BIOCHEMICAL EFFECTS OF COMPLEX III Qo SITE RESPIRATORY CHAIN INHIBITOR KRESOXIM-METHYL ON MAMMALIAN CELLS

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Motivations

The ultimate concern over pesticides in the environment is their toxic impact on nontarget organisms, including humans. For over the last two decades, the toxicological research has focused on pesticide-induced oxidative stress in terms of monitoring alterations in various biochemical and molecular compositions.

A relative new group of agricultural fungicides, strobilurins, represent a major class of plant protection products and world's largest selling fungicides. They act as complex III Qo site inhibitors and thus disrupting electron transport in the respiratory chain. This electron leakage generates superoxide and potentially results in increased oxidative stress. The present study was aimed at investigating the biochemical mechanisms involved in the toxic action of kresoxim-methyl, a widely used agricultural fungicide of the stobilurins group on murine neuroblastoma cells (N2a).

Materials and Methods

-Cell culture: cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Figure 1A), harvested for subculture using trypsin/EDTA digestion and grown in a humidified atmosphere containing 5% CO_2 and 95% air at 37℃. Before experiment, the cells were pre-cultured until confluence was reached.

-Concentrated stock solutions of the fungicide (Figure 1B) were prepared in dimethyl sulfoxide (DMSO) and diluted with DMEM to the desired concentrations. The final concentration of DMSO did not exceed 0.5% v/v.

-Assessment of cellular viability was conducted by MTT reduction assay.

-For determination of enzyme activities at the end of each treatment time, total cell lysates were prepared by solubilizing cells in lysis buffer and total protein (mg/ml) and enzyme activities were assayed in the extracts.

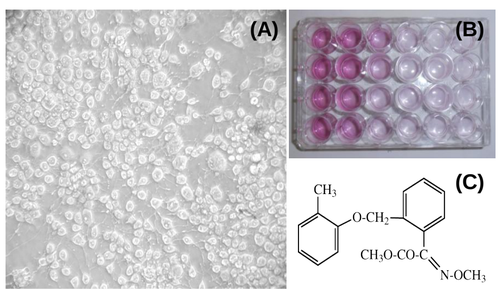

Figure 1. A, Phase-contrast microphotograph of neuroblastoma N2a cells; B, Kresoxim-methyl molecular structure; C, Formazan formation through the MTT proliferation assay.

Table 1. Non-lethal doses of kresoxim-methyl for the two time treatments. (N=6)

Incubation time	1 hour		24 hours	
Fungicide concentration (μg/ml)	50	60	0.1	0.2
Viability (% control)	96,149	95,354	115,875	96,8101
SEM	1,407	1,639	14,916	3,450

Results

-Morphological changes: microphotographs were taken from live cells plated in tissue culture plates, with an inverted phase contrast microscope. No morphological changes could be observed after 1h treatment with 50 μg/ml or 60 μg/ml and after 24h treatment with 0.1 μg/ml or 0.2 μg/ml (non-letal doses). At higher doses and after 24 h incubation with 50μg/ml, dead cells were clearly visible in the cultures (Figure 2C), and the cellular extension (dendrites) seen in untreated N2a cells (Figure 2A), were extracted, and cells appeared rounded and detached.

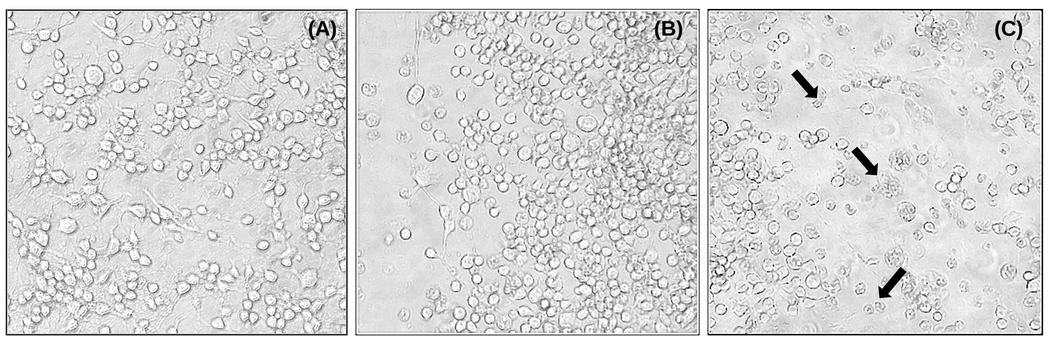

Figure 2. Microphotographs of neuroblastoma cells incubated during 24 h with the following treatments: A, serum free DMEM containing 0.1% DMSO (vehicle control); B, serum free DMEM containing 1 μg/ml kresoxim-methyl in DMSO; C, serum free DMEM containing 50 μg/ml kresoxim-methyl in DMSO.

-Extracellular H_2O_2:

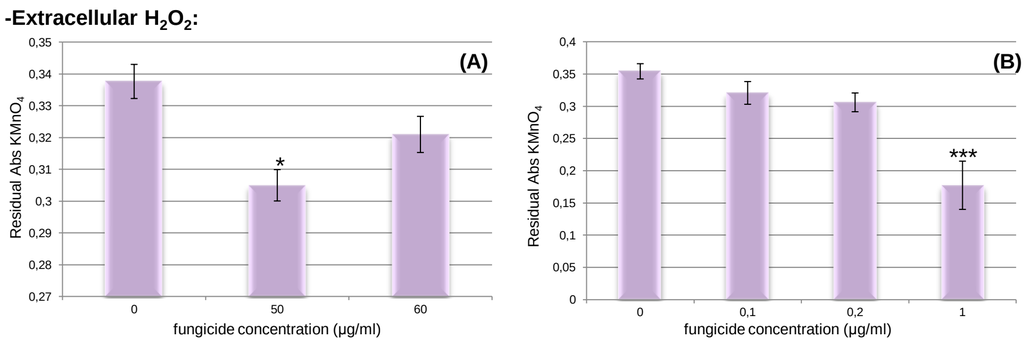

Figure 3. Hydrogen peroxide release in the extracellular phase by non-lethal doses. Sixty minutes (A) and 24h (B) after fungicide addition, H_2O_2 was measured in the medium using the potassium permanganate test. Higher levels of H_2O_2 result in less $KMnO_4$. Values represent the mean (SEM) of six independent measurements. The asterisks (*) indicate values that are significantly different from the control (*P<0.05 and *** P<0.001 by one way ANOVA).

-Enzyme activities:

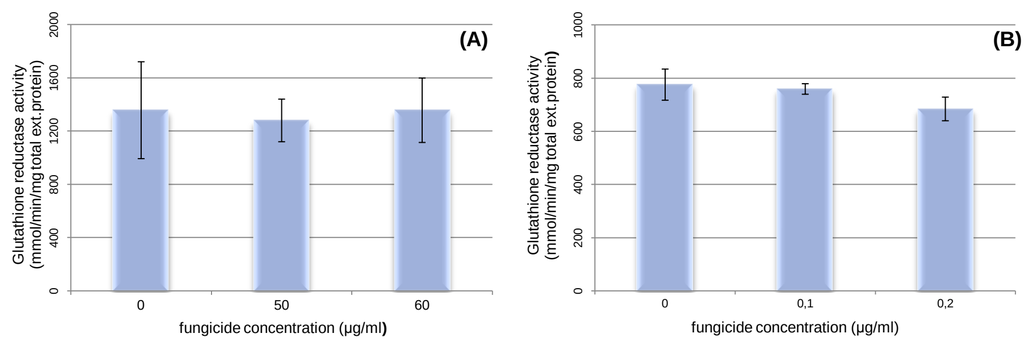

Figure 4. Glutathione reductase activity by non-lethal doses. Sixty minutes (A) and 24h (B) after fungicide addition. GR was measured through monitoring NADPH oxidation. Values represent the mean (SEM) of six independent measurements. No significantly different values were observed for both incubation periods.

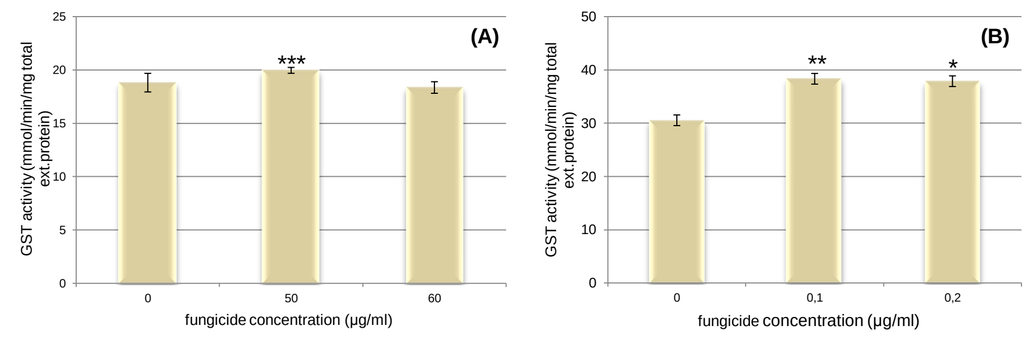

Figure 5. Glutathione tranferase activity by non-lethal doses. Sixty minutes (A) and 24h (B) after fungicide addition GST was measured by observing CDNB conjugation with GSH. Values represent the mean (SEM) of six independent measurements. The asterisks (*) indicate values that are significantly different from the control (**P<0.01 and *** P<0.001 by one way ANOVA).

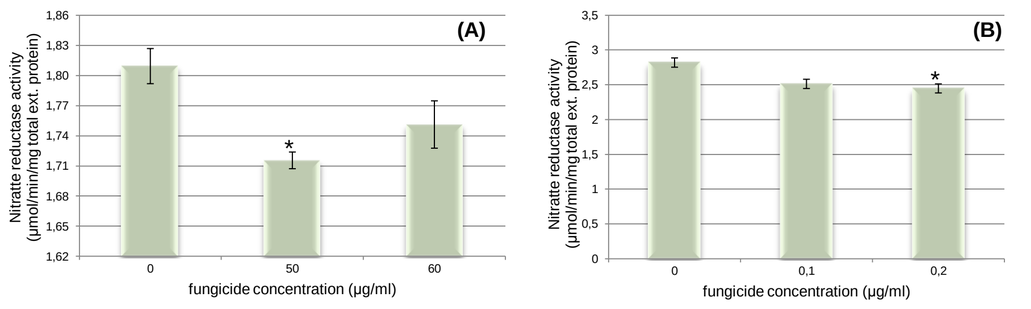

Figure 6. Nitrate reductase activity by non-lethal doses. Sixty minutes (A) and 24h (B) after fungicide addition NR was measured through quantization of nitrite using Griess Reagent. Values represent the mean (SEM) of six independent measurements.. The asterisks (*) indicate values that are significantly different from the control (P<0.05 by one way ANOVA).

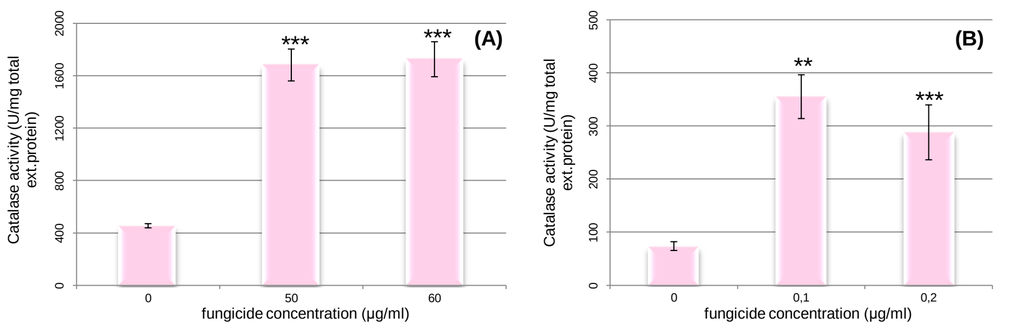

Figure 7. Catalase activity by non-lethal doses. Sixty minutes (A) and 24h (B) after fungicide addition CAT was measured using enzyme-catalyzed decomposition of H_2O_2, using potassium permanganate. Values represent the mean (SEM) of six independent measurements. The asterisks (*) indicate values that are significantly different from the control (**P<0.01 and *** P<0.001 by one way ANOVA).

-Statistical analysis:

Data were processed using Microsoft Excel and StatistIcal analysis was performed using GraphPad InStat Version 3.05 software.

Conclusions

-Results show that exposing the cells to small, non–lethal doses of the fungicide for both time periods induced oxidative stress

-Hydrogen peroxide leakage into culture medium was increased for the 1h incubation period at 50μg/ml. For the 24h treatment no significantly different values were observed at non-lethal doses.

-All assayed enzymes showed differences in activity except GR. Treated cells exhibited higher GST and CAT activities at all concentration and time treatments except 60μg/ml. NR activity was decreased after all treatments

-CAT activity had a roughly four-fold increase for both time and concentration experimentations. These results could explain the limited variations on extracellular H_2O_2 at the sub-lethal doses.

Future work

-Due to the observed sensitivity of the N2a cells to kresoxim-methyl this cell line will be used as the biological component for the construction of a biosensor for fungicide detection.

-Evaluation of the oxidative status of other cell lines exposed to the fungicide and/or other fungicides of the strobilurin group.

-More stress indicators for a more integrated study on the effects of the strobilurin fungicide on mammalian systems.

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