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The Role of Flupyradifuron in Inducing Apoptosis in HMC3 Cells: An In Vitro Analysis

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Abstract

Objective

Flupyradifurone, a neonicotinoid insecticide, is widely applied in pest management. The present study aimed to elucidate the underlying mechanisms of flupyradifurone-induced toxicity in HMC3 microglial cells.

Material and Method

Cell viability was assessed by the MTT assay following exposure to flupyradifurone. In parallel, the expression of key apoptotic pathway genes, including MDM2, p53, and CASP9, as well as the apoptosis- and cell viability–associated microRNA hsa-miR-21-5p, was evaluated using RT-PCR.

Results

The results demonstrated that flupyradifurone induced dose-dependent cell death in HMC3 cells. RT-PCR analysis revealed a significant downregulation of MDM2, miR-21-5p, and an upregulation of p53 and CASP9 after 24 h of treatment.

Conclusion

This study reveals that FDP causes cell death in HMC3 cells, and this process occurs through apoptosis.

Keywords: Apoptosis, Casp9, flupyradifurone, hsa-miR-21-5p, MDM2, p53.

Introduction

The widespread application of pesticides has led to their persistence in soil and subsequent transport into aquatic systems via surface runoff, thereby contributing to water pollution. Through bioaccumulation in the food chain, these compounds can ultimately enter the human body and pose potential risks to human health. Ensuring food safety and protecting public health, therefore, requires a comprehensive and quantitative assessment of the toxic effects of pesticide residues on non-target organisms in the environment (1). Pesticides are generally classified based on their target organisms, with the principal categories including insecticides, herbicides, fungicides, bactericides, and rodenticides. Among these, insecticides represent one of the most widely applied groups. According to the Food and Agriculture Organization (FAO) of the United Nations, the most commonly used classes of insecticides worldwide are chlorinated organophosphates, hydrocarbons, pyrethroids, and carbamates (2). Over the past two decades, the fields of public health and pest

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management have undergone substantial growth (3). Within this context, insecticides targeting nicotinic acetylcholine receptors have exhibited the most rapid development and widespread adoption compared to other insecticide classes (2).

Neonicotinoid insecticides, which are highly efficacious and selective, have become the most effective insecticides in pest control. Neonicotinoids act as nicotinic acetylcholine receptor (nAChR) agonists (4). Synthesised by Bayer and officially registered and listed in China in 2018, flupyradifurone (FDP) is a novel butenolactone insecticide with low mammalian toxicity that acts on nAChR (5). nAChRs are heteromeric complexes composed of several subunits that are expressed in various brain cell types, including neurons, endothelial cells, astrocytes, and microglia (6). These receptors regulate a wide range of physiological processes, such as cell proliferation, neuronal differentiation, and apoptosis (7).

Flupyradifurone (FDP) has been shown to increase reactive oxygen species (ROS) levels while reducing the enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) (1). Environmental toxins are also known to induce mitochondrial dysfunction and oxidative stress through the overproduction of free radicals. Such mitochondrial impairment diminishes ATP synthesis, thereby damaging intracellular components and ultimately leading to cell death (8). Under physiological conditions, protein aggregation is prevented in healthy neurons by cellular quality control mechanisms (9). However, elevated oxidative stress disrupts protein homeostasis by impairing the ubiquitin–proteasome system (UPS), which is responsible for the degradation of misfolded or damaged proteins (8, 10). In addition, neuroinflammatory processes further exacerbate oxidative stress. Collectively, these mechanisms promote neuronal degeneration and are implicated in the pathogenesis of neurodegenerative disorders such as Parkinson's disease (8).

A review of the existing literature indicates that, while several studies have examined the effects of flupyradifurone (FDP) on different organisms, limited research has focused on its cytotoxic and molecular mechanisms in humans. By investigating the effects of FDP on human HMC3 microglial cells, the present study aims to fill this knowledge gap and contribute to the risk assessment of off-target biological toxicity.

Material and Method

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

The MTT (Sigma, USA) tetrazolium reduction assay represents the inaugural homogeneous cell viability assay developed for a 96-well format suitable for high-throughput screening. As evidenced by the thousands of published articles, the MTT assay technology has been widely adopted and continues to be popular in academic laboratories. The MTT assay described in the study by Riss et al. was performed in accordance with the methodology outlined in the present study. (11). In brief, HMC3 cells (ATCC, USA) were incubated with Dulbecco's Modified Eagle Medium (DMEM) (Capricorn, Germany) supplemented with 100 IU/ml penicillin, 10 µg/ml streptomycin (Sigma-Aldrich, USA), and 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA). The cells were seeded in 96-well plates at a density of 1x104 cells/well and incubated overnight before treatment with FPD (Sivanto). The cells were then treated with FPD at concentrations of 31.25 µg/mL to 1000 µg/mL for 24 hours. The optical densities were measured at a wavelength of 570 nm, employing a multiscan plate reader (Synergy HTX BioTek, USA).

Analysis by Quantitative Real-time PCR

A real-time polymerase chain reaction (PCR) analysis was conducted by exposing HMC3 cells to the determined half-maximal inhibitory concentration (IC50): 53.63 µg/mL and 31.25 µg/mL (lower dose) for 24 hours. According to the manufacturer's protocol, total RNA was isolated from HMC3 cells using the Hibrigen RNA Isolation Kit (Hibrigen, Turkey). The purity and quantity of the RNA samples were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The cDNA synthesis was conducted in accordance with the protocol outlined in the A.B.T.™ cDNA Synthesis Kit (Atlas Biotechnology, Turkey). The sequences of selected mRNAs are available on the website of the US National Center for Biotechnology Information. Before analysis, the primer sequences were tested. p53 F: TCTACAAGCAGTCACAGCACAT, p53 R: CAACCTCAGGCGGCTCATAG, MDM2 F: TGGCGTGCCAAGCTTCTCTGT, MDM2 R: ACCTGAGTCCGATGATTCCTGCT, CASP9 F: GTGACCCCAGAATTGACCCT, CASP9 R: ACCATGAAATGCAGCGAGGA, ACTB F: CATGTACGTTGCTATCCAGGC, ACTB R: CTCCTTAATGTCACGCACGAT. The present study employed the CFX96 real-time quantitative polymerase chain reaction (qPCR) instrument (Bio-Rad, CA, USA). The A.B.T.™ 2X SYBR-Green MasterMix (Atlas Biotechnology, Turkey) was utilised for real-time qPCR. In accordance with the manufacturer's guidelines, real-time qPCR conditions were established. The expression of the ACTB gene was used to normalise the results.

For miRNA translation into cDNA, a stem-loop primer

and miR cDNA synthesis kit were employed for miRNA conversion into cDNA, in accordance with the manufacturer's instructions (A.B.T.™ miRcDNA synthesis kit, Turkey). The sequences of the U6 (F:5′- 5′-CGCTTCGGCACATATACTA-3′; R: 5′-CGCTTCACGAATTTGCGTGTCA-3) gene region were utilised for the purpose of normalising the hsa-miR-21-5p (F:5′-GGGGTAGCTTATCAGACTGATG-3′; R:5′-TGTCGTGGAGCGGCAATTG-3′. The Cq values obtained were normalised and calculated using the formula 2-ΔΔ Cq (12, 13).

miRNA Selection / Biological Processes/ Interaction With Genes

The selection of hsa-miR-21-5p was determined using the mirPath software from DIANA TOOLS (https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath#mirnas=hsa-miR-21-5p&methods=Tarbase&selection=0). The miRNA in question has been demonstrated to influence the expression of certain genes within the p53 signalling pathway ($p < 0.001$). Fig 1 shows the genes targeted by hsa-miR-21-5p. The fold changes of biological processes related to hsa-miR-21-5p were obtained using Funrich enrichment analysis tool v.3.1.3 (http://funrich.org/download). The interactions between the p53, MDM2, and CASP9 genes associated with biological functions were searched using the GeneMANIA prediction server (http://genemania.org).

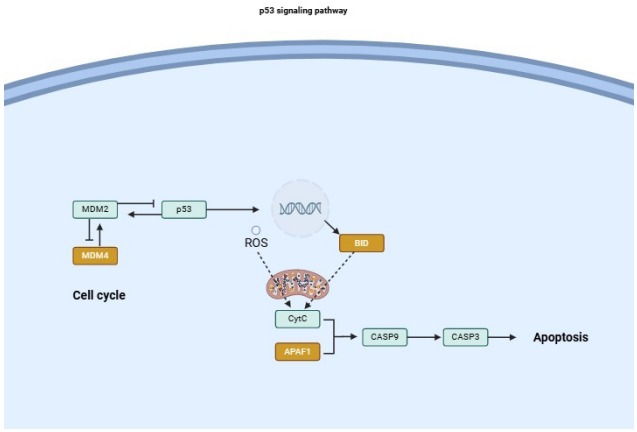

Figure 1
The p53 signaling pathway of hsa-miR-21-5p and its target genes are indicated by brown boxes.

Statistical Analysis

In the statistical analysis, the results of the expression tests were compared between the groups. The Shapiro-Wilk W test was employed to analyse the normal distribution of the numerical parameters. One-way analysis of variance (ANOVA) was utilised for the statistical analysis. These analyses and IC50 calculation were conducted using GraphPad Prism v. 8.0.1 (GraphPad Software, USA). The level of significance was set at $p < 0.05$.

Results

FDP Reduces HMC3 Cell Viability

The effect of FDP at varying concentrations on HMC3 cell viability was evaluated, and the IC_{50} was determined to be 53.63 µg/mL (Fig 2). A dose-dependent reduction in cell viability was observed with increasing FDP concentrations.

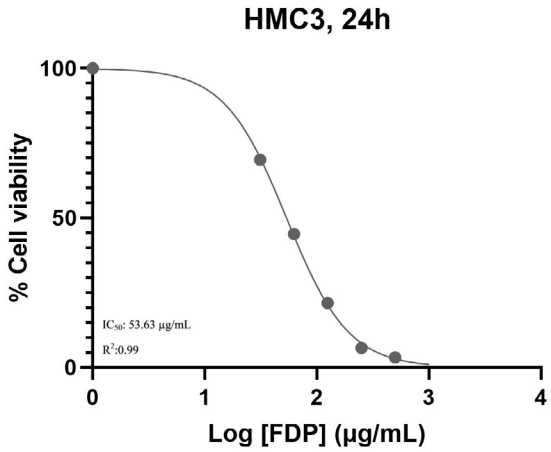

Figure 2
MTT results of HMC3 microglia cells treated with flupyradifurone for 24 hours

Effects of FDP on miRNA and Gene Expression

Gene expression analysis revealed that p53 and CASP9 were significantly upregulated in the IC_{50} group compared to the control group ($p < 0.05$). In contrast, the expression levels of MDM2 and hsa-miR-21-5p were significantly downregulated in the IC_{50} group relative to controls ($p < 0.05$). These findings indicate that p53, MDM2, and CASP9 actively participate in the intrinsic apoptotic pathway and mediate cellular responses to environmental and abiotic stimuli. Furthermore, the results demonstrated that p53 and MDM2 are involved in the regulation of the cell cycle (Fig 3).

Discussion

FDP is a novel butenolide insecticide that targets nAChRs and features a unique bioactive butenolide pharmacophore with considerable market potential (1). Previous studies have reported that FDP influences oxidative stress levels in various organisms, including

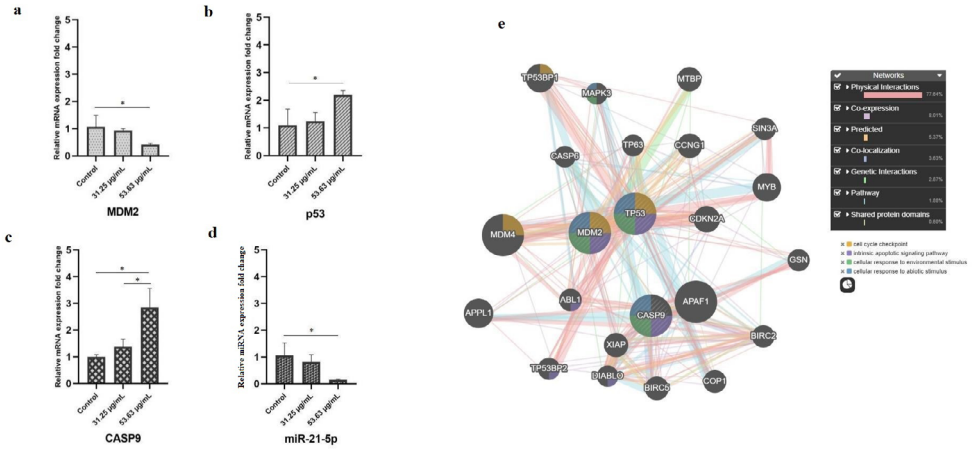

Figure 3

a) MDM2 mRNA expression fold change (* $p < 0.05$), b) p53 mRNA expression fold change (* $p < 0.05$), c) CASP9 mRNA expression fold change (* $p < 0.05$), d) miR-21-5p expression fold change (* $p < 0.05$), e) biological process based p53, MDM2, CASP9 interaction networks.

zebrafish, honeybees, and earthworms. Specifically, exposure to FDP was associated with increased oxidative stress markers such as SOD, CAT, and ROS in zebrafish (1), ROS/RNS in honeybees (4), and DCFH-DA, MDA, SOD, CAT, and ROS in earthworms (14). In addition, apoptotic markers were altered in response to FDP-induced oxidative stress, including Bax, Bcl-2, and CASP9 in zebrafish (1), and CASP3 in honeybees (4).

FDP, acting as an nAChR agonist, has been shown to induce nervous system impairment and alterations in signaling pathways in earthworms. Substantial evidence further indicates that exposure to FDP leads to oxidative cellular damage and triggers apoptosis (4). Exposure to FDP has been reported to disrupt cellular lipid homeostasis, potentially leading to abnormal fatty acid β-oxidation and excessive energy expenditure in women, as well as increased triglyceride accumulation in men (15). In addition, a study investigating the cytotoxic and genotoxic effects of FDP in human lymphocyte cultures demonstrated significant increases in chromosomal aberrations (CA) and micronucleus (MN) formation at medium (170 µg/mL) and high (340 µg/mL) concentrations compared to the control group This is evidence that FPD may have cytotoxic and genotoxic effects in human lymphocytes (16). These findings provide evidence that FDP may exert both cytotoxic and genotoxic effects in human lymphocytes.

The relationship between MDM2 and TP53 is based on the hypothesis that MDM2 acts as a negative regulator of TP53. Several studies have provided evidence that strengthens and supports the antagonistic role of MDM2 for TP53. The tumour suppressor functions of TP53 include cell cycle arrest in response to stress, cell senescence, and regulation of the expression of genes that lead to apoptosis (17).

Research on various pesticides has demonstrated their adverse effects on living organisms. For example, etalfluralin, a member of the dinitroaniline herbicide family, is widely used as an effective weed control agent. In a study on zebrafish larvae, etalfluralin exposure was shown to increase reactive oxygen species (ROS) production, induce inflammation and mitochondrial dysfunction, and upregulate the expression of apoptosis-related genes, including p53, Casp9, and Casp3 (18). Tebuconazole (TEB), a triazole fungicide, is widely used for controlling fungal growth on seeds, vegetables, and fruits. Exposure to TEB has been shown to increase lipid peroxidation and DNA damage, as well as elevate the expression of p53, p21, and CASP9 in H9c2 cardiomyoblast cells (19). One study investigating the effects of chlorpyrifos, an organophosphate pesticide, on murine microglial cells (BV-2) demonstrated that exposure increased oxidative stress markers, including NO, MDA, and $O_2^{\cdot}$, and upregulated the expression of genes encoding pro-inflammatory cytokines, such as IL-1β and NLRP3 (20).

Microglia play a critical role in maintaining a healthy nervous system by clearing cellular debris, abnormal proteins, and invading pathogens, including bacteria and viruses, which could otherwise be harmful to the

organism. However, when their clearance capacity is impaired, the structure and function of the nervous system are compromised (21).

MicroRNAs (miRNAs) are a class of non-coding RNAs that play critical roles in regulating gene expression across a variety of diseases (22). Among them, miR-21 was one of the first mammalian miRNAs to be identified and is involved in the onset and progression of multiple diseases. Apoptosis, a genetically regulated form of cell death, allows organisms to adapt to environmental changes, and its dysregulation can contribute to a range of central nervous system (CNS) disorders (23). Administration of a miR-21-5p inhibitor in rats has been reported to cause significant loss of hippocampal neurons, accompanied by increased CASP3 and Bax levels and decreased Bcl-2 expression, highlighting its role in apoptosis (24). Conversely, elevated miR-21 levels have been shown to promote cell growth, survival, and proliferation, while concurrently suppressing immune regulation and apoptosis (25). Furthermore, miR-21 has been implicated in mitigating apoptosis through its involvement in pathophysiological processes such as oxidative stress (26). Dysregulation of miR-21 and its target genes has also been suggested to increase susceptibility to various CNS disorders and may serve as a diagnostic biomarker for conditions including myasthenia gravis, multiple sclerosis, Parkinson's disease, and epilepsy (23). Fig 4 illustrates the fold changes of biological processes associated with hsa-miR-21-5p.

In this study, the human brain microglial cell line HMC3 was employed to analyse the effect of FDP. The results demonstrated that the treatment with FDP induced apoptosis by causing DNA damage in a dose-dependent manner, thus affecting cell viability. Consequently, a significant alteration in the expression levels of MDM2, p53, and CASP9, which are associated with apoptosis, was observed in FDP-treated HMC3 microglial cells in comparison to the control group ($p < 0.05$). The expression level of miR-21 was significantly decreased in FDP-treated HMC3 microglial cells compared to the control ($p < 0.05$).

This study has some limitations. First, the experiments were conducted only in the HMC3 microglial cell line, and the effects of flupyradifuron on the p53–MDM2 axis cannot be directly generalized to other central nervous system cell types or in vivo conditions. Furthermore, the cell line may reflect immune response and tumor-suppressive signaling pathways to a limited extent compared to the natural microglia population. The study evaluated only specific concentrations and exposure times, failing to cover all variations in molecular responses in terms of dose and time. Therefore, further studies involving primary microglia and animal models are needed to extend the findings.

The findings of this study demonstrate that FDP exerts cytotoxic effects on human microglial HMC3 cells by inducing mitochondrial dysfunction and apoptosis in a dose-dependent manner. FDP exposure resulted in significant alterations in apoptosis-related gene

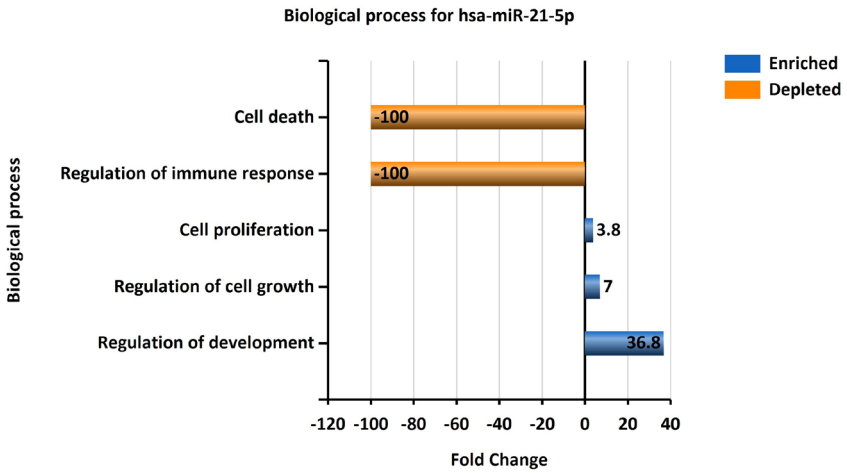

Figure 4
Expression of miR-21-5p-related changes in biological process as fold change using Funrich enrichment software v.3.1.3.

expression, characterized by the upregulation of p53 and CASP9 and the downregulation of MDM2 and hsa-miR-21-5p. These results contribute to the growing body of knowledge regarding the molecular mechanisms by which neonicotinoid insecticides may affect microglial function. Further in vivo studies are warranted to validate these findings and to better assess the potential risks of FDP exposure to human health.

Conflict of Interest Statement
There is no conflict of interest to declare.

Ethical Approval
This article does not contain any studies on human or animal subjects.

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Availability of Data and Materials
Data available on request from the authors.

Artificial Intelligence Statement
The authors declare that they have not used any type of generative artificial intelligence for the writing of this manuscript, nor for the creation of images, graphics, tables, or their corresponding captions.

Authors Contributions
O.S: Conceptualization, methodology, investigation, administration, visualization, data curation, formal analysis writing-original draft.

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