"Analysis of diclofenac-induced physiological and developmental disorders of *Chlamydomonas reinhardtii* cells with particular emphasis on mitochondria functioning"
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Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the commonly detected groups of pharmaceuticals in the aquatic environment [1]. They are produced and used in large quantities and entering the environment can pose an ecological threat [2]. NSAIDs pose a danger not only to animals but also to higher plants and algae, which have many biochemical pathways analogous to animal cells [3]. An example of these substances is diclofenac (DCF), which is currently listed as a priority environmental threat [4,5]. DCF, like other NSAIDs, was originally designed as a therapeutic substance for humans and animals; therefore, its impact on animal organisms is now well-researched [6–8]. Studies concerning the toxic effects of DCF on plant organisms are rare, however growth inhibition and disturbances in photosynthesis are reported, which indicates the phytotoxicity of this substance [9–11]. The mentioned phytotoxic effects of DCF were observed in studies conducted on the single-celled planktonic green alga *Chlamydomonas reinhardtii* (**article nr 1**), which is a model organism in biochemical, physiological, molecular, and toxicological analyses on both population and cellular levels [12–14]. Studies described in the article „Diclofenac alters the cell cycle progression of the green alga *Chlamydomonas reinhardtii*" (**article nr 1**) were conducted in one of the top algae research centres - ALGATECH, in Třeboň, Czech Republic, thanks to participation in the Iwanowska Program, financed by the Polish National Agency for Academic Exchange. The obtained results allowed us to demonstrate that one of the reasons of DCF phytotoxicity is the disruption of the development of *C. reinhardtii* cells, specifically, the extension of the time it takes for individual cells to reach cell cycle commitment points and a delay in DNA replication. Moreover, I observed DCF-dependent reduction in overall photosynthetic efficiency, chlorophyll *a* and carotenoid content in cells, and increased starch content in cells treated with DCF, particularly at the end of the cell cycle. This observation indicated disruption in metabolic processes leading to excessive storage of photosynthesis products, rather than their use for energy acquisition and synthesis of new macromolecules. As a result of the above changes, individual cell growth was slower, the cells later reach cell cycle commitment points and the number of daughter cells was reduced. The ultimate effect of DCF's action was a decrease in the population density.

Analogous anti-proliferative effects of DCF are described in numerous studies involving animal cells [7,15–17], but, to my knowledge, it has not yet been thoroughly studied for plant cells, so the work discussed here adds new information to the literature data on the phytotoxicity of this substance. It is believed that the toxicity of DCF and other NSAIDs in animal cells involves

the overproduction of reactive oxygen species (ROS), leading to oxidative damage to lipids and proteins, as well as disruptions in cellular energetics (respiratory processes) [8], leading to inhibition of cell growth and cell divisions [17]. In contrast to animal cells, the toxic effect of DCF in plant cells is rarely discussed in the context of mitochondria functioning. It is typically attributed to the inhibition of the photosynthesis process [9,11]. In light of the above, in the next stage of study **(article nr 2)** I decided to investigate the impact of DCF on photosynthesis in *C. reinhardtii* cells, comparing the effects of DCF with those of atrazine, a triazine herbicide with a well-known mechanism of action on photosynthetic processes. The obtained results allowed us to demonstrate that both substances induced a different cellular response, with the toxic effect of DCF appearing to be less associated with photosynthesis, as compared with atrazine's toxic effect. Importantly, I observed a slight stimulation of oxygen consumption in the dark by *C. reinhardtii* cells treated with DCF and the overexpression of the gene encoding catalase - an antioxidant enzyme primarily located in the mitochondria of *C. reinhardtii*. The results of the conducted and described studies thus indicated that processes occurring in the mitochondria of *C. reinhardtii* cells may play a significant role in the phytotoxicity of DCF, and DCF's impact on mitochondrial function could be an important factor disrupting the growth and development of individual cells, and consequently, the entire population.

The above conclusions formed the basis for further research, in which a significant component was the optimization of the method for determining the mitochondrial membrane potential (MMP), as a sensitive tool for assessing mitochondrial function [18] and the adaptation of the protocol for estimating MMP to be used for planktonic algal cells. In **article nr 3,** I presented an optimized protocol for reliable estimation of MMP values in *C. reinhardtii* cells. I also demonstrated that the JC-1 fluorochrome (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) can be effectively used for assessing MMP as a stress marker, including stress induced by DCF. This method was employed in the research presented in **article nr 4.**

During the research, the results of which are described in the paper published in 2024 **(article nr 4),** I focused on the parameters of the mitochondrial electron transport chain functioning in DCF-treated *C. reinhardtii* cells. In the case of this organism, as well as the higher plant cells, there are two alternative electron transport pathways: the pathway based on cytochrome oxidase complex IV and the pathway uses the alternative oxidase (AOX). The "cytochrome" electron transport pathway is the main mechanism responsible for the translocation of protons from the mitochondrial matrix to the intermembrane space, thus enabling ATP synthesis by ATP synthase. AOX, present in the electron transport chain of plants and algae, is

considered an important mitochondrial component of the cellular stress response due to its ability to maintain an appropriate balance between reduced and oxidized forms of ubiquinone [19].

To analyze the impact of DCF on mitochondrial function and to assess the significance of complex IV and AOX in DCF toxicity, I used specific inhibitors of individual respiratory chain components: salicylhydroxamic acid (SHAM) (an inhibitor of the AOX pathway) and potassium cyanide (KCN) (an inhibitor of the complex IV). The comparison of DCF's effects with those of SHAM and KCN, including extended statistical analysis of the physiological parameters (discriminant analysis and correlation matrices for MMP, oxygen consumption in the dark, and cell volumes), clearly indicated the non-specific action of DCF on the studied processes. The observed decrease in MMP values and low levels of ROS in mitochondria suggested that DCF uncouples oxidative phosphorylation and electron transport due to damage to the mitochondrial membranes. This assumption was confirmed by analyses of cell images obtained through electron microscopy and confocal microscopy. The mitochondria in the microscopic images of DCF-treated cells had elongated shapes, irregular forms, and degraded inner membrane cristae. These observations suggest the abnormal division of these organelles or their degeneration, similar to what is described in the case of animal mitochondria treated with indomethacin [20]. A comprehensive analysis of the results described in **article nr 4** allowed me to conclude that the tendency for increased oxygen consumption in the dark and the uncoupling of oxidative phosphorylation result from non-specific damage to the mitochondrial membrane, which is likely also the cause of the observed low levels of mitochondrial ROS.

The ultrastructural analysis also revealed other changes induced by DCF, namely the disappearance of the pyrenoid in chloroplasts and an increase in the deposition of chloroplastic starch. This corroborates the results quantitatively described in **article nr 1** and supports the suggestion that DCF causes a shift in metabolic balance towards carbohydrate storage in cells rather than their utilization for energy production (respiratory processes).

At this point, I would like to emphasize that I obtained very interesting results thanks to the use of a confocal microscope, which allowed for the visualization of mitochondrial status using the JC-1 fluorochrome. Analysis of the images obtained in this way revealed that in the population treated with DCF, two cell populations could be observed: cells similar to control cells, with a strong fluorescent signal indicative of a high membrane potential, and cells displaying significantly weaker, more green fluorescence, indicating a low MMP. This result is not only new and previously unreported in the literature but also very important for interpreting quantitative results obtained during MMP analyses at the population level, based on JC-1 fluorescence intensity measurements. This finding clearly indicates that the low MMP observed at the

population level in quantitative measurements is due to the presence of a fraction of cells with severely disrupted metabolism, rather than a uniform decrease in the vital parameters of all cells in the population. It can be assumed that DCF more strongly affects cells that have slight metabolic or developmental disturbances, which are not visible under control conditions but make these cells more sensitive to the toxic substance. This is consistent with the results obtained in **article nr 1**, where I demonstrated that approximately 30% of cells in the DCF-treated population died within the first few hours of exposure to the substance; these are probably cells with minor metabolic defects that weaken their resistance to stress. I believe that the observed mitochondrial dysfunction could be one of the causes of the physiological and developmental disturbances in cells and changes in the cell cycle described earlier.

The most important conclusions drawn from the studies can be summarized as follows:

1. DCF affects physiological processes and the development of *Chlamydomonas reinhardtii* cells by delaying the attainment of cell cycle commitment points, leading to a reduction in the number of daughter cells, ultimately resulting in the inhibition of population growth.
2. The anti-proliferative effect of DCF is largely associated with disturbances in processes occurring in the mitochondria of *C. reinhardtii* cells.
3. DCF non-specifically affects mitochondria, inducing the uncoupling of oxidative phosphorylation and electron transport due to damage to mitochondrial membranes.

In summary, I can state that a significant factor in the phytotoxic action of DCF on *Chlamydomonas reinhardtii* cells is the disruption of mitochondrial functioning caused by the non-specific interaction of this substance with membranes, leading to destruction of mitochondrial membrane structure, which is evident in microscopic images of treated cells. Due to the disruption of membrane structure, uncoupling of oxidative phosphorylation is observed, as indicated by a decrease in MMP values. Given the close interaction between mitochondria and chloroplasts in plant organisms and its important role in maintaining redox balance, overall cellular metabolism, and stress tolerance, changes in mitochondrial functioning significantly impact all physiological processes occurring in the cell. Consequently, this can lead to abnormal cell growth and development, delayed attainment of cell cycle commitment points, a reduction in the number of daughter cells, and ultimately, the inhibition of population growth.

Literature

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The scientific articles included in the doctoral dissertation:

1. Harshkova, D.; Liakh, I.; Bialevich, V.; Ondrejmišková, K.; Aksmann, A.; Bišová, K. 2021. Diclofenac Alters the Cell Cycle Progression of the Green Alga *Chlamydomonas reinhardtii*. *Cells* 10, 1936. https://doi.org/10.3390/cells10081936 (IF=5,1, punkty MNiSW= 140)
2. Harshkova, D., Majewska, M., Pokora, W., Baścik-Remisiewicz, A., Tułodziecki, S., Aksmann, A., 2021. Diclofenac and atrazine restrict the growth of a synchronous *Chlamydomonas reinhardtii* population via various mechanisms. *Aquatic Toxicology* 230, 1-11. https://doi.org/10.1016/j.aquatox.2020.105698 (IF=4,1, punkty MNiSW= 140)
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