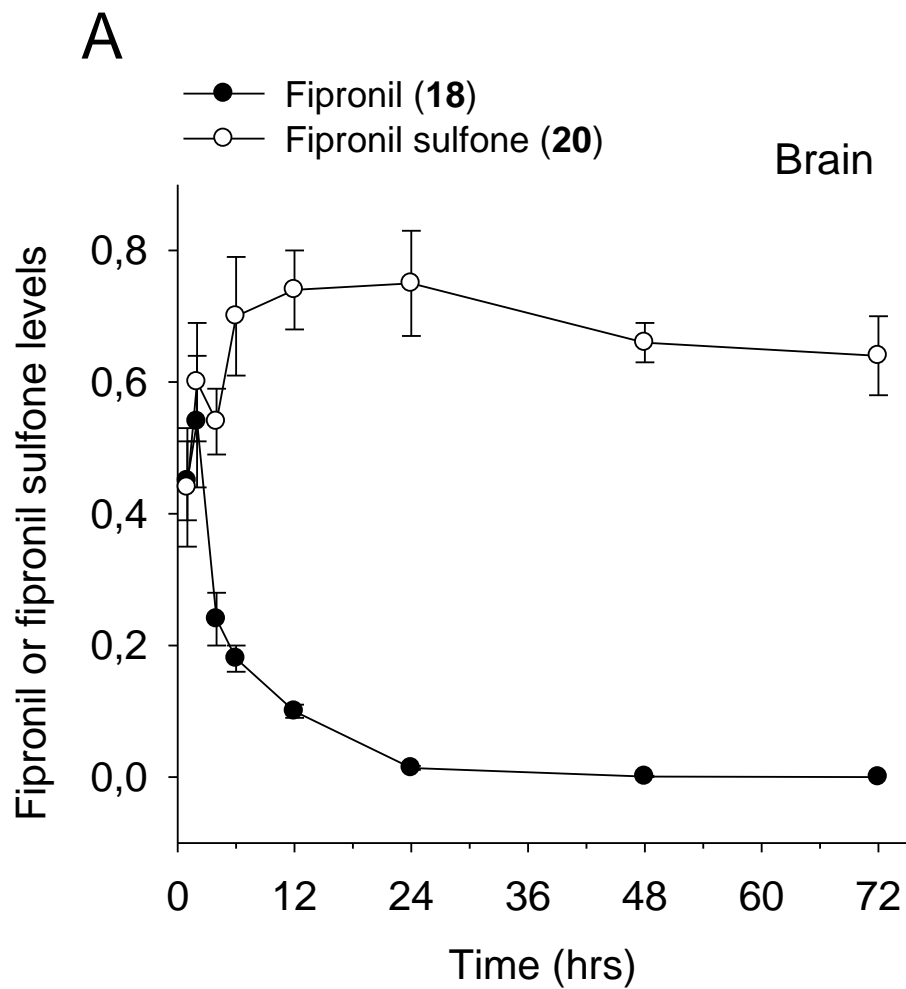


Figure 11A, 11B



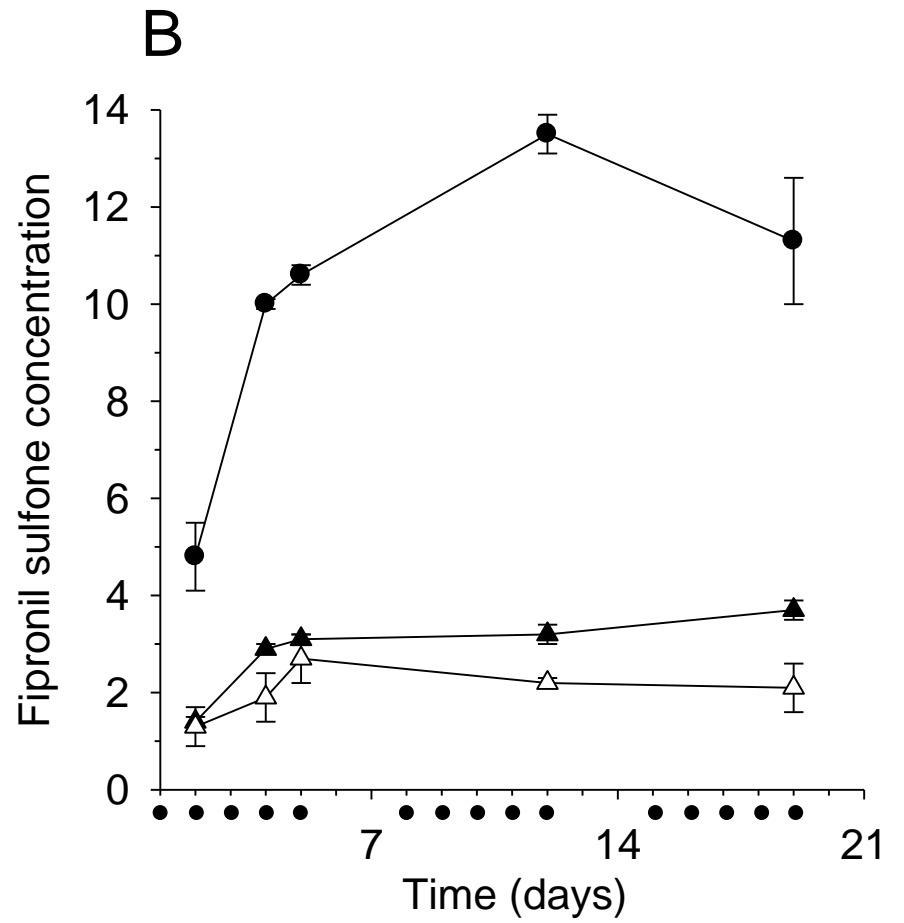
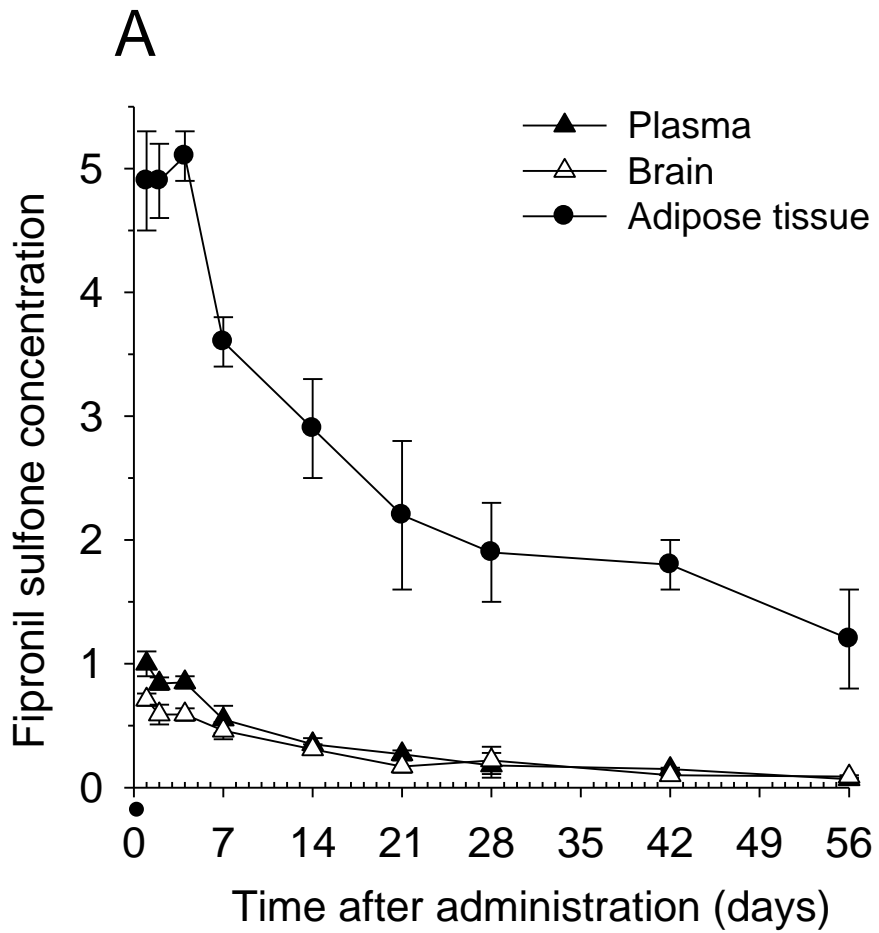


Figure 12A, 12B