Chemosphere 104 (2014) 91–96

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Determination of toxicity of neonicotinoids on the genome level using chemogenomics in yeast

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HIGHLIGHTS

- Additives are responsible for side effects of formulations including neonicotinoids.
- Chemogenomics provided a possible mechanism of toxicity for inhibition of spermatogenesis.
- Neonicotinoids negatively affect cell wall organization and biogenesis in yeast.

ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 14 October 2013
Accepted 16 October 2013
Available online 18 November 2013

Keywords:
Neonicotinoids
Additives
Side effects
Chemogenomics
Yeast *Saccharomyces cerevisiae*
Spermatogenesis

ABSTRACT

Neonicotinoid insecticides are an important contribution to plant protection products. At the same time, their environmental impact on non-target organisms is often problematic. It has been shown that the toxicity of formulations of neonicotinoid insecticides can originate from non-neonicotinoid additives. In the present study we used chemogenomics to analyse side effects of purified neonicotinoids, additives and formulations on the genome-wide scale. We show that the additives in formulations have more pronounced effects than the active components, and that these effects could explain previously observed negative effects of neonicotinoid insecticides on spermatogenesis in animals. We also demonstrate that cell wall organization and biogenesis in yeast is negatively affected by neonicotinoid substances.

1. Introduction

In spite of known harmful effects on the environment and negative experiences with pesticides in the past, we can at present not imagine how sufficient quantities of high quality food could be provided without using plant protection products. Since pests relatively quickly develop resistance to existing insecticides, the discovery of new insecticidal compounds with unique modes of action is essential for efficient crop protection in the future. In the development of new insecticides, research is aimed at synthesizing molecules that exhibit both efficient and spectrally broad protection against insects, while acting upon a specific target site. Discoveries of crop-protecting compounds with high efficiency and broad spectrum of pest control, coupled with action at a novel target site, are however relatively rare (Cordova et al., 2006). Neonicotinoids are relatively new systemic insecticides which are chemically similar to nicotine – the toxin present in tobacco. All commercially available neonicotinoids (imidacloprid, acetamiprid, clothianidin, dinotefuran, nitenpyram, thiacloprid and thiamethoxam) resemble nicotine and epibatidine, both of which are potent antagonists of nicotinic acetylcholine receptors (nAChRs). Neonicotinoids have partially positive charge and can irreversibly bind to nAChRs. The binding affinity of neonicotinoids is higher for nAChRs from insects than those from mammals (Matsuda et al., 1998; Roberts, 1999).

The toxicity of pesticides relies on their affinity to key biological molecules, but other molecular targets for pesticide toxicity cannot be excluded. In addition to the active compound, commercial formulations usually contain solvents or compounds which improve properties important for easier application and better penetration. These compounds are typically considered as inert and are usually excluded from the assessment of possible adverse effects on non-target organisms. It should be additionally stressed that also legislation regulating the placement of pesticides on the market focuses

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http://dx.doi.org/10.1016/j.chemosphere.2013.10.063

only on active (parent) compounds and neglects complete formulations and transformation products, although reports exist in the literature that for non-target organisms the composition of formulations can be more important than active substances (Anderson and Roberts, 1983; Neves et al., 2001).

Aquatic toxicology has made an immense contribution to our understanding of how natural and man-made substances affect the living environment. Consequently, it has also played a central role in the development of policies and strategies for environmental protection, providing the scientific basis for many of the standards and quality objectives now applied in waste management and water pollution control. To perform toxicity experiments, usually a base set of test species from different taxonomic groups, which are most frequently used for toxicity identification of chemicals and biocides, is selected, e.g. bacteria *Vibrio fischeri*, algae *Desmodesmus subspicatus,* crustacean water flea *Daphnia magna* and fish *Danio rerio*. Few studies however exist where the formulations of neonicotinoids were tested on aquatic organisms (Jemec et al., 2007; Tišler et al., 2009; Malev et al., 2012). In general, much more studies have dealt with the toxicity assessment of pure neonicotinoid on different terrestrial (Drobne et al., 2008; Trebše et al., 2009) and aquatic organisms (Jemec et al., 2007). Available data indicate that imidacloprid can be highly toxic to some aquatic crustaceans, but generally less toxic to fish (TDC Environmental, 2003; Jemec et al., 2007). Also, the LD50s for mammals and birds are much higher than for invertebrates (Anatra-Cordone and Durkin, 2005). Recent controversies regarding neonicotinoids were largely linked to their effect on pollinators, especially bees (Stokstad, 2013). Acute and chronic toxicity of imidacloprid and its metabolites is high in bees, as the acute toxicity (LD50) has been found to be 60 ng/organism (Suchail et al., 2001), and the chronic toxicity test revealed 50% mortality at approximately 8 d. For acetamiprid, LD50 for bees is approximately 400-fold higher compared to imidacloprid (Iwasa et al., 2004). For thiamethoxam, LD50 toxicity data for bees in contact with the insecticide was found to be 24 ng/organism (Senn et al., 1998), and this active compound severely affected behaviour of bees causing decreased foraging success and lower survival rate (Henry et al., 2012).

At the molecular level, some research on the action of neonicotinoids has been focused on anti-inflammatory and analgesic effect in mice (Tomizawa et al., 2001). However, the majority of studies addressing the environmental safety of the neonicotinoids, mainly imidacloprid, was focused exclusively on the function of acetylcholine receptors (Buckingham et al., 1997; Anatra-Cordone and Durkin, 2005), and the data on side effects of neonicotinoid formulations are lacking. In contrast to such focused approach, toxicogenomics and chemogenomics offer methodologies to identify also other potential target molecules affected by these substances (reviewed in Dos Santos et al., 2012). Yeast *Saccharomyces cerevisiae* has been successfully used as a model organism for the study of the toxicity of pesticides even before the advent of genomics approaches (Cabral et al., 2003). In the functional genomics era this model organism has become extremely popular because of the similarity in key biological processes with homologous processes in other eukaryotes, including humans, and because of a wide spectrum of available genome-wide techniques and the most thoroughly annotated genome among all eukaryotes (Andrusiak et al., 2012; Mattiazzi et al., 2012; Roemer et al., 2012). An example of using chemogenomics in yeast for the identification of both primary and secondary targets of a pesticide is the study of Dias et al. (2010), wherein the effects of the agricultural fungicide Mancozeb were assessed. In this study, cellular processes such as oxidative stress response, protein degradation and carbohydrate metabolism have been identified as part of the response to Mancozeb in yeast, and these results contributed to the understanding of the toxicity of this compound in humans.

In the present study, the focus was on the most used neonicotinoid insecticides imidacloprid, acetamiprid, and thiamethoxam. Apart from the active substances, the effects of complete formulations were analysed: imidacloprid containing formulation Confidor, acetamiprid and its formulation Mospilan, and thiamethoxam and its formulation Actara. Dimethyl sulfoxide (DMSO) and N-methyl-2-pyrrolidone (NMP) were also tested as the known additives to Confidor and other pesticide formulations. Active substances were isolated from commercial formulation, and their stability in media during the experiment was confirmed by classical analytical methods. The growth rates of single deletion mutants of yeast *S. cerevisiae* were measured in the presence of the tested compounds/formulations to demonstrate that (i) in most cases additives in the formulations exert more pronounced effects than active substances in yeast cells, and (ii) neonicotinoid active substances have a common negative impact on the cell wall organization and/or biogenesis in yeast.

2. Materials and methods

2.1. Yeast strains

S. cerevisiae gene deletion collection (EUROSCARF), constructed in the BY4741 background strain (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 xxxΔ::Kan*^{r}) and representing all non-essential yeast genes, was used in the study. The strains were arrayed on 14 individual plates of 384 colonies per plate, together comprising 5376 colonies and 4293 mutated genes, where control (“wild-type”; i.e. background strain without additional deletions) strains (*MATa his3Δ::Kan*^{r} *leu2Δ0 met15Δ0 ura3Δ0*) were placed on the rim rows and columns.

2.2. Media and reagents

Control and master plates contained 1% (w/v) yeast extract (Sigma, USA), 2% (w/v) peptone (Sigma, USA), 2% (w/v) D-(+)-glucose (Fluka, Germany), 2% (w/v) agar (Sigma, USA) and 200 mg L^{-1} antibiotic geneticin (Gibco-Invitrogen, USA). In the test plates, different formulations or active substances were added to the medium of the upper composition, one per test.

Mospilan 20 SP (Nippon Soda Co., Japan), Confidor 200 SL (Bayer, Germany) and Actara 25 WG (Syngenta Crop Protection AG, Switzerland) pesticides were analysed in this study. Purified neonicotinoids and Mospilan, Confidor and Actara were added before autoclaving since prior testing (see Section 2.4 below) demonstrated stability of their active substances under autoclaving conditions. Dimethyl sulfoxide (DMSO; molecular biology grade)

Table 1
The neonicotinoid pesticides (‘Formulations’) addressed in our study. Active compounds and additives are listed. Substances in bold have been experimentally tested in the present study.

Formulation	Active compound	Additives
Mospilan 20 SP (Nippon Soda Co., Japan)	**Acetamiprid** (20%)	Surfactants (not specified; 5%) Carrier (not specified; 75%)
Actara 25 WG (Syngenta Crop Protection AG, Switzerland)	**Thiamethoxam** (25%)	Diatomaceous Earth (N/A) Crystalline Silica, Quartz (N/A) Starch (N/A)
Confidor 200 SL (Bayer, Germany)	Imidacloprid (17.1%)	**Dimethyl sulfoxide** (N/A) **N-Methyl 2 pyrrolidone** (N/A)