# DETAILED CYTOTOXICITY ASSESSMENT OF THE FORMULATED HERBICIDE ROUNDUP CLASSIC AND ITS CONSTITUENTS

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## Abstract

Cytotoxicity of the globally market-leading herbicide ROUNDUP CLASSIC formulation and its components such as the active ingredient glyphosate and the formulating agent POEA (a mixture of polyethoxylated tallow amines) were investigated on the murine neuroectodermal stem cell-like (NE-4C) and osteoblastic (MC3T3-E1) cell lines. The cytotoxic and genotoxic effects on cell viability and cell cycles were evaluated based on the results of flow cytometry, enzymatic-assays, and alkaline single cell gel electrophoresis (Comet) assays, furthermore, the effects on cell morphology and dynamic mass redistribution of cellular contents were assessed with the use of the label-free Epic BenchTop optical biosensor on MC3T3-E1 cells adhered on the surface of the biosensor. Differences in the sensitivity of the investigated cell lines were detected, while the MC3T3-E1 cell line indicated less sensitivity to the effects of the treatments. Furthermore, differences were also observed in the sensitivity of the performed assays. The order of the inhibitory potency of the investigated compounds was as follows: glyphosate IPA salt << ROUNDUP CLASSIC < POEA. The applied Epic technique provides an effective tool for the real-time detection of cytotoxicity.

## Introduction

Recently, the intensive use of various plant protection products and the consequent human exposure has been associated with several toxic effects including carcinogenicity, therefore the identification of potential hazards and the estimation of the exposures gained outstanding importance during the risk assessment of pesticides [1–3]. Due to the continuous high application rate of glyphosate, the world market-leading herbicide active ingredient exerts substantial environmental impacts as a ubiquitous surface water contaminant [4–6] and can cause unintended exposure to non-target organisms and humans due to the presence of its residues in the different environmental matrices, food and feeds [7–9]. The side-effect including genotoxic and endocrine-disrupting effects of glyphosate and its formulations have been demonstrated by several studies [10,11], thus strong criticism was expressed when the scheduled EU registration revision of glyphosate (and ROUNDUP) was postponed several times, although there is no uniform opinion regarding to the effects of glyphosate on human health. The US Environmental Protection Agency and the European Food Safety Authority classified glyphosate as a "probably not carcinogenic to humans" compound, while the International Agency for Research on Cancer has identified glyphosate as a "probably carcinogenic to humans" (2A) [7]. The present study aimed to assess the cytotoxic effects including the effects on cell viability, genotoxicity, apoptosis, and cell cycle of glyphosate IPA salt, its formulation (ROUNDUP CLASSIC), and the formulating agent POEA by several methods such as MTT assay, flow cytometry, alkaline Comet assay on the murine neuroectodermal stem cell-like (NE-4C) and osteoblastic (MC3T3-E1) cell lines. Furthermore, the effects of the investigated compounds

on the whole-cell responses of MC3T3-E1 cells were determined with the use of the optical biosensor Epic BT, and the comparison of the sensitivity of the cell lines and the different methods was also performed based on the results of the measurements.

## Experimental

The assessment of the cytotoxic effects of ROUNDUP CLASSIC, and its components were performed on two murine neuroectodermal stem cell-like (NE-4C) and osteoblastic (MC3T3-E1) cell lines based on different 96-well microplate-based methods. Cell viability was tested by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and by flow cytometry. The determination by flow cytometry was performed with the use of the Muse Cell Analyzer (Merck Millipore, Budapest, Hungary), which is a microcapillary cytometry instrument equipped with a fluorescence detector for single-cell analysis and used with different assay kits specific for given cell characteristics, including viability, DNA damage, apoptosis, as well as cell signalling. DNA damages were also detected by the alkaline Comet assays using single-cell gel electrophoresis suitable for the indication of DNA damages in the cells (e.g., damaged fragments from the nucleus). Effects of apoptosis were determined by flow cytometry with the use of two kits suitable for the measurement of annexin levels and caspase activity, while cell cycle analysis was conducted by flow cytometry after staining with propidium iodide [12]. The assessment of the effects on the whole-cell responses of MC3T3-E1 cells was performed with the use of the optical biosensor Epic BT and in 384-well Epic cell assay microplates. The bottom of each well of the microplate contained a 2×2mm RWG sensor, and MC3T3-E1 cells adhered on the surface of the biosensor in serum-containing and serum-free assay media. Responses of the biosensor were recorded for 1 h in real-time with a very high resolution. After the biosensor analysis, actin filaments in the cytoskeleton, focal contacts, as well as the nucleus of the cells were visualized by staining and microscopy, and the 3D structures of cells were assessed by digital holographic microscopy (HoloMonitor M4) [13]. The reported ROUNDUP CLASSIC concentrations are specified to actual dilutions (expressed as mass-per-volume %) of the formulation, while the concentrations of glyphosate and POEA are specified as ROUNDUP CLASSIC equivalent concentrations (mass-per-volume % concentrations of the diluted formulation containing the corresponding concentrations of the given components). Based on the measurements the sensitivity of the cell lines and the different methods was compared.

## Results and discussion

Based on the results of the cell viability tests, MTT assays indicated, that all of the investigated compounds affected the viability of the cells. The exposure to ROUNDUP CLASSIC significantly decreased the viability of NE-4C cells. The calculated 24 h $IC_{50}$ values on NE-4C cells were found to be 0.652 ± 0.006%, 0.00995 ± 0.00010%, and 0.00315 ± 0.00007% for glyphosate IPA salt, ROUNDUP CLASSIC, and POEA, respectively. The cytotoxicity of POEA was 200-fold higher for POEA compared to the active ingredient in NE-4C cells. According to the results of flow cytometry, high cytotoxicity was also detected for the formulation and POEA compared to glyphosate IPA salt. The calculated 24 h $IC_{50}$ values were in accordance with the result of the MTT assays on NE-4C cells. Glyphosate IPA salt resulted in substantially lower inhibition of cell viability on MC3T3-E1 cells, compared to the cytotoxic effects of the formulation and POEA. The tendency showed by the 24 h $IC_{50}$ values calculated for NE-4C cells agreed with the trend of the calculated values for MC3T3-E1 cells (24 h $IC_{50}$ = 1.2495± 0.0024%, 0.0187 ± 0.0007%, 0.00936 ± 0.00085% for glyphosate IPA salt, ROUNDUP CLASSIC, and POEA, respectively after the 24-h exposure. Based on the cell viability measurement on NE-4C cells, the sensitivity was higher by the flow cytometric measurement, than in MTT assays, and the

performed testing methods indicated acute physiological effects on both cell lines. Compared to MC3T3-E1, NE-4C cells indicated 1.1–2-fold higher sensitivity to all tested compounds in both viability tests. The effects indicated the explicit cytotoxicity of POEA in good accordance with the scientific literature.

During the evaluation of DNA damages, visualization of the fragmented DNA was performed by Comet assay based on electrophoretic separation, while specific staining of breaks in the double-stranded DNA damages were assessed by flow cytometry. The result of Comet assays indicated 2910-fold and 2247-fold higher levels of DNA migration for POEA, compared to glyphosate IPA salt, and ROUNDUP CLASSIC, respectively. The calculated lowest genotoxic dose (LGD) values were the following: 0.0259%, 0.00002%, and 0.0000089% for the active ingredient, ROUNDUP CLASSIC, and POEA, respectively. Based on the results, MC3T3-E1 cells were less sensitive to DNA damaging effects, than NE-4C cells, and the 24 h LGD values for POEA, ROUNDUP CLASSIC, and POEA were 271-, 120- and 3.2-fold higher for MC3T3-E1 than for NE-4C cells, respectively. Flow cytometric assays for DNS damage (double-stranded breaks) indicated 127- and 3.9-fold higher DNA migration was detected for POEA compared to glyphosate IPA salt and the formulation, respectively, after the 24-h exposure, and the detected LGD values were 0.0376%, 0.00117% and 0.000295% for the active ingredient, ROUNDUP CLASSIC, and POEA. DNA damage was observed also in the negative control in the absence of p53 tumor suppressor protein in the NE-4C cell line. The Comet assay was found to be more sensitive, than flow cytometry for NE-4C cells. The trend of the LGD values on the MC3T3-E1 cell line was similar to the values calculated for NE-4C cells. Compared to the effects of glyphosate IPA salt and the formulation, 401-fold and 8.4-fold higher DNA-damage levels were demonstrated for POEA, respectively. Our results indicated a higher frequency of single-stranded DNA breaks than double-stranded breaks.

The results of the assays for apoptosis evaluated by flow cytometry based on both annexin levels and caspase activity demonstrated the effects on the important regulatory pathway of cell growth and proliferation. Based on the determined annexin levels, the calculated 24 h $IC_{50}$ values for the ratio of total apoptotic cells were 0.246 ± 0.0134%, 0.00238 ± 0.00003%, and 0.00092 ± 0.00005% for glyphosate IPA salt, ROUNDUP CLASSIC, and POEA, respectively on NE-4C cells. A 2.6- fold and a 273-fold higher rate of apoptotic cells was observed for POEA compared to ROUNDUP CLASSIC and the active ingredient, respectively. After the 24-h exposure, the results of the caspase activity measurements, the determined 24-h $IC_{50}$ values for the ratio of apoptotic and dead cells were 0.568 ± 0.043%, 0.00748 ± 0.00012% and 0.00099 ± 0.00002% for glyphosate IPA salt, ROUNDUP CLASSIC, and POEA, respectively, and the highest level of apoptotic and dead cells was observed also for POEA, while 573-fold and 7.5-fold higher level was determined for the formulating agents than for glyphosate IPA salt and ROUNDUP CLASSIC, respectively. The tendency of the calculated 24 h $IC_{50}$ values on the MC3T3-E1 cell line corresponded to the trend of values determined for NE-4C cells and the highest level of apoptotic cells was detected also for POEA. After the 24-h treatment, the ratio of the dead cells increased, while the number of viable cells decreased in a dose-dependent manner. Based on the results, the formulating agents induced apoptosis at a lower concentration compared to ROUNDUP CLASSIC. The results of the applied methods indicated the lower genotoxicity of the active ingredient compared to the formulation and POEA. The observed difference between the two methods for the evaluation of apoptosis, while the determination based on the annexin levels evaluates the level of all apoptotic cells separately along with the dead cells, but the combined level of only the caspase-activated apoptotic cells and the dead cells indicated together by caspase activity assay.

Based on the cell cycle analysis performed by flow cytometry, in the negative control, the majority of NE-4C cells (~46%) were in the growth ($G_0/G_1$) phase after 24-h exposure, while a

decrease was observed for the investigated compounds. The decrease showed a monotonous dose-dependence for the formulation and POEA at the range of 0.0007–0.0026 ROUNDUP CLASSIC equivalent concentration, while the highest effect of glyphosate IPA salt was observed at the lowest tested concentration and gradually reaches the level of control at higher concentrations. The beginning of DNA replication (S phase) was not affected by glyphosate IPA salt based on the ratio of the cells, but decreased cell ratio was observed for ROUNDUP CLASSIC and POEA treatments. In contrast, in the cell division ($G_2$/M) phase the cell ratio increased after the treatments, but the highest increase was observed for the active ingredient at the lowest concentration, and a gradually decreased was detected at higher concentrations. Glyphosate IPA salt increased the ratio of NE-4C cells with the increase of the concentration, but the growth of the cell stopped in the $G_0/G_1$ phase due to the not optimal conditions, and only a small part of the cells get through the checkpoint control to the S phase and then to the $G_2$/M phase, thus cell ratio is lower in these phases compared to the control. In the negative control, a higher cell ratio (~80%) was detected in the $G_0/G_1$ phase for MC3T3-E1, than for NE-4C cells. The high relative ratio of cells in the resting ($G_0$) and first gap ($G_1$) phase is unique to MC3T3-E1 cells [14], but the cell ratio in $G_0/G_1$ phase decreased in ROUNDUP CLASSIC- and POEA-treated cells. The ratio of cells in the S phase was also not affected by the treatments, while the ratio of the cells increased in the $G_2$/M after exposure [12].

The effects of glyphosate IPA salt, ROUNDUP CLASSIC, and POEA were assessed on MC3T3-E1 cells with the use of the optical biosensor Epic BT, where the magnitude of the signal well correlated with the concentration of the investigated compounds. The lowest signals were observed for ROUNDUP CLASSIC and POEA at the highest concentrations. Glyphosate exposure (equivalent to 0.1% ROUNDUP CLASSIC) in a serum-containing medium resulted in late phase response on the cells, possibly due to cell cytoskeletal reactions (the effect was not significant under serum-free conditions). Dose-dependent responses of MC3T3-E1 cells were detected after the exposure of the investigated compound in serum-containing and serum-free media as well. Significant differences were not detected in the calculated $IC_{50}$ values for the formulation and POEA in both assay media, while the $IC_{50}$ values for glyphosate IPA salt were lower in the serum-containing medium. The results indicated the possible cytotoxic effect of ROUNDUP CLASSIC may attribute to the presence of POEA and possibly affected by the active ingredient. According to the visual assessments, elongated cell shape was detected in glyphosate-exposed cells compared to the control group, and reduced distribution of F-actin was observed in the treated groups. The results indicated the possible effects of glyphosate on the morphology of the cytoskeleton and cell adhesion. Based on the visualization, POEA exposure resulted in cytoskeletal collapse, cellular plasma membrane permeabilization, and cell death indicating higher toxicity compared to the effects of the active ingredient and ROUNDUP CLASSIC, furthermore necrosis of the cells was also observed as a result of POEA treatment. The detected effect on morphological changes in MC3T3-E1 cells of ROUNDUP CLASSIC was similar to the effects of POEA, suggesting that, the effect of the formulating agent prevails in the formulation. HoloMonitor technique demonstrated the inhibition of the proliferation in cells exposed to 0.02% ROUNDUP CLASSIC, and the cells became rounded, furthermore, the average optical thickness of the cells increased over time indicating induced apoptosis. POEA treatment (equivalent to 0.02 % ROUNDUP CLASSIC) resulted in a massive reduction of cell area after 5 min, and caused necrosis after 1 h of exposure. At a lower concentration of glyphosate IPA salt (equivalent of 0.02% Roundup solution) significant morphological changes were not detected compared to the control, but glyphosate caused impairments in the cell mobility and cell growth [13].

## Conclusion

The results of the performed cytotoxicity assays, the higher toxicity of the formulation and the formulating agent POEA were demonstrated compared to the individual effects of the active ingredient on both of the investigated murine cell lines, however, significant differences were observed in the sensitivity of the cell lines based on the results of the applied assays. Generally, the order of the cytotoxic potency of the tested compounds was the following: glyphosate IPA salt << ROUNDUP CLASSIC < POEA for both cell lines, but the higher cytogenotoxic effects observed for the formulation can be explained primarily by the presence of POEA. Based on the cell viability, genotoxicity tests the potential inhibitory and adverse effects of glyphosate, ROUNDUP CLASSIC, and POEA were demonstrated, and additional genotoxic risk for the ecosystem and human health was indicated. During the measurements, the applied Epic BT biosensor provides real-time, unique, and accurate information about the cytotoxicity of the different contaminants, with shorter assay time and the possibility of the early detection of the cytotoxic effects.

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