“Disorders of the photosynthesis process as an important element of diclofenac phytotoxicity”

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Human activity contributes to the increasing environmental pollution with biologically active substances, among which increasing attention is paid to pharmaceuticals and their metabolites. The most commonly detected environmental pharmaceutical contaminants include antibiotics, lipid-regulating drugs, hormonal drugs, antiepileptic drugs and beta-blockers, and non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs, available without a prescription, are commonly used in human and veterinary medicine to relieve inflammation and pain, and their quantity in the environment is constantly increasing. Among them, diclofenac (DCF) deserves special attention, due to its frequent detection in freshwater systems (McGettigan and Henry, 2013), ineffective removal by sewage treatment plants (Zhang et al., 2008), slow degradation in the environment (Fent et al., 2006; Wu et al., 2015) and not fully understood impact on non-target organisms, in 2013 was placed by the European Commission on the list of substances subject to monitoring (Directive 2013/39/EU). Since humans have used DCF for years, its impact on mammals is well known. DCF is a weak acid that acts by inhibiting cyclooxygenases (COX-1 and COX-2) involved in prostaglandins synthesizing from arachidonic acid (Bácsi et al., 2016). The adverse effects of DCF observed in mammals include, among others, gastrointestinal complications, neurotoxicity, cardiotoxicity, hepatotoxicity, nephrotoxicity, hematotoxicity, genotoxicity, teratogenicity, bone fractures, and skin allergies (Sathishkumar et al., 2021). In turn, knowledge about the effects of DCF on plants is very limited. There are reports of its phytotoxicity and its high harmfulness compared to other anti-inflammatory drugs (e.g. acetylsalicylic acid or ibuprofen). Available literature data allow us to assume that one of the causes of DCF phytotoxicity is disturbances in the light-dependent phase of photosynthesis, which has been indicated in several works (Hájková et al., 2019; Kummerová et al., 2016; Vannini et al., 2018). The research results published so far did not indicate the exact cause of the observed disorders, which became the inspiration and a good basis for the research described in this doctoral thesis.

The research aimed to understand the mechanism of inhibition of light-dependent phase of photosynthesis under the influence of DCF, through a detailed analysis of the changes occurring in the photosynthetic apparatus exposed to this substance. In the research the unicellular green alga *Chlamydomonas reinhardtii* was used as a model organism for studying basic issues of cell biology, molecular biology, and toxicological studies. As a second research object, spinach (*Spinacia oleracea*), popular in photosynthesis research, was used. To indicate

the probable mechanism of DCF action, it was decided to use a reference substance with a precisely known effect on the photosynthetic apparatus, i.e. atrazine (ATR). ATR is a triazine herbicide that inhibits photosynthesis by binding to the plastoquinone B binding site of the D1 protein, blocking the transfer of excitation energy from PSII to the PSI reaction center, which consequently leads to excessive production of reactive oxygen species (ROS) and oxidative damage in the cell.

In the first stage of the study (**Majewska et al. 2018**), the effective concentration of the tested substances, causing 50% inhibition of *C. reinhardtii* population growth (EC50) was estimated, which was 421.35 μM for DCF (134.0 mg/L) and 0.360 μM (77.6 mg/L) for ATR, respectively. Then, after 24 hours of exposure to toxicants, their effect on the green algae cells was examined. In the case of both substances, the intensity of photosynthesis, estimated based on the amount of oxygen produced by the cells, decreased by about 40%. Since this measurement gives only a general picture of the course of the photosynthesis process, the next step was to analyze the curve of induction and quenching of chlorophyll *a* fluorescence *in vivo* (OJIP test), which provided detailed information on the efficiency and condition of the photosynthetic apparatus. ATR was shown to cause a decrease in the probability of electron transfer further than QA (ψ0) and a decrease in the maximum electron transport efficiency in the photosynthetic chain (φE0), which was consistent with reports that its mechanism of action is blocking electron flow beyond photosystem II (PSII). DCF, on the other hand, seemed to directly affect PSII reaction centers, as a decrease in the maximum quantum efficiency of primary photochemical reactions (φP0) and an increase in nonphotochemical excitation energy dissipation (φD0) were observed. Additionally, the fraction of active PSII reaction centers (RCM) was significantly lower than in the control, but the energy flux through one active RC, in particular energy absorption (ABS/RC), energy trapping (TR0/RC), electron transport (ET0/RC, RE0/RC) and nonphotochemical energy dissipation (DI0/RC) increased. This suggested that some RCs were transformed into "silent" reaction centers, dissipating excess absorbed energy as heat (Krüger et al., 1997; Strasser et al., 2004), whereas those RCs that remained active functioned properly. Furthermore, it could be assumed that the xanthophyll cycle was involved in dissipating excess excitation energy (Demmig-Adams et al., 1996), as indirectly evidenced by the increased carotenoid content in cells exposed to DF.

The effect of disturbances in photosynthetic electron transport is often the overproduction of reactive oxygen species (ROS) and the associated oxidative stress (Czarnocka and Karpiński, 2008). Indeed, both DCF and ATR caused increased production of hydrogen peroxide in algal cells, with the effect of ATR being much stronger (**Majewska et al.**

2018). Interestingly, the activity of ascorbate peroxidase (APX), an enzyme responsible for the decomposition of hydrogen peroxide in chloroplasts, increased only in cells treated with ATR, which is consistent with the described mechanism of action of this substance. An increase in APX activity was not observed in *C. reinhardtii* cells treated with DCF, which suggested that the chloroplast was not the main source of hydrogen peroxide overproduction under these conditions, and the mechanism of DCF-induced photosynthesis disturbances required further studies.

The results obtained in the first stage of the study (**Majewska et al. 2018**) were related to *C. reinhardtii* population, in which cells are at different stages of development. Moreover, the observed changes concerned cells exposed to the tested substances for 24 hours, which did not allow us to determine how quickly toxic effects appear. For this reason, in subsequent experiments (**Majewska et al. 2021**), it was decided to use a synchronous population of this green alga, in which all cells are in the same developmental phase, and to take samples at short time intervals. Therefore, the synchronized population of zoospores was treated at the beginning of the cell cycle with DCF and ATR at concentrations determined in previous studies (**Majewska et al., 2018**), after which samples were taken for analysis every hour. This made it possible to analyze the effect of DCF and ATR at the level of a single cell and observe the early effects of their action. Interesting part of the study were analyses of the transcripts level of genes encoding enzymes responsible for neutralizing reactive oxygen species in chloroplasts, the increased expression of which is considered a marker of oxidative stress (Chankova et al., 2014): FSD1 encoding iron superoxide dismutase (Fe-SOD), MSD3 encoding manganese superoxide dismutase (Mn-SOD) and APX1 encoding ascorbate peroxidase (APX). Exposure of cells to ATR already after the first hour after treatment caused an increase in transcripts of all these genes, while in cells treated with DCF, the level of transcripts of genes encoding APX and Mn-SOD decreased, and the level of transcript of the gene encoding Fe-SOD increased, but only after several hours and to a much smaller extent than in the case of ATR. This was consistent with the conclusions drawn in a previous study (**Majewska et al., 2018**) that ROS overproduction in chloroplasts exposed to DCF is not the main cause of the phytotoxic effect of DCF in plant cells. This is also evidenced by the fact that the amount of chlorophylls *a* and *b* in cells decreased only 7 h after DCF treatment, in contrast to cells treated with ATR, which caused such an effect after 1 hour. Strong overproduction of ROS in chloroplasts of cells exposed to ATR could lead to the oxidation of pigment molecules and the destruction of their structure (Nguyen et al., 2021), while in the case of DCF, this effect appeared much later and was much weaker.

A very important element of the above studies was the analysis of the parameters of the curve of induction and quenching of chlorophyll *a* fluorescence *in vivo*, which allowed us to show that the disruption of photosynthetic electron transport, leading to excessive production of ROS due to the action of ATR and the transformation of some reaction centers into "radiators" of non-photochemical energy dissipation in cells treated with DCF, were visible after 1 hour of exposure. This means that both substances quickly penetrate the cells and reach the chloroplast, but the way they act on the photosynthetic apparatus and their effects are different. At this stage, it could already be stated that although DCF does not affect photosynthesis as strongly as ATR, its inhibitory effect on the photosynthetic apparatus is also significant and worth further research. Since the experiments described above were conducted on whole cells of green algae, the obtained results were the sum of all the biochemical processes occurring in these cells, which may lead to an erroneous interpretation of some of the results. This is particularly important due to the similarities between the processes of photosynthesis and respiration, because both processes use the electron transport chain and produce energy in the form of ATP. Moreover, the close cooperation of chloroplasts with mitochondria has been proven, ensuring not only the optimal functioning of the cell in the "physiological" state but also mitigating the effects of energy metabolism disorders under stress conditions (Kromer, 2003; Raghavendra and Padmasree, 2003). Therefore, to separate the effect of DCF on photosynthesis from its impact on other cellular organelles, especially mitochondria, on which DCF may have a negative impact (Gómez-Lechón et al., 2003; Syed et al., 2016), it was decided to conduct further studies on isolated chloroplasts and thylakoids of spinach (*Spinacia oleracea*), a recognized model in photosynthetic research.

In this series of experiments (**Majewska et al., 2024**), chloroplasts and thylakoids isolated from spinach cells were exposed to various concentrations of DCF (ranging from 125 to 4000 μM), and after 15 minutes of incubation, the parameters of the curve of induction and quenching of chlorophyll *a* fluorescence *in vivo* were analyzed. It was shown that changes in the efficiency of the spinach photosynthetic apparatus were of the same nature as those obtained for whole cells of the green alga *C. reinhardtii* (**Majewska et al., 2018, 2021, 2024),** and the differences in the phytotoxicity of this substance depended on the dose and whether whole chloroplasts or only thylakoid membranes were exposed. The lowest concentration of DCF used (250 μM), which was used to treat chloroplasts, had a relatively small effect on the parameters of chlorophyll *a* fluorescence *in vivo*. The quantum yield of primary photochemical reactions (φP0), electron transfer probability beyond QA (ψ0), and maximum quantum yield of electron transport (φE0) decreased by about 5% compared to the control. At the highest DCF

concentration used (4000 μM), the values of these parameters were reduced by 15 to 20%. On the other hand, the quantum yield of non-photochemical energy dissipation (φD0) increased by 5% to 25% depending on the DCF concentration used. It was also found that the fraction of active PSII reaction centers (RCM) decreased by 5% to 20% with increasing DCF concentration. Nevertheless, those RCs that remained active seemed to function properly, since the absorption of light energy, its trapping, and electron transport by one active RC did not show significant changes, except for about a 30% increase in non-photochemical excitation energy dissipation. The obtained results showed that isolated chloroplasts exhibit relatively low sensitivity to DCF compared to intact *C. reinhardtii* cells (**Majewska et al. 2018, 2021**). This could be due to the fact that not only the chloroplasts themselves but also mitochondria are exposed to DCF, which contributes to the increased toxicity of this compound in whole cells. Another concept, presented in the work of Hájková et al. (2019), assumed that DCF does not cross the barrier of the chloroplast membrane and, consequently, cannot cause significant disruption of the photosynthetic apparatus. To verify this assumption, it was decided to examine isolated chloroplasts using confocal microscopy, as direct visualization of DCF interactions with chloroplasts has not been described in the literature so far. The study showed that after 15 minutes of exposure, only the lowest concentration of DCF used in the experiments (125 μM) did not cause significant changes in the structure of chloroplasts. Chloroplasts exposed to 1000 μM DCF became irregular, grana appeared disorganized, and showed much weaker fluorescence. In contrast, at the highest concentration (4000 μM) chloroplast envelopes were clearly damaged, releasing the stroma to the outside and allowing for direct DCF–thylakoid interactions.

In the next part of the study, it was decided to allow DCF molecules to interact directly with the photosynthetic electron transport chain elements by conducting experiments with isolated spinach thylakoids. As expected, thylakoids were more sensitive to DCF than chloroplasts. When they were treated with DCF at the highest concentration (4000 μM), the quantum yield of primary photochemical reactions (φP0), the probability of electron transfer beyond QA (ψ0) and the maximum quantum yield of electron transport (φE0) decreased, compared to the control, by 70, 80 and 30%, respectively, and the quantum yield of nonphotochemical energy dissipation (φD0) increased by 250%. The fraction of active PSII reaction centers decreased with increasing DCF concentration, from 20% at 1000 μM to 90% at 4000 μM, and the values of parameters for specific energy flows through one reaction center increased significantly. The results, both in the case of studies on chloroplasts and thylakoids, indicated the transformation of some reaction centers into energy radiators. Importantly, the

lack of specific interaction with a specific element of the electron transport chain and the degradation of the chloroplast membrane clearly indicated a non-specific effect of the drug on the photosynthetic membranes. The lipophilicity of many NSAIDs is one of the most important factors behind their toxic effects (Ferreira Marlene Lu et al., 2005; Giraud et al., 1999; Tomisato et al., 2004) and it can be stated that the same is for DCF.

Summarizing all the studies conducted, it can be concluded that the harmful effect of DCF on the photosynthetic apparatus results from its non-specific interaction with photosynthetic membranes, causing their degradation, which leads to disruption of the electron transport chain, which in turn reduces the efficiency of photosynthesis, an extremely important metabolic pathway of plants. The non-specificity of the effect of DCF on biological membranes is even more worrying because it suggests the possibility of a similar effect of the discussed substance on other cellular organelles. Considering that plants are an important element in ecosystems, being primary producers, and the fact that the amount of medicinal substances, including DCF, in the environment is constantly increasing, the conducted studies significantly expand our knowledge in this area and draw attention to the impact of anthropogenic pollution on plant organisms.

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2. Majewska, M., Harshkova, D., Pokora, W., Baścik-Remisiewicz, A., Tułodziecki, S., Aksmann, A. 2021. Does diclofenac act like a photosynthetic herbicide on green algae? Chlamydomonas reinhardtii synchronous culture-based study with atrazine as reference. *Ecotoxicology and Environmental Safety*, *208*, 1-10. DOI:10.1016/j.ecoenv.2020.111630 (Q1, IF 6,2)
3. Majewska, M., Kapusta, M., Aksmann, A. 2024. Diclofenac Interacts with Photosynthetic Apparatus: Isolated Spinach Chloroplasts and Thylakoids as a Model System. *Plants*, *13*(16), 1-13. DOI:10.3390/plants13162189 (Q2, IF 4,0)