# Unravelling the Genotoxic Potential of Agrochemicals on Fish Cell Line

**Concise summary**

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Pests and diseases, the main factors causing low agricultural productivity, are mainly controlled by chemical means. Despite the advantages of applying chemical pesticides in improving food quantity and quality, negative effects on human health and serious environmental problems challenge these benefits (Aktar et al., 2009; Damalas & Eleftherohorinos, 2011; Bénit et al., 2019). Pesticide is potential chemical pollutants, designed to selectively eliminate variety of pests. The mode of action is strongly connected to their chemical structures which are widely diversified (Abhishek et al., 2014; Bénit et al., 2019). Indeed, pesticides in our surroundings do not appear singly and usually occurs as complex mixtures and their combined effect may exhibit toxicity to the targeted and non-targeted organisms including humans (Sarkar et al., 2008). India is an agrarian country, where agriculture is the lifeline of farming community. To keep pace with increasing demands of food for growing population the indiscriminate use of pesticides has led to the contamination of environment and food commodities in the country (Bedi et al., 2013).

Agrochemicals and chemical fertilizers are widely used under Green Revolution to protect the crops from pests and enhance yield, thereby increasing the productivity and economical gain of the crop yield to meet the high demand for food due to the fast growing population (Gill & Raine, 2014). Aquatic ecosystems that run through agricultural areas have high probability to get contaminated by runoff and ground water leaching by a variety of chemicals used in agricultural operations. Fish is the economically most important non-target species that are adversely affected by severe agrochemical pollution (Pandey et al., 2005; Jacquin et al., 2020).

In India, the pesticides were introduced in sixties and extensively used for agriculture and vector control purposes due to low cost and high effectiveness (Mishra & Sharma, 2011; Witczak et al., 2021). As the results of the widespread use and the lack of safe management of pesticides in developing

countries, various compartments of the environment are contaminated and exposure to pesticides is a concern toward the general population (Mehta et al., 2020). For screening purposes, there is increasing interest in the development of *in vitro* methods to replace conventional animal toxicity tests, because the maintenance is difficult and *in vivo* bioassay are more tedious to perform. The ultimate goal is to achieve an alternative system that allows rapid testing of candidate compounds, formulations and finished products which enables the accurate prediction of toxic They are preferred as it reduces time and is cost-effective (Ilboudo et al., 2014; Fischer et al., 2019) Cell lines provide unlimited material resources because the cells can be readily maintained at relatively low costs with minimum labour input. Cell lines also provide a population of cells to generate consistent samples and reproducible results (Segner, 2004; Abdul Majeed et al., 2013; Caron-Beaudoin et al., 2018; Fischer et al., 2019).

The environmental risk assessment of chemicals in traditional toxicity testing is mostly based on in vivo single compound experiments and has been well explored on all representatives of the tropic levels viz. producer and consumer level. However, *In-Vivo* testing is extremely time-consuming and costly, requiring much maintenance and a high number of animals, which is ethically debated. Thus, interest in *In-vitro* methods has been growing greatly in the recent years for economical, practical and ethical reasons, and the use of cell lines as alternatives to *in vivo* testing is being seriously considered (Kasi Elumalai, 2012; Nagpure et al., 2016, Schug et al., 2020). The use of cell lines has many advantages. It avoids the testing of contaminants on living animals or even the regular sampling of cells for primary cultures. Their maintenance is less demanding since the only requirements are cell medium and an incubator at the right temperature and $CO_2$ concentration which is even unnecessary in the case of piscine cell lines. These methods are cost affecting and non-invasive, and the testing in itself uses very limited amounts of the test chemicals and creating little toxic waste. Results present little variability since the cell lines are relatively homogeneous and used in a very controlled environment, the complex interactions happening in a whole organism being avoided.

Literature survey done till date has plethora of references for screening the toxic potential of agrochemicals which are limited to *in-vivo* conditions. That too with either single or in combination of the pesticides. Baring the previous *In-Vivo* studies from our lab which has well established the toxic potential of all the classes of agrochemicals viz: Insecticide (IMI), Curzate (CZ) , Micronutrient (MN) and Pyrezonsulfuron ethyl (PE) by reporting the alteration of hematological, histological, blood biochemical parameters, behaviour alteration and neuroendocrine response as well (Sadekarpawar, et al, 2010, 2015; Upadhyay et al., 2014; Pandya et al., 2016). However, there is a lacuna in our understanding with regards to the molecular mechanism. Thus to fill the gap the present study was undertaken to unravel the genotoxic potential of agrochemicals (PE, CZ, MN and IMI) in *In-Vitro* system. To evaluate these obscure aspects of the loss of normal cell orchestration, cell death, cell proliferation and other genetic markers which will make us to understand the disturbed machinery.

In the present study and attempt is made to understand the alterations in the expressions of the universal proliferative markers when exposed to diverse class of agrochemicals. It has been shown that ICG cells are suitable candidates for evaluating *In-Vitro* acute cytotoxicity of harmful chemicals and heavy metals (Taju et al., 2014). Here we extend the use of ICG cells to evaluate *In-Vitro* toxicity of agrochemicals like IMI, CZ, MN and PE. The half maximal inhibitory concentration ($IC_{50}$) is a measure of the potency of a chemical in inhibiting a specific biological or biochemical function (Yilmaz et al., 2012). $IC_{50}$ is a quantitative measure that indicates how much of a particular inhibitory substance (agrochemicals) is needed to inhibit, *In-Vitro*, a biological component by 50%. The $IC_{50}$ value obtained by MTT assay in the present study concluded that of all the agrochemicals tested; IMI to be highly toxic compared to CZ, MN, PE. The assessment of viability can also point to a cell's survival and, in some cases, cell multiplication. Cell cytotoxicity and proliferation are generally used for screening to detect whether the toxicants have effects on cell proliferation or display direct cytotoxic effects. The study also reported a dose dependent alteration in cell viability in which the maximum reduction was

observed for IMI followed by CZ>PE>MN compared to control. Furthermore, There was a significant decrease observed in gene expression of pcna, cyclin A and cyclin E. There was a significant decrease in proliferation markers like pcna and cyclin genes indicating the toxicity of AGs is mediated by expression of proliferation-related genes and cell cycle progression genes in ICG cell line ***(Chapter 1).***

Apoptosis is a type of genetically regulated programmed cell death that controls the development of multicellular organisms and tissues by eliminating physiologically redundant, physical damaged, and abnormal cells (Galluzzi et al., 2012;.Ilboudo et al., 2014). Studies focusing on the genes and signals regulating apoptosis have played an important role to determine the cell death pathway (Liu & Levine, 2015). Cell death is discriminated into two main forms: apoptosis and necrosis. In contrast to necrosis, apoptosis is a regulated, energy-dependent form of cell death leading to phagocytosis of cellular remnants by neighbouring cells. Initiation is induced by various stimuli, including the binding of ligands to cell surface receptors of the tumor necrosis factor family, damage of DNA integrity by various stress factors like toxicants or major changes of the homeostasis of cells. A main theme in transduction of many of these signals further downstream is the oligomerization and interaction of proteins with death effector domains. These proteins with conserved structural modules like death and death effector domains have a number of different functions in the cell, including connecting membrane-bound receptors to cytosolic effector caspases (Ziegler & Groscurth, 2004; Fábio et al., 2021). After establishing the impact on the proliferation on ICG cell line on exposure of agrochemicals our next target was to analyze the morphological alterations in the cell line on exposure of AGs and to evaluate whether AGs have interfered with the antioxidant balance and resulted in generation of the reactive oxygen species (ROS) by DCFDA staining as well as quantifying the enzymatic and non-enzymatic antioxidant parameters. Moreover, to understand the type of cell death the AO/EB double staining and FACS were performed and to analyse the altered mechanistic pathway leading to cell death the quantification of apoptotic markers were analysed.

The results concluded that the exposure of all AGs in general have altered the morphology of the ICG cells, DCFDA staining and alterations in the enzymatic and non enzymatic parameters have proved that AGs have induced oxidative stress. AO/EB double staining along with FACS analysis and the expression of the bax, bcl2. Caspase-3, tnfα and nfķb genes confirm that the AGs are effective through intrinsic mechanistic pathwayof Apoptosis. Overall, putting all the results it can be concluded that the exposure of all AGs in general have altered the morphology of the ICG cells. DCFDA staining and alterations in the enzymatic and non enzymatic parameters have proved that AGs have induced oxidative stress that leads to generation of ROS. Intracellular ROS functions as a trigger of signaling molecules to initiate downstream events in regulating cell differentiation, cell cycle, and apoptosis. A high level of ROS can cause oxidative damage to cardiolipin, resulting in dissociation of the cytochrome c and subsequent release into the cytosol, which is followed by mitochondrial depolarization (Tanaka *et al.,* 2012). Once Cytochrome C is released into the cytosol, it binds to Apaf-1 to form an apoptosome, resulting in activation of Caspase cascade and cell death **(*Chapter 2*)**

In recent times, the relationship between oxidative DNA damage and homeostasis of DNA methylation modification is found (Jiang et al., 2020). Induced oxidative stress by xenobiotics leads to DNA damage which further interrupts the binding of DNA methyltransferases (DNMTs) to CpG islands in DNA templates, resulting in abnormal methylation of cytosine in CpG dinucleotides (Jiang et al., 2020). DNA that contains 5-methylcytosine (5-mdC) can recognize proteins by enriching methylated nucleotides. 5-mdC blocks the binding of transcription factors to DNA templates, leading to chromatin compression and gene silencing (Vanaja et al., 2018; Thakur and Chen, 2019). DNA methylation is considered to be one of the most crucial epigenetic regulators of gene expression. However, epidemiological evidence shows that changes in DNA methylation are associated with exposure to multiple trace metals in the environment, including Pb, As and Ni (Li et al., 2020). Furthermore, *in vivo* and *in vitro* assays have also demonstrated that exposure

to metals and toxicants might have an impact on global DNA methylation patterns (Sanchez et al., 2017; van der Ven et al., 2017; Wang and Yang, 2019). Thus, abnormal DNA methylation status can be a valuable tool to assess the adverse epigenetic effects of trace metals on organisms.

Previous studies from our lab has reported the ability of these AGs to damage liver, Kidney, Muscle and gills and have induced enzymatic responses in *O.mossambicus* and *L.rohita*, associated with the disruption of normal fish behaviour and physiology in in vivo conditions A major occurrence in cells exposed to toxic chemicals is DNA damage. Nucleotide sequence can be altered when DNA lesions occurs at specific sites of the gene, setting off the process of mutation and some other cellular responses (Lord et al., 2012). The exposure of AGs has led to formation of nuclear abnormalities in which micronucleus formation, bi-nucleated and lobed nucleated cells were highest at HD of IMI followed by CZ and PE suggesting its genotoxic potential of AGs in ICG cells. The significant alteration in expression and sequences of p450 and dnmt was observed in ICG cells exposed to HD of IMI suggesting the probability of having its role in epigenetic changes and causing the alteration in regulation for xenobiotic toxicity (***Chapter 3***).

Imidacloprid exhibited binding in diversified protein classes including nuclear receptor, cytochromes, enzymes, proteases, Kinases, GPCRs, and transporters. Whereas rest compounds like pyrazosulfuron ethyl, cymoxanil, and Mancozeb exhibited very little binding probability with proteins. A total of 396 genes were found to be in close association with the candidate genes whose gene expression was studied. Out of which 18 were found to function as controlling state change of other genes, while seven were found to be involved in controlling the expression and the remaining 371 were designated as state change genes. The interaction showed that casp3 had controlling state change with bcl2 and bax, while the expression pattern control was only found between bcl2 and casp3. The interaction also revealed that casp9, tnf (Tumor Necrosis Factor), fasl (Fas Ligand), ptk2 (protein tyrosine kinase 2) had a control state change. In addition, tnf with this interaction showed that it was controlling the

expression of nfkb. The interaction further revealed that it showed the controlling the state change of hdac2 (histone deacetylase 2) hence regulating the epigenetic changes (***Chapter 4***).

In a nutshell, the present study shows that among all the agrochemicals validated, Imidacloprid has a very strong binding affinity with ligand-gated ion channel, cyclins, and bax, bcl2 for the apoptosis pathway. The present study also enlists the new possible targets of Imidacloprid, curzate which needs to be accounted in the in-silico databases. Pathway analyses strongly suggest that alteration of candidate genes like ccne1,ccna4, pcna, bax,bcl2, casp3 tnf and nfkb will lead to change in the expression pattern of downstream genes and their protein products and ultimately leads the cell into apoptosis. All the evidence i.e. from morphological examination, gene expression to the *in-silico* analysis suggests that cells are highly under stressed and the use of test pesticides i.e. imidacloprid, curzate should be controlled.