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Toxicity evaluation of new agricultural fungicides in primary cultured cortical neurons

Jorge Regueiro [a,b], Nair Olguín [a], Jesús Simal-Gándara [b], Cristina Suñol [a,*]

[a] Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona (IIBB), Consejo Superior de Investigaciones Científicas (CSIC), IDIBAPS, CIBERESP, Barcelona, Spain

[b] Department of Analytical Chemistry and Food Science, Nutrition and Bromatology Group, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, Ourense, Spain

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ABSTRACT

Fungicides are crucial for food protection as well as for the production of crops of suitable quality and quantity to provide a viable economic return. Like other pesticides, fungicides are widely sprayed on agricultural land, especially in wine-growing areas, from where they can move-off after application. Furthermore, residues of these agrochemicals can remain on crops after harvest and even after some food processing operations, being a major exposure pathway. Although a relatively low toxicity has been claimed for this kind of compounds, information about their neurotoxicity is still scarce.

In the present study, nine fungicides recently approved for agricultural uses in the EU – ametoctradin, boscalid, cyazofamid, dimethomorph, fenhexamid, kresoxim-methyl, mepanipyrim, metrafenone and pyraclostrobin – have been evaluated for their toxicity in primary cultured mouse cortical neurons. Exposure to 0.1–100 μM for 7 days *in vitro* resulted in a dose-dependent toxicity in the MTT cell viability assay. Strobilurin fungicides kresoxim-methyl (KR) and pyraclostrobin (PY) were the most neurotoxic compounds (lethal concentration 50 were in the low micromolar and nanomolar levels, respectively) causing a rapid raise in intracellular calcium $[Ca^{2+}]_i$ and strong depolarization of mitochondrial membrane potential. KR- and PY-induced cell death was reversed by the calcium channels blockers MK-801 and verapamil, suggesting that calcium entry through NMDA receptors and voltage-operated calcium channels are involved in KR- and PY-induced neurotoxicity. These results highlight the need for further evaluation of their neurotoxic effects *in vivo*.

1. Introduction

In recent years, the agricultural industry has experienced strong market and technological competition among the producing countries, resulting in increased production yields while reducing costs for both producers and consumers. In this regard, pesticides are of paramount importance since they allow controlling insect or fungal infestations or growth of weeds, either to handle immediate infestations or to anticipate long-lasting problems (Regueiro et al., 2015). Among them, fungicides are used to manage fungal disease organisms on growing crops, or used as a post-harvest treatment to prevent fungi or molds causing food to rot during storage or transport. Therefore, fungicides have become essential for food protection as well as for maintaining healthy crops, and consistent yields of high quality produce. In fact, fungicides accounted for over 52% of all plant protection products used in the European Union (EU) in 2003, with an estimated consumption above 100 kt (Eurostat, 2007). Like other pesticides, fungicides are widely sprayed on agricultural land and therefore they can move-off of fields after application, becoming a potential health risk not only for farm workers, but also for residents and children by exposure through the air, soil and water environment (Kamel and Hoppin, 2004). Furthermore, residues of these agrochemicals can remain on crops after harvest and even after some food processing operations (Kaushik et al., 2009).

Because of the growing health concerns about the use of ethylene-bis-dithiocarbamate (EBDC) fungicides, especially maneb and mancozeb (Bjørling-Poulsen et al., 2008; Domico et al., 2006), a number of fungicides showing new modes of action have been released in the recent years. These include compounds which affect cell wall biosynthesis in fungal cells (dimethomorph), sterol biosynthesis in membranes (fenhexamid), amino acid and protein synthesis (mepanipyrim), fungal respiration (ametoctradin, boscalid, cyazofamid, kresoxim-methyl and pyraclostrobin) or even with unknown mode of action (metrafenone) (FRAC, 2014). Some of them have been very recently included in EU list of authorized

* Corresponding author.
E-mail address: csenqi@iibb.csic.es (C. Suñol).

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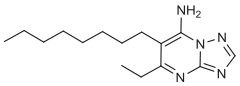
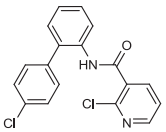
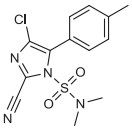
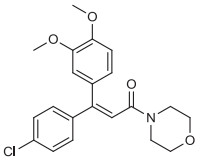
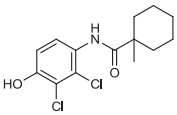
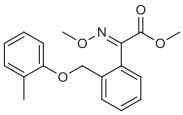
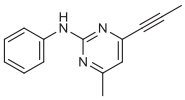
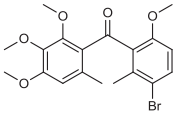
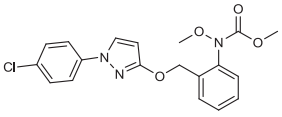
Table 1
Structures, characteristics and modes of action of the studied fungicides.

Fungicide	Structure	Chemical group	Log K_{ow}[a]	Mode of action[b]	Approval year[c]	ADI (mg/kg bw/d)
Ametoctradin (AM)		Triazolo-pyrimidylamine	4.4	Respiration inhibition of complex III: cytochrome bc1(ubiquinone reductase) at Qo site, stigmatellin binding sub-site	2013	10
Boscalid (BO)		Pyridine-carboxamide	2.96	Respiration inhibition of complex II: succinate dehydrogenase inhibitors	2008	0.04
Cyazofamid (CY)		Cyano-imidazole	3.2	Respiration inhibition of complex III: cytochrome bc1(ubiquinone reductase) at Qi site	2003	0.17
Dimethomorph (DI)		Cinnamic acid amide	2.68	Cell wall biosynthesis: cellulose synthase	2007	0.05
Fenhexamid (FE)		Hydroxyanilide	3.51	Sterol biosynthesis in membranes: 3-keto reductase in C4-de-methylation (erg27)	2001	0.2
Kresoxim-methyl (KR)		Strobilurin	3.40	Respiration inhibition of complex III: cytochrome bc1(ubiquinol oxidase) at Qo site (*cyt b gene*)	1999	0.4
Mepanipyrim (MY)		Anilino-pyrimidine	3.28	Amino acid and protein synthesis: methionine biosynthesis (*cgs gene*)	2004	0.02
Metrafenone (ME)		Aryl-phenyl-ketone	4.3	Unknown: actin disruption (proposed)	2007	0.25
Pyraclostrobin (PY)		Strobilurin	3.99	Respiration inhibition of complex III: cytochrome bc1(ubiquinol oxidase) at Qo site (*cyt b gene*)	2004	0.03

[a] (Tomlin, 2004).
[b] (FRAC, 2014).
[c] (European Commision, 2009); Log K_{ow}: octanol-water partition coefficient; ADI: acceptable daily intake.

plant protection products (Table 1) according to Commission Regulation (EC) No 1107/2009 concerning the "placing of plant protection products on the market" (European Commision, 2009). The main properties of these new fungicides are their broad spectrum of action, their special penetration and redistribution properties and their long-term stability, which results in better long-term efficacy, allowing the use of lower concentrations of active substances (Wightwick et al., 2010). These fungicides are used to treat and prevent a broad range of fungal diseases such as late blight and downey mildew on potatoes, eyespot and powdery mildew on cereals, gray mold on strawberries, grapes, tomatoes and cucumbers, or scab on apples and pears (PPDB, 2014). They have also been proposed as effective agents against the fungal production of aflatoxins (Sakuda et al., 2014).

Recent studies suggest that exposure to certain agrochemicals, including fungicides such maneb and mancozeb, may be involved in the development of neurodegenerative disorders such as Parkinson's disease (Bjørling-Poulsen et al., 2008; Costello et al., 2009). Although a relatively low toxicity is claimed for the newly formulated fungicides, which acceptable daily intakes (ADI) have been established from 0.02 to 10 mg/kg body weight (Table 1), little is known regarding their contribution to neuronal toxicity. Thus, the aim of the present work was to investigate the toxic effects of nine recently approved fungicides with different modes of action on primary cultured cortical neurons.

2. Materials and methods

2.1. Materials

Pregnant NMRI mice (16th gestational day) were obtained from Charles River/Iffa Credo (Saint Germain-sur-l'Arbreste, France). Plastic multi-well culture plates were purchased from Nunc (Rockilde, Denmark). Fetal bovine serum (FBS) was from Gibco (Invitrogen, Barcelona, Spain). Dulbecco's modified Eagle's minimum essential medium (DMEM) was obtained from Biochrom (Berlin, Germany). Isofluorane (FORANE) was from Abbot