



Cytotoxic effects of Roundup Classic and its components on NE-4C and MC3T3-E1 cell lines determined by biochemical and flow cytometric assays

Marianna Oláh^a, Enikő Farkas^b, Inna Székács^b, Robert Horvath^b, András Székács^{a,*}

^a Agro-Environmental Research Centre, Institute of Environmental Sciences, Hungarian University of Agriculture and Life Sciences, Herman Ottó u. 15, H-1022 Budapest, Hungary

^b Nanobiosensors Laboratory, Institute of Technical Physics and Materials Science, Centre for Energy Research, Konkoly-Thege M. u. 29-33, H-1121 Budapest, Hungary

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ABSTRACT

Cytotoxic effects of the market leading broad-spectrum, synthetic herbicide product Roundup Classic, its active ingredient glyphosate (in a form of its isopropylamine (IPA) salt) and its formulating surfactant polyethoxylated tallowamine (POE-15) were determined on two murine cell lines, a neuroectodermal stem cell-like (NE-4C) and a high alkaline phosphatase activity osteoblastic cell line (MC3T3-E1). Cytotoxicity, genotoxicity, effects on cell viability and cell cycles were examined in five flow cytometry tests, the two former of which were compared by the enzymatic-assay and the alkaline single cell gel electrophoresis (Comet) assay. All of the tests indicated the NE-4C cells being more sensitive, than the MC3T3-E1 cell line to the treatments with the target compounds. Higher sensitivity differences were detected in the viability test by flow cytometry (7–9-fold), than by the MTT assay (1.5–3-fold); in the genotoxicity test by the Comet assay (3.5–403-fold), than by the DNA-damage test (9.3–158-fold); and in the apoptosis test by the Annexin V dead cell kit (1.1–12.7-fold), than by the Caspase 3/7 kit (1–6.5-fold). Cell cycle assays indicated high count of cells (~70%) in the G0/G1 phase for MC3T3-E1 cells, than in NE-4C cell (~40%) after 24 h. The order of the inhibitory potency of the target substances has unequivocally been POE-15 > Roundup Classic > glyphosate IPA salt.

1. Introduction

Glyphosate is the world market leading herbicide active ingredient. Glyphosate-based herbicides (GBHs) introduced into the US market in 1974, became leading plant protection products (PPPs) within 5 years, and their market has been substantially boosted by the introduction of genetically modified (GM) glyphosate-tolerant crops [1–4]. The global annual sales volume was estimated at 826 thousand metric tons in 2014 [4,5] expected to further grow, and the global glyphosate market is projected to reach USD 12.54 billion by 2024 [6]. Globally, there are more than 750 commercial GBHs on the market.

Acting on plants by blocking the biosynthesis of aromatic amino acids (tyrosine, tryptophane, phenylalanine) through the inhibition of the shikimic acid biosynthesis pathway [7,8], it is phytotoxic to practically all vegetal organisms with the exception of glyphosate-resistant weeds evolved by natural selection of genetically modified glyphosate-tolerant GM crops [9–12]. Due to its ongoing immense application volume, currently, glyphosate exerts a substantial environmental load, became a ubiquitous surface water contaminant [13–18],

and can result in unintended exposure of humans and other non-target organisms through its residues in environmental matrices and food [3, 4, 11, 19, 20].

The intensive uses of pesticides and consequent human exposure have been associated with numerous toxic effects including carcinogenicity [21–24]. As a result, hazard identification and exposure estimation gained importance in risk assessment of pesticides [25, 26]. Glyphosate or GBHs have also been found to exert side-effects of concern. Based on literature data cytotoxic, genotoxic and hormonal effects in *in vitro* (eukaryote and prokaryote) of glyphosate and GBHs have been analysed in numerous studies [27–30]. Studies demonstrated that glyphosate can act as an endocrine disruptor (*in vivo*) and induce reproductive damage in adulthood in rats [31–33]. Serious concerns have been raised, particularly in relation to human pregnancy and birth defects (via the above mentioned mechanisms) among agricultural workers and consumers [34].

Therefore, strong criticism was expressed when the scheduled EU registration revision of glyphosate (and Roundup) was postponed in 2012–2015 and postponed again later [35]. In 2015, the International

* Corresponding author.

E-mail address: szekacs.andras@uni-mate.hu (A. Székács).

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Agency for Research on Cancer (IARC) classified glyphosate as probably carcinogenic to humans (Group 2 A), based on “limited evidence” in human experiments and “sufficient evidence” in animal-experiments [36,37]. Nonetheless the European Food Safety Authority (EFSA) [31], the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) [38] and the European Chemicals Agency [39] did not confirm the IARC conclusion, and ECHA’s Committee for Risk Assessment (RAC) concluded that no classification for carcinogenicity is warranted under the Classification, Labelling and Packaging (CLP) Regulation, however, the harmonised classification for serious eye damage and toxic to aquatic life should be maintained [40]. In turn, the European Commission (EC) re-approved the authorization of glyphosate for 5 years in 2017 [41], and established the Assessment Group of Glyphosate (AGG), consisting of four Member States (France, Hungary, the Netherlands, Sweden) acting jointly for the forthcoming assessment of glyphosate beyond its current approval until 15 December 2022.

The discrepancy between the IARC and EFSA positions originated, among others, from two factors: the range of experimental data considered in the evaluations was different (all peer-reviewed reports or only accredited studies), and physiological effects of GBHs in some cases have been erroneously attributed to glyphosate, and not to its formulating agent POE-15. As a result, the two agencies interpreted the carcinogenicity of glyphosate in humans differently: IARC considered the association between exposure to glyphosate and non-Hodgkin lymphoma as “limited evidence in humans”; while in its conclusion, EFSA considered the evidence as “very limited”. The IARC position was explained in detail [42], and the difference between the IARC and EFSA statements was discussed [43] and challenged [44,45].

Due to the above, increased attention has been focused also on the toxicity and potential side-effects of the substances used for the formulation of pesticides. Glyphosate is commonly formulated with polyglucosides and polyethoxylated substances as adjuvants [46]. Polyethoxylated tallowamine (POE-15) is a main surfactant in herbicide Roundup Classic, the chemical acts as a tackifier by helping its adsorption on plant surfaces and uptake by plants, and it also facilitates its uptake to cells through disturbing membrane (cell wall) processes. Increased toxicity of POE-15-formulated GBHs compared to glyphosate has been documented in the scientific literature [47,48], and POE-15 and other co-formulants alone also exert numerous side effects, for example genotoxicity, cytotoxicity [28, 47, 49–53]. GBHs containing POE-15 were banned in the EU in 2016 [54], and the ban was extended to polypropoxylated tallowamines and a number of other co-formulants in 2021 [55]. In this study the main test chemicals were related to a group of worldwide used herbicides, the formulated herbicide preparation Roundup Classic, its active ingredient glyphosate, and its formulation surfactant POE-15. In light of the apparent contradiction between IARC on the one side and EFSA, ECHA and the JMPR on the other side, cyto- and genotoxicity of glyphosate and its formulated products are very important to be examined in *in vitro* toxicity assays to provide further experimental data for a sound, evidence-based risk assessment for licensing and regulation of both the active ingredient alone and its formulations.

The present investigation was aimed to examine cytotoxic effects of the target substances, glyphosate isopropylamine salt (G IPA), polyethoxylated tallowamine (POE-15) and Roundup Classic (R), by several methods on two murine cell types: an osteoblastic cell line (MC3T3-E1) with high alkaline phosphatase activity and a neuroectodermal stem cell-like (NE-4C) both successfully used in previous studies [51,56,57]. Thus, cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and by flow cytometry; DNA damage was assessed by the alkaline Comet assay and by flow cytometry; cell death/apoptosis was evaluated by flow cytometry on the basis of both annexin levels and caspase activity; and cell cycle analysis was performed by flow cytometry upon staining with propidium iodide (PI). Roundup Classic concentrations reported in the current study are specified to actual dilutions (expressed as

mass-per-volume %) of the formulated herbicide. Concentrations of glyphosate and POE-15 are specified both in actual concentrations of the given compound (expressed e.g. as $\mu\text{g}/\text{ml}$) and in “Roundup Classic equivalent concentrations” (mass-per-volume % concentrations of diluted Roundup Classic containing the corresponding concentrations of these substances).

2. Materials and methods

2.1. Chemicals

Solid reagents for culturing media were purchased from Reanal Laborvegyzser Kereskedelmi Kft (Hungary, Budapest). All other reagents were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA), unless stated otherwise. Glyphosate (N-phosphonomethyl glycine, G, CAS No: 1071-83-6) was also purchased from Sigma-Aldrich Co. LLC in the form of its isopropyl ammonium salt (CAS No: 386411-94-0), as the active ingredient in most glyphosate-based herbicides. Formulation Roundup Classic (R, MON2139, Monsanto, Hungary, approval 02.5/915/2/2010) was available as a commercial herbicide. Co-formulant POE-15 under trade name Emulson AG GPE 3SS was obtained from Lamberti S.p.A. (Abbazate, Italy). Cell culture media (pH 7.4) for NE-4C and MC3T3-E1 cells were minimum essential medium (MEM) containing 4 mM glutamine, 5% fetal bovine serum (FBS) (Biowest SAS, France), 100 U/ml penicillin and 100 mg/ml streptomycin solution, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B, as well as α -modified MEM (α -MEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin solution, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B, respectively. Stock solutions of G IPA, R and POE-15 were prepared freshly in appropriate cell culture medium and filtered through a 0.22 μm filter (Merck MF-Milipore, Budapest, Hungary).

2.2. Cell cultures

The NE-4C cell line originated from primary brain cell cultures prepared from the fore- and mid-brain vesicles of 9-day-old transgenic mouse embryos lacking functional p53 tumour suppressor protein [58, 59] was obtained from Dr. Emilia Madarász at the Institute of Experimental Medicine of the Hungarian Academy of Sciences (Budapest). The MC3T3-E1 cell line (99072810 Sigma-Aldrich) originated from C57BL/6 mouse calvaria [60,61]. Cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C. On reaching 80% confluence, cells were passaged by using 0.05% (w/v) trypsin, 0.02% (w/v) EDTA solution. These cell lines were selected partly because their dose-dependent physiology, surface adhesion, dynamic mass redistribution and morphology responses to glyphosate and GBHs have been elucidated in our earlier studies using biosensor platforms [51,56], within which the integrin-specific cell adhesion modifying activity of glyphosate has been demonstrated on MC3T3-E1 cells [57], and both cell lines showed high response levels to these substances compared to other cell lines reported in the scientific literature [4].

2.3. MTT assay

Cell viability was also assessed by an *in vitro* biochemical assay, the succinate dehydrogenase activity test carried out as described previously [51,62] using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as a substrate. During the assay, the mitochondrial succinate dehydrogenases of viable cells transform MTT into a blue formazan product.

NE-4C and MC3T3-E1 cells at 5×10^4 cells/ml concentration were plated in 96-microwell plates (ThermoFisher Scientific, Waltham, MA, USA). After the preincubation phase, cells were exposed to various concentrations of G, R or co-formulant (POE-15) for 24 h, in volumes of 200 $\mu\text{l}/\text{well}$. Optical densities were determined using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA)

at a test wavelength of 570 nm and a reference wavelength of 620 nm. Cell viability was calculated as relative percentage of the untreated control [62]. In our experiments, 3 independent treatments were performed at 6 concentrations in triplicates to determine cell viability.

2.4. Comet assay

Single-cell gel electrophoresis (SCGE) is a useful technique developed for studying DNA damage. The treated cells are suspended in a thin agarose gel on a microscope slide, and after lysis and electrophoresis, the gel is stained with a fluorescent DNA binding dye (ethidium bromide) [63,64]. If DNA damage occurs in the cells, the migration of the chromosomal DNA and its damaged fragments from the nucleus appears in a shape of a comet [65], and the more intensive DNA damage is, usually the larger the comet becomes. In the current study alkaline lysis version of the Comet assay was used, followed by electrophoresis under alkaline conditions (pH 12.1). DNA damage was assessed by the “tail moment” parameter developed by Olive et al. [66] – a combination of two parameters already in use – tail length in micrometers and percentage of DNA content in a damaged comet tail (tail intensity). The cells were seeded in 6-well plates at a density of 1.5×10^5 cells/well for 24 h, and then exposed to various concentration of G, R or POE-15 in supplemented MEM medium for 24 h. The negative and positive controls were only MEM and 0.01% hydrogen peroxide plus MEM, respectively. Following the steps of the Comet assay, the samples were finally stained with 50 μ l of a 20 μ g/ml solution of ethidium bromide in the dark at room temperature, and main assay descriptors (tail moment, percentage of DNA in tail and percentage of DNA in the head) were measured with a fluorescence microscope (Nikon Eclipse E600 or Olympus IX73) using the LUCIA™ Comet Assay 3.5 software (Laboratory Imaging, s.r.o., Praha, the Czech Republic). The use of software in particular also provides a range of different parameters (head DNA (%), tail DNA (%), integral intensity, head radius, tail length, tail moment, head area, tail area) many of which relevant to determine the extent of DNA damage. These parameters are preferred over the manual scoring method due to the ease of interpretation of data during statistical analyses. In our experiments, we used tail moment because it describes the value of the damaged DNA complex, but the percentage of tail DNA is also a reliable parameter [67]. In our experiments, 3 independent treatments were performed at 4 concentrations in triplicates (50 cells in one slide measured) to determine DNA damage.

2.5. Flow cytometry

The Muse™ Cell Analyzer (Merck Millipore, Budapest, Hungary), is a microcapillary cytometry device equipped with a fluorescence detector for single-cell analysis. The instrument is used with several assay kits by the manufacturer utilising fluorescent reagents specific for given cell characteristics, including viability, apoptosis and DNA damage, as well as cell signalling. Cells were pre-treated for each assay type similarly. Thus, NE-4C and MC3T3-E1 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and grown for overnight in a humidified incubator followed with the cell treatment with different concentration of the compounds. Floating and adherent treated cells were collected after 24 h incubation. All the kits were applied according to the manufacturer's instructions and the samples were analysed using appropriate software module. In our experiments, treatments were performed at 6 concentrations in triplicates to determine given cell characteristics.

2.5.1. Cell viability determined by flow cytometry

After incubation with the compounds for 24 h, the cells were collected in microcentrifuge tubes (ThermoFisher Scientific, Waltham, MA, USA) and were subjected to flow cytometry using the Muse™ Count and Viability Kit (MCH100102, Merck Millipore, Budapest, Hungary), detecting optical density in all treatments at 532 nm wavelength, and calculating viable cell count (cells/ml), total cell count (cells/ml) and %

viability of the samples by the Muse™ Count and Viability Software Module. Results were expressed as a percentage of the negative control.

2.5.2. DNA damage determined by flow cytometry

Cells were treated with the test substances at lower concentrations than in the Count and Viability kit to assure that viability of the cells tested did not decrease below 60%. Cells were prepared according to the descriptions of Muse™ Multi-colour DNA-damage kit (MCH200107, Merck Millipore, Budapest, Hungary) instruction. This kit includes two conjugated antibodies, ATM protein kinase and histone H2X.A to measure DNA damage in the samples tested. When DNA damage occurs, the ATM protein kinase is phosphorylated and activates downstream gene products e.g., histone H2X.A, the most important indicator of the level of breaks in double-stranded DNA.

2.5.3. Apoptosis/Caspase 3/7 activity determined by flow cytometry

Apoptosis status analysis of the cells was carried out by the Muse™ Annexin V and Dead Cell and Muse™ Caspase 3/7 Assay Kits (MCH100105 and MCH100108, Merck Millipore, Budapest, Hungary). The overall apoptotic status of the cells treated with the test substances was monitored by the Muse™ Annexin V and Dead Cell kit. The method allows distinction among four cell populations: non-apoptotic (alive), early apoptotic, late apoptotic and dead (with nuclear debris). The Muse™ Caspase 3/7 kit detects sub-populations of the cells detected by Muse™ Annexin V and Dead Cell kit on the basis of the activity of caspase 3/7 enzymes, conserved cysteine proteases executing programmed cell death (apoptosis) by cleaving their substrate proteins. The Muse™ Caspase 3/7 kit also provides distinction among four cell populations: non-apoptotic (alive), apoptotic cells exhibiting caspase 3/7 activity, late apoptotic/dead cells and necrotic cells.

2.5.4. Cell cycle determined by flow cytometry

The distribution of cells within a cell population among different phases of the cell proliferation cycle is an informative indicator, whether cell division of the population has been affected upon exposure to the test substances. The proportion of cells in the beginning DNA replicating (S), cell division (G2/M) and growth (G0/G1) phases was detected by the Muse™ Cell Cycle Assay Kit (MCH100106, Merck Millipore, Budapest, Hungary), utilising staining of the DNA content of the cells by PI, showing a characteristic increase during DNA replication and subsequent decreases relative to cell size also detected, as seen in the DNA content index and histogram, as well as cell size index determined by the assay. Percentage of cells in G0/G1, S and G2/M phases were determined using the Muse™ Cell Cycle Software Module.

2.6. Statistical analysis

Statistical analyses were conducted using the R Statistical program 3.5.1 (R Development Core Team, 2018) and OriginLab OriginPro 7.0 data analysis and graphing software system (Origin(Pro), Version 7.0 (OriginLab Corporation, Northampton, MA, USA). Results are reported as mean \pm standard deviation (SD). Mean values were calculated as the average of the replicates. In case of independent treatments, replicates were considered individually i.e., mean values were determined as the average of the replicates in all treatments (not as the average of the averages from each treatment). When necessary, single outliers were selected by boxplots statistics using the boxplot.stats function in the R Statistical program. IC₅₀ values were calculated by non-linear regression using a logistic (5-parameter) sigmoid dose-response equation by Rodbard [68], with p values \leq 0.05 considered statistically significant. During statistical analysis, the effects of the treatments on the cell cycle were analysed with the use of general linear models. The normality of the data and the applicability of the fitted model were checked in each case with diagnostic plots (residual variances, QQ plot, Cook's distance plot).

3. Results and Discussion

3.1. Cell viability

MTT assay and flow cytometry have been used to distinguish dead and alive cells on the basis of the differential staining of the dyes applied in the tests to intact and damaged cell membranes.

3.1.1. MTT assay

The MTT assay was carried out to measure cell viability in a biochemical (enzyme) assay for each substance, G IPA, POE-15 and R. Obtained data demonstrated, that all compounds studied inhibit the viability of the cells (Fig. 1A,B). Roundup Classic markedly decreased NE-4C cell viability at concentrations above 0.0032%. 24 h IC₅₀ values on NE-4C were found to be 0.652 ± 0.006%, 0.00995 ± 0.00010%, and 0.00315 ± 0.00007% for G IPA, R, and POE-15 (Fig. 1A), respectively. Cytotoxicity appears to be approximately 200-fold higher for POE-15 than for G IPA after 24 h treatments on NE-4C cell line.

On MC3T3-E1 osteoblastic cells, 24 h IC₅₀ values were, determined similarly as above, were found to be 0.7256 ± 0.0068%, 0.0101 ± 0.0004%, and 0.00639 ± 0.00003% for G IPA, R, and POE-15, respectively (Fig. 1B). Cytotoxicity appears to be approximately 110-fold higher with POE-15 than with G IPA after 24 h treatments. IC₅₀ values determined for cytotoxicity and other biological effects determined for glyphosate, polyethoxylated tallowamine (POE-15) and Roundup Classic are summarized in Table 1.

3.1.2. Flow cytometric cell viability assay

After 24 h of exposure to the substances tested, NE-4C cells were collected and analysed using Muse™ Cell Analyzer. G IPA concentrations were chosen to 4-fold lower than the concentration used in agricultural applications (G IPA content in 2% Roundup solution), because stem cells are more sensitive, than carcinoma cells. The concentrations used for G IPA were calculated on the basis of literature data [51]. High levels of cytotoxicity, approximately 500-fold higher, than that for G IPA, was detected for POE-15 and Roundup Classic.

On NE-4C cells, 24 h IC₅₀ values determined similarly as above

(Fig. 1C), were found to be 0.595 ± 0.009%, 0.00469 ± 0.00008%, and 0.00115 ± 0.00007% for G IPA, R and POE-15, respectively in Roundup Classic equivalents. Similarly to reported values in the scientific literature [51,63,69], results observed in the present study confirmed that R and POE-15 exerted cytotoxic effects on NE-4C cells at very high dilutions, substantially below anticipated agricultural exposures. The cytotoxicity appears to be approximately 495-fold higher with POE-15 than with G IPA after 24 h treatments.

MC3T3-E1 cells were treated and analysed similarly as NE-4C cells. G IPA caused substantially lower inhibition of cell viability than R or POE-15. After 24 h of exposure, IC₅₀ values on MC3T3-E1 osteoblastic cells, determined similarly as above (Fig. 1D), were found to be 1.2495 ± 0.0024%, 0.0187 ± 0.0007%, 0.00936 ± 0.00085% for G IPA, R and POE-15, respectively. These IC₅₀ values indicate that this kit system assesses NE-4C cells appear to be more sensitive than the MTT assay.

In our viability studies, all test substances exerted acute physiological effects on both cell lines, NE-4C and MC3T3-E1 cells. The NE-4C cell line was found 1.1–2-fold more sensitive to all target substances than MC3T3-E1 in both viability tests (MUSE viability kit, MTT assay). In agreement with previous findings, our studies also clearly demonstrated the explicit cytotoxicity of POE-15, exerted in other studies on cellular respiration and membrane integrity between 0.00155% and 0.0097% at Roundup Classic equivalent concentration [47,48]. The in vitro data on cytotoxicity of glyphosate and GBHs indicate that the most sensitive cell lines appeared to be human hepatopoietic Epstein-Barr virus transformed lymphocyte Raji cells [52], human peripheral white blood cells [69], regenerative fin cell lines from Pond loach (*Misgurnus anguillicaudatus*) [70], human epithelial HaCaT keratinocyte cells [71] and murine neuroectodermal stem-cell-like line NE-4C [51]. In contrast, cell types with lower apparent sensitivity were JEG3 [28,47,49,72], murine osteoblastic cells (MC3T3-E1) [56], human embryonic kidney cells (HEK293) [4,28,47,49].

3.2. DNA damage

DNA damage was assessed by Comet assay and by flow cytometry. The former method is based on electrophoretic separation and

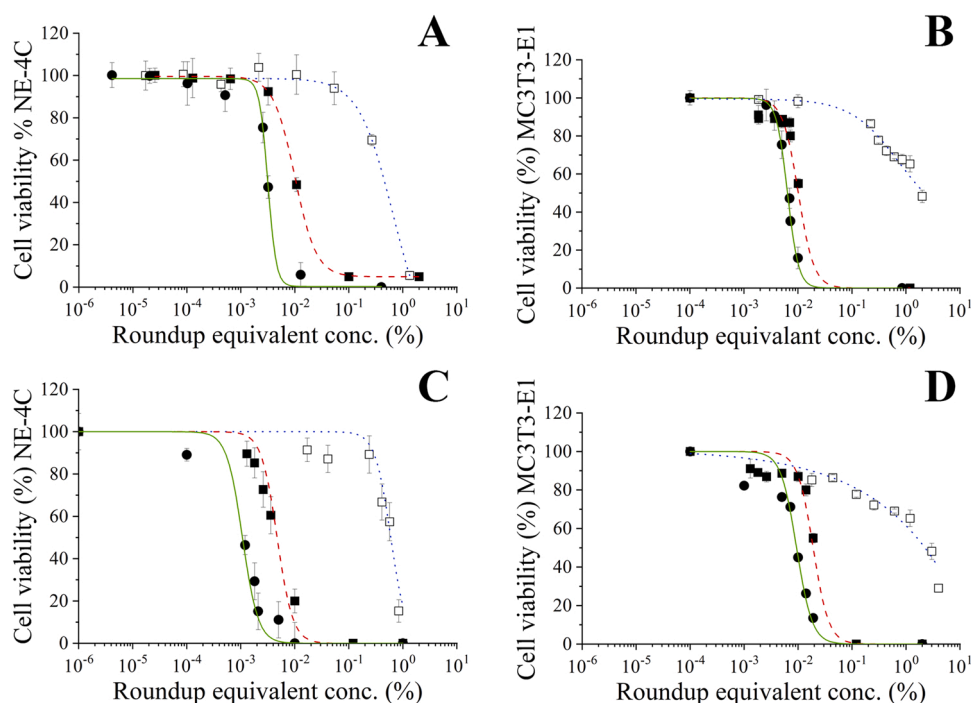


Fig. 1. Concentration-dependent effects on cell viability upon 24 h of exposure to G IPA (□), R (■) and POE-15 (●) determined by the MTT assay on NE-4C (A) and MC3T3-E1 (B) cells or by the Muse™ Count and Cell Viability test on NE-4C (C) and MC3T3-E1 (D) cells. Data are shown as a mean ± SD.

Table 1

Half-maximal inhibitory concentration (IC₅₀) for cytotoxicity and apoptosis and lowest genotoxic dose (LGD) values for genotoxicity disruption determined for glyphosate, polyethoxylated tallowamine (POE-15) and Roundup Classic.

IC ₅₀ or LGD values ^a (expressed as % Roundup Classic equivalent concentration ^b and as mg/ml or µg/ml)							
Cell line	glyphosate (%)	(µg/ml)	POE-15 (%)	(µg/ml)	Roundup Classic ^c (%)	glyphosate (µg/ml)	POE-15 (µg/ml)
NE-4C^d							
Cell viability							
MTT (biotest)	0.652 ± 0.006	3168.72	0.00315 ± 0.00007	5.72	0.00995 ± 0.00010	48.36	18.06
Cell Analyzer kit (Muse)	0.595 ± 0.009	2891.70	0.00115 ± 0.00007	2.09	0.00469 ± 0.00008	22.79	8.51
DNA damage							
Comet (biotest)	0.0259	125.87	0.0000089	0.043	0.00002	0.09	0.03
DNA Damage kit (Muse)	0.0376	182.55	0.000295	0.54	0.00117	5.68	2.12
Programmed cell death							
Annexin V Dead Cell kit (Muse)	0.246 ± 0.134	1195.56	0.00092 ± 0.00005	1.67	0.00238 ± 0.00003	11.57	4.32
Caspase 3/7 kit (Muse)	0.568 ± 0.043	2760.48	0.00099 ± 0.00002	1.80	0.00748 ± 0.00012	36.35	13.58
MC3T3-E1^d							
Cell viability							
MTT (biotest)	0.7256 ± 0.0068	3526.42	0.00639 ± 0.00003	11.60	0.0101 ± 0.0004	49.09	18.33
Cell Analyzer kit (Muse)	1.2495 ± 0.0024	6072.57	0.00936 ± 0.00085	16.99	0.0187 ± 0.0007	90.88	33.94
DNA damage							
Comet (biotest)	0.0835	405.97	0.0024125	4.38	0.00224	10.89	4.07
DNA Damage kit (Muse)	0.0375	182.15	0.0000935	0.17	0.000786	3.82	1.43
Programmed cell death							
Annexin V Dead Cell kit (Muse)	0.2731 ± 0.0045	1327.27	0.01169 ± 0.00048	21.22	0.0167 ± 0.0013	81.16	30.31
Caspase 3/7 kit (Muse)	0.6412 ± 0.0339	3116.23	0.00649 ± 0.00012	11.78	0.0073 ± 0.0001	35.48	13.25

^a The IC₅₀ value refers to the concentration at which the substance studied exerts half of its maximal inhibitory effect in the cytotoxicity and apoptosis assays. The LGD value refers to the lowest dose at which the substance studied causes a positive response in the genotoxicity assay.

^b Mass-per-volume percent concentrations of diluted Roundup Classic containing the corresponding concentrations of these substances

^c Percentage concentrations of the formulated herbicide and actual concentrations of the active ingredient and the formulant in Roundup Classic at the given mass-per-volume concentration are indicated

^d Cell lines – NE-4C: established from the cerebral vesicles of 9-day-old mouse embryos lacking the functional p53 genes; MC3T3-E1: osteoblast precursor cell line derived from *Mus musculus* (mouse) calvaria

visualisation of the fragmented DNA, while specific staining of breaks in the double stranded DNA are detected in the latter.

3.2.1. Comet assay

Tail moments (the rate of DNA fragmentation) were visualised on a fluorescence microscope and were calculated automatically using the LUCIA™ Comet Assay 3.5 software. Tail moment is the product of the

tail DNA content and the mean distance of migration in the tail. For NE-4C cells, tail moment values ranged from 21.9 to 104.6 for the test substances, while tail intensity and tail length ranged from 42.8% to 63.8% and from 43.6 to 114.1 µm, respectively. For MC3T3-E1, tail moment values ranged from 2.15 to 38.1 for the test substances, while tail intensity and tail length ranged from 7.12% to 28.6% and 6.64–45.9 µm, respectively. After 24 h of exposure on NE-4C cells,

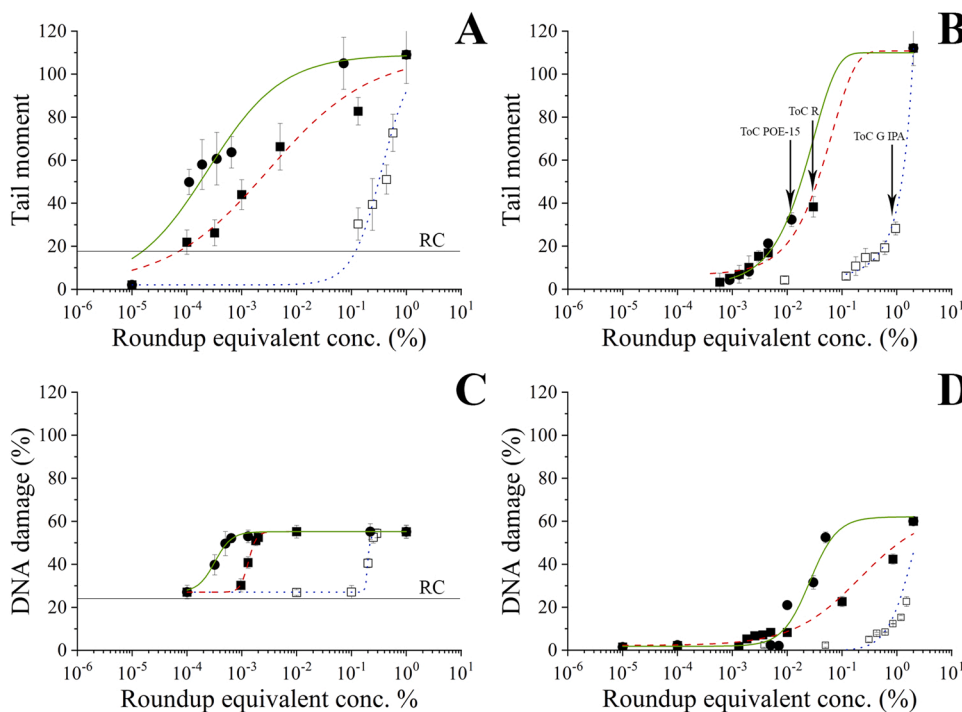


Fig. 2. Concentration-dependent effects on cell DNA damage upon 24 h of exposure to G IPA (□), R (■) and POE-15 (●) determined by Comet assay on NE-4C (A) and MC3T3-E1 (B) cells or by the Muse™ Multi-colour DNA-damage kit on NE-4C (C) and MC3T3-E1 (D) cells. Data are shown as a mean ± SD. ToC: threshold of cytotoxicity for R and POE-15 (ToC R and ToC POE-15) and G IPA (ToC G IPA). Above these thresholds, direct cytotoxicity exerted impeded the determination of the tail moments.

determined similarly as above on Fig. 2A, the lowest genotoxic dose (LGD) values were 0.0259%, 0.00002% and 0.0000089% for G IPA, R and POE-15, respectively in Roundup Classic equivalents. The high sensitivity of the Comet assay has been used for an initial screening of potential genotoxicity of G IPA, Roundup and POE-15 [50,52]. High level of DNA migration was detected for POE-15, approximately 2910-fold and 2247-fold higher than for G IPA and Roundup, respectively. On MC3T3-E1 cells, the 24 h LGD values, determined similarly as above and depicted on Fig. 2B, were 0.0835%, 0.00224%, 0.0024125% for G IPA, R, POE-15, respectively in Roundup Classic equivalents. High levels of DNA migrations were detected for POE-15, and were found to be 34-fold higher than for G IPA, respectively. A comparison of the results obtained on DNA migrations in NE-4C and MC3T3-E1 cells indicates that NE-4C cells are more sensitive to DNA damaging effects than MC3T3-E1 cells. Thus, the 24 h LGD values of POE-15, R, and G IPA were found to be 271-, 120- and 3.2-fold higher for MC3T3-E1 compared to the NE-4C cells, respectively. LGD values determined for genotoxicity and other biological effects determined for glyphosate, polyethoxylated tallowamine (POE-15) and Roundup Classic are summarized in Table 1.

3.2.2. Flow cytometric assay for double stranded breaks

Histone H2X.A and ATM protein kinase together can describe DNA damage (double stranded breaks). Muse™ Multi-colour DNA damage kit can describe DNA-damage in percentage. After 24 h of exposure on NE-4C cells were determined similarly as above on Fig. 2C, LGD value was 0.0376%, 0.00117% and 0.000295% for G IPA, R, POE-15, respectively in Roundup Classic equivalents. High level of DNA migrations detected for POE-15, which is 127-fold higher, than for G IPA, and 3.9-fold higher than R. In our result, we cannot detect DNA damage above 55%, because we have seen cell death upper than 55%. We observed DNA-damage in negative control in absence of p53 tumour suppressor protein in the NE-4C cell line, the effect of which having been more apparent in the results by the DNA damage kit than in the Comet assay. For NE-4C cells Comet assay is more sensitive, than the flow cytometry double strands breaks kit. After 24 h exposure the LGD value on MC3T3-E1 cells were determined similarly as above were found to be 0.0375%, 0.000786% and 0.0000935% for G IPA, R, POE-15 (Fig. 2D). High level of DNA-damage detected for POE-15, which is 401-fold higher, than for G IPA, and 8.4-fold higher than R, in this flow cytometry Muse™ Multi-colour DNA damage kit.

RC: residual cytotoxicity. Observed DNA damage in the negative control due to the absence of the p53 tumour suppressor in the NE-4C cell line.

Our assessment indicated marked DNA damage measured by tail moment in cells exposed to G IPA, R, and POE-15 for 24 h, similarly to previous reports on other cell lines [50,52,73], also correlating with the reported teratogenic effects of GBHs in amphibians, mammals (rats) and teratogenic birth defects in rats demonstrated to be exerted through the retinoic acid signalling pathway [74–76]. Differences in the effects detected by the two assay types are due to the fact that the Comet assay detects single strand DNA breaks and like alkali-labile sites, while the flow cytometric assay measures double stranded DNA breaks. Single stranded DNA breaks are more frequent than double stranded ones, which is reflected in the differences seen in LGD values between the Comet assay and the DNA damage kit version. Nonetheless, the aim of this study has been to demonstrate the extent of DNA damage observed, and not to compare the two methods to each other.

3.3. Flow cytometric assays for apoptosis and caspase 3/7 activity

The rate of cell death or apoptosis was evaluated by flow cytometry on the basis of both annexin levels and caspase activity determined in the treated cell cultures. Programmed cell death is an important regulatory pathway of cell growth and proliferation. Caspases are important regulatory elements in the programmed cell death in response to pro-apoptotic signals, as Caspase 3 and Caspase 7 enzymes are the

executioners of apoptosis [56,77,78].

The Muse™ Annexin V dead cell kit was used to determine the ratio of total apoptotic cells, after 24 h of exposure. The IC₅₀ values on NE-4C, determined similarly as above and depicted on Fig. 3A, were found to be 0.246 ± 0.0134%, 0.00238 ± 0.00003% and 0.00092 ± 0.00005% for G IPA, R, POE-15, respectively in Roundup Classic equivalents. A high level of apoptotic cells was detected for POE-15, being 273-fold and 2.6-fold higher than for G IPA and R, respectively, using the flow cytometry Muse™ Annexin V dead cell kit.

The Muse™ Caspase 3/7 kit was used to determine the ratio of apoptotic and dead cells, after 24 h of exposure, determined similarly as above and depicted on Fig. 3C, the IC₅₀ values on NE-4C cells were 0.568 ± 0.043%, 0.00748 ± 0.00012% and 0.00099 ± 0.00002% for G IPA, R, POE-15, respectively, in Roundup Classic equivalents. High levels of apoptotic and dead cells were detected for POE-15, being 573-fold and 7.5-fold higher than for G IPA and R, respectively, using the flow cytometry Muse™ Caspase 3/7 kit. IC₅₀ values determined for apoptosis disruption and other biological effects determined for glyphosate, polyethoxylated tallowamine (POE-15) and Roundup Classic are summarized in Table 1.

For the Annexin V and dead cell kit, IC₅₀ values on the MC3T3-E1 cell line were, 0.2731 ± 0.0045%, 0.0167 ± 0.0013% and 0.01169 ± 0.00048% for G IPA, R, POE-15, respectively, in Roundup Classic equivalents. A high level of apoptotic cells was detected for POE-15, being 24-fold and 1.4-fold higher than for G IPA and R, respectively, using the flow cytometry Muse™ Annexin V dead cell kit. The results show that after treatments for 24 h the number of viable cells decreased and the ratio of dead cells increased in a dose-dependent manner. The results also demonstrated that POE-15 induces apoptosis at lower concentration than in R (Fig. 3B, D).

In the Caspase 3/7 kit applied on MC3T3-E1 cells the IC₅₀ values expressed in Roundup Classic equivalents were calculated and resulted in 0.6412 ± 0.0339%, 0.0073 ± 0.0001% and 0.00649 ± 0.00012%, for G IPA, R, POE-15, respectively, in Roundup Classic equivalents (Fig. 3D). High levels of apoptotic and dead cells were detected for POE-15, being 99-fold and 1.1-fold higher than for G IPA and R, respectively, using the flow cytometry Muse™ Caspase 3/7 kit.

These results correlated with those of the previous test kit systems; indicating that G IPA exerts lower toxicity, than R or POE-15. A substantial difference is seen between the results of the two methods for the assessment of apoptosis, the Annexin kit and the Caspase 3/7 kit. This is explained by the fact that the former shows separately the level of all apoptotic cells along with the dead cells, while the latter indicates the combined level of only the caspase activated apoptotic cells and the dead cells together.

The flow cytometric assays for the assessment of cell viability/apoptosis allow differentiation of cell subpopulations: the Muse™ Annexin V dead cell kit can distinguish among non-apoptotic (live) cells, early apoptotic cells, as well as late stage apoptotic and dead cells; while the Muse™ Caspase 3/7 kit sets apart non-apoptotic (live) cells, apoptotic cells, late stage apoptotic and dead cells, as well as necrotic (dead) cells. As seen, the differentiation among the subpopulations is somewhat different in the two assay types, moreover, the setting of the gates to establish the quadrates of the subpopulations in the fine tuning of the plot is to some extent arbitrary and therefore subjective. Therefore, the cumulated fraction of apoptotic (including early and late stage apoptosis) and dead cells appeared to be most accurate for the calculation of IC₅₀ values.

Apoptotic levels 60–70% of NE-4C cells were detected using the flow cytometry Muse™ Annexin V dead cell kit, for exposure to POE-15 approximately 2.15 orders of magnitude difference than for G IPA, and 2.5-fold higher than R. In addition, higher level (80–100%) of apoptotic and dead cells detected for POE-15, which is approximately 2.5 orders of magnitude difference, than for G IPA, and 7.5-fold higher than R using flow cytometry Muse™ Caspase 3/7 kit. Similarly, elevated levels were observed in both flow cytometry assays for the MC3T3-E1

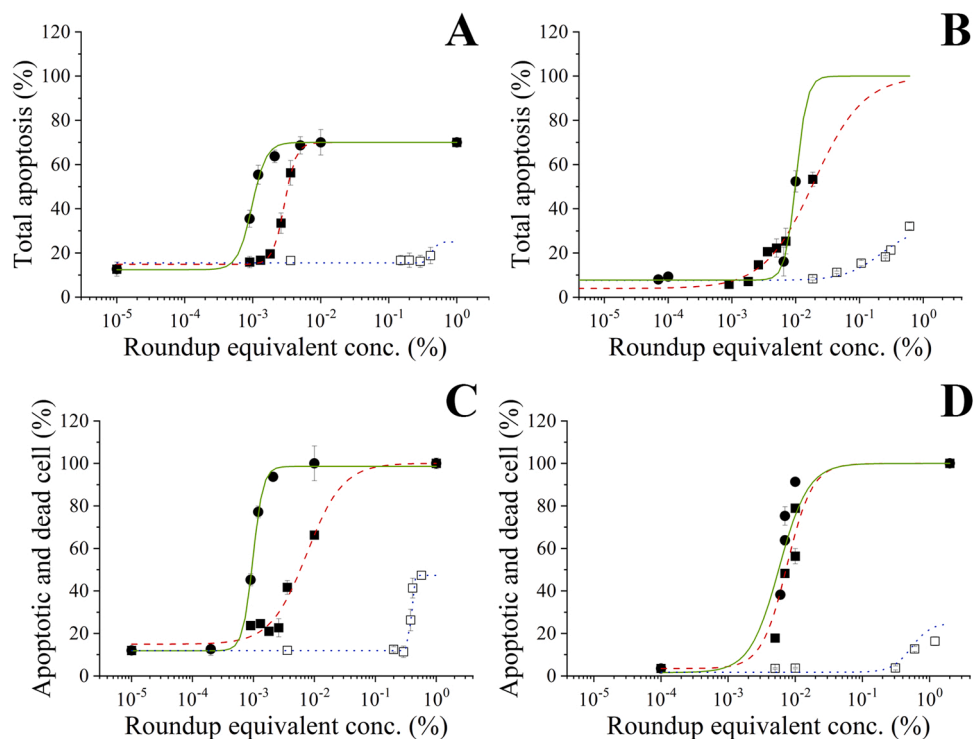


Fig. 3. Concentration-dependent effects on cell apoptosis upon 24 h of exposure to G IPA (□), R (■) and POE-15 (●) determined by Muse™ Annexin V and Dead cell kit on NE-4C (A) and MC3T3-E1 (B) cells or by Muse™ Caspase 3/7 on NE-4C (C) and MC3T3-E1 (D) cells. Data are shown as a mean ± SD.

cell line as well.

3.4. Flow cytometric assay for cell cycle analysis

Cell cycle analysis was performed by flow cytometry upon PI staining. The Muse™ Cell cycle assay kit uses a premixed reagent that

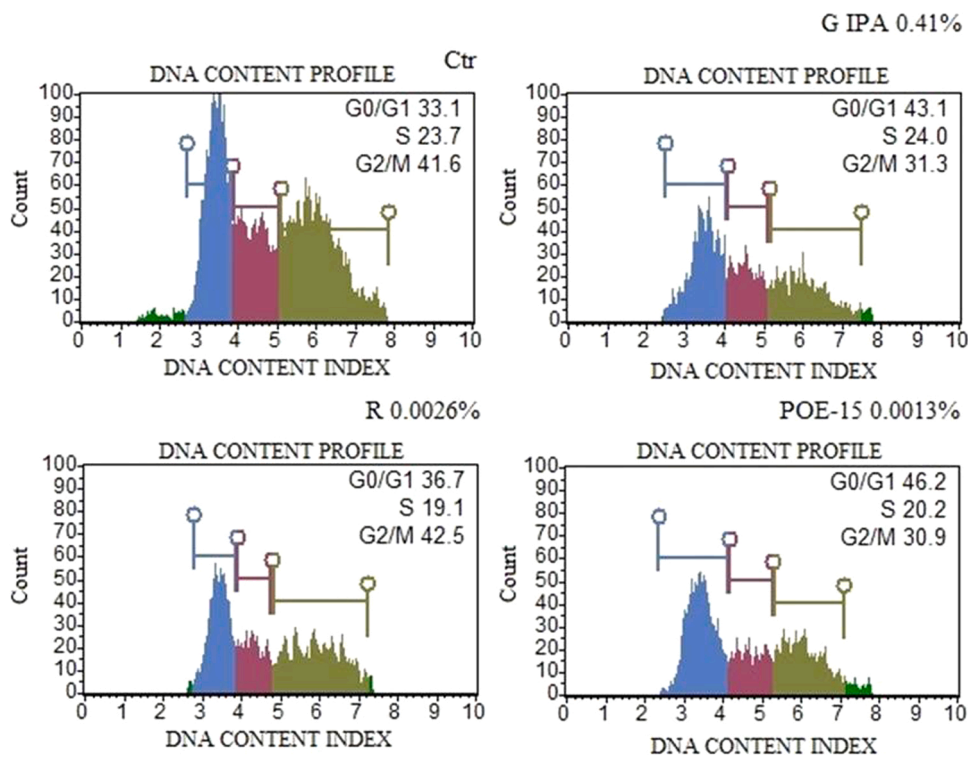


Fig. 4. Concentration-dependent effects on the cell cycle of NE-4C cells after 24 h of exposure to glyphosate IPA-salt (G IPA), Roundup Classic (R) and POE-15 (P) determined by the Muse™ Cell Cycle Assay kit. Top: control treatment after 24 h, G IPA (0.41% Roundup Classic equivalent) after 24 h. Bottom: Roundup (0.0026%) after 24 h, POE-15 (0.013% Roundup Classic equivalent) after 24 h.

contains PI as a nuclear DNA intercalating stain and RNase A in a proprietary formulation.

Cell cycle regulation is a very complex system based on cyclin-dependent protein kinases. The phases of the cell cycle are not interchangeable, they follow each other in strict order. There are 3 checkpoints where it is settled whether the cell can move from its current stage to the next one [79]. The first of these checkpoints, called the restriction point occurs at the end of the G1 phase, when the integrity of the DNA is verified. The second point, the G2 checkpoint appears at the end of the G2 phase, when DNA duplication is ensured for integrity. Finally, checkpoint M is in the metaphase of division, when it is assessed whether the chromosomes had been arranged in a plane and whether the separation of the chromatids proceeded accurately. PI stains cells at different stages of the cell cycle differently due to their differential DNA content, therefore it allows discrimination among the phases. The specificity of DNA staining is amplified by the presence of RNase. NE-4C cells and MC3T3-E1 cells were treated for 24 h with G IPA, R and POE-15 (concentrations for G IPA and POE-15 expressed as Roundup Classic equivalents), and were then analysed with Muse™ Cell Analyzer using the Muse™ Cell Cycle Assay kit. DNA damage to NE-4C neutral stem cell line was observed in the negative control because these p53 knockout cells are incapable of repairing incidental mutations due to the lack of the p53 tumour suppressor gene in them [80]. Treatment concentrations for cell cycle analysis were chosen on the basis of our results of the genotoxicity tests to avoid cell cycle variability due to excessive genotoxicity. Nonetheless, apparent toxicity to NE-4C cells up 24 h of exposure occurred in treatments with G at 0.82 Roundup Classic equivalent and POE-15 at 0.0026 Roundup Classic equivalent that rendered cell cycle determination inadequate in these two cases. Fig. 4 shows the distribution of cells in different phases as a function of the cell number and the DNA content index. Eukaryotic cell lines are diploid, and the overall genome mass of eukaryotic cells is approximately 7 pg. Therefore, DNA content indices in Fig. 4 are plotted in the 0–7 pg range.

After 24 h, the negative (untreated) control of NE-4C cells indicate that the majority (~46%) of cells in the G0/G1 phase (Fig. 4). The ratio of these cells appear to decrease in response to treatments with G IPA, R and POE-15 (Fig. 5). This decrease occurs to show a monotonous dose-dependence in the case of R and POE-15 in the range of 0.0007–0.0026 Roundup Classic equivalent, while the effect of G is interestingly the strongest at the lowest concentration applied (0.2 Roundup Classic equivalent) and gradually approaches the control level at higher concentrations (0.29–0.41 Roundup Classic equivalent). The ratio of cells in the S phase doesn't appear to be affected by exposure to G IPA (0.2–0.41 Roundup Classic equivalent), but is decreased by treatments with R or POE-15 (0.0007–0.0026 Roundup Classic equivalent). In contrast, the ratio of cells in the G2/M phase increased upon the treatments, in a concentration-dependent manner for R and POE-15, but with a highest increase for exposure to G at the lowest concentration (0.2 Roundup Classic equivalent) and a gradually decline at higher concentrations (0.29–0.41 Roundup Classic equivalent) (Fig. 5). The NE-4C cells try to grow in the G0/G1 phase until the conditions are optimal for them to double and later mitosis. Therefore, in the case of G IPA, the proportion (%) of cells increases with increasing dose, the cells try to wait for the optimal condition, which is not realized, so in the G0/G1 phase they stop the cell cycle and stay inside. Only a few cells get through the checkpoint control in the cell cycle to the S phase and then to the G2/M phase, which is also shown in Fig. 5 that the proportion of cells in these phases is lower than in the control.

The negative (untreated) control of MC3T3-E1 cells after 24 h shown an even higher count (~80%) of cells in the G0/G1 phase, than that seen for NE-4C cells (Fig. 6). Such high relative ratio of cells in the resting (G0) and first gap phase (G1) is a unique feature of the MC3T3-E1 cells [81–83]. This ratio (G0/G1) appears to decrease consistently in almost all cases with R (0.0025–0.01%) and POE-15 (0.0004–0.0016% Roundup Classic equivalent) (Fig. 7). In contrast, the ratio of cells in the S phase does not appear to be affected by the treatments, while that of

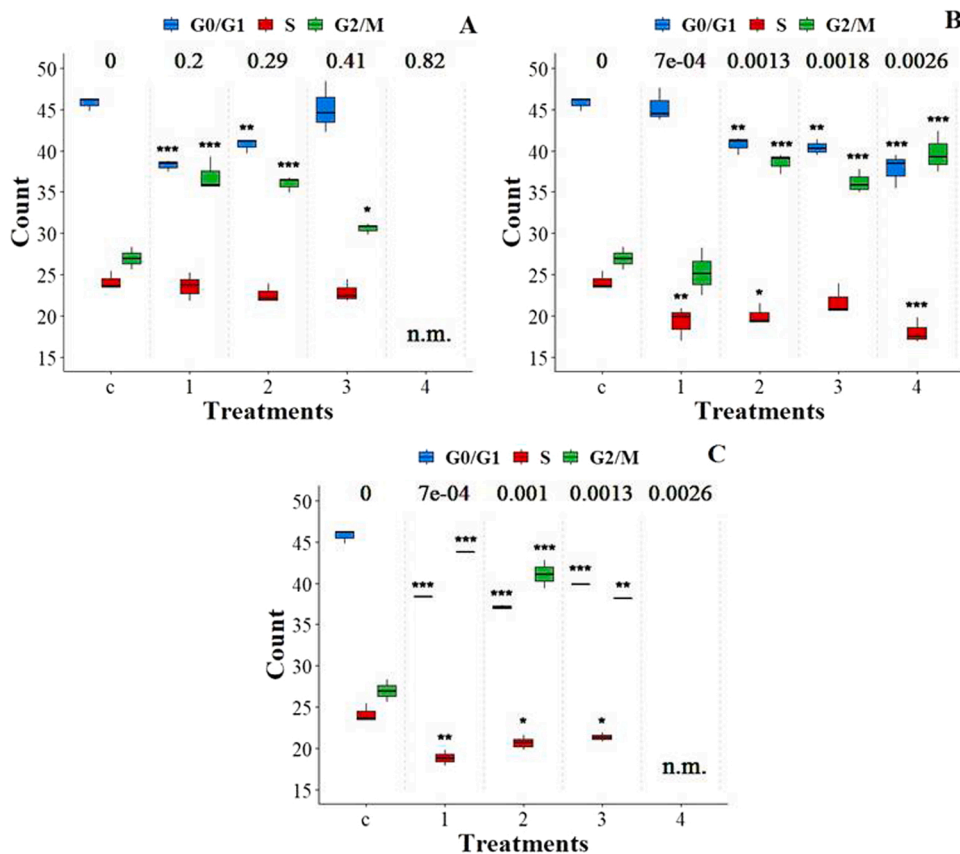


Fig. 5. Concentration-dependent effects on the cell cycle of NE-4C cells after 24 h of exposure to glyphosate IPA-salt (G IPA) (A), Roundup Classic (R) (B), and POE-15 (C) determined by Muse™ Cell Cycle Assay kit. Control treatment after 24 h, G IPA (0.2–0.82% Roundup Classic equivalent) after 24 h, Roundup (0.0007–0.0026%) after 24 h, POE-15 (0.0007–0.013% Roundup Classic equivalent) after 24 h. Data are shown as a mean±SD. Statistical analyses were performed with the R Statistical program v.4.0.0. (R Development Core Team, 2020). Asterisks indicate levels of significant differences from the control (*: p < 0.05, **:p < 0.01, ***:p < 0.001). n.m. indicates data not measurable due to excessive cell mortality.

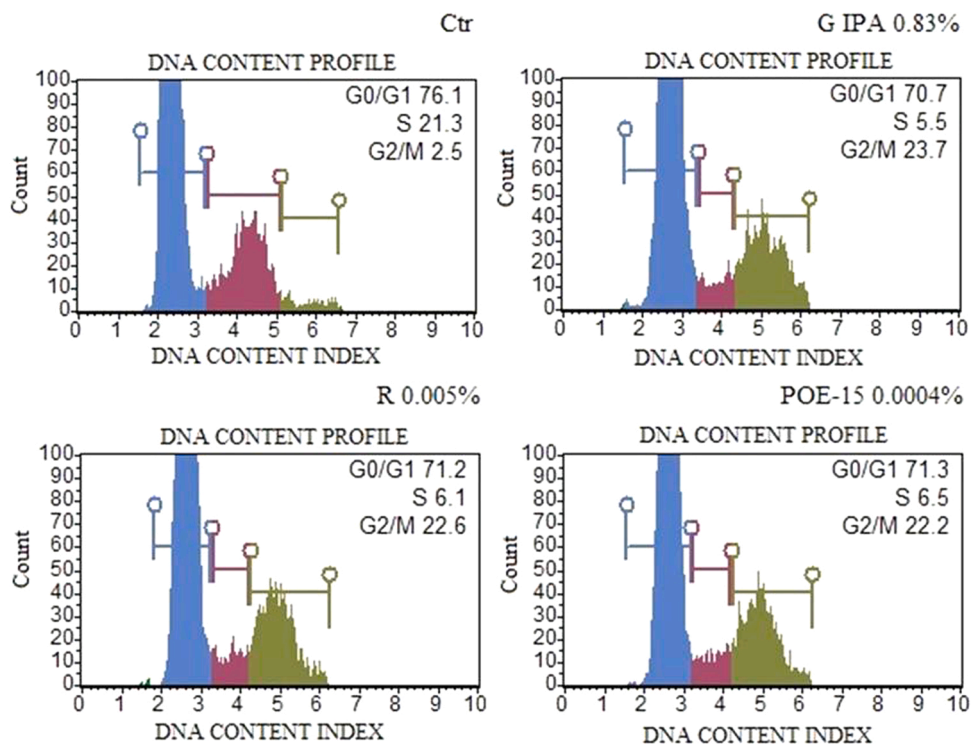


Fig. 6. Concentration-dependent effects on the cell cycle of MC3T3-E1 osteoblastic cells after 24 h of exposure to glyphosate IPA-salt (G IPA), Roundup Classic (R) and POE-15 (P) determined by the Muse™ Cell Cycle Assay kit. Top: control treatment after 24 h, G IPA (0.83% Roundup Classic equivalent) after 24 h. Bottom: Roundup (0.005%) after 24 h, POE-15 (0.0004% Roundup Classic equivalent) after 24 h.

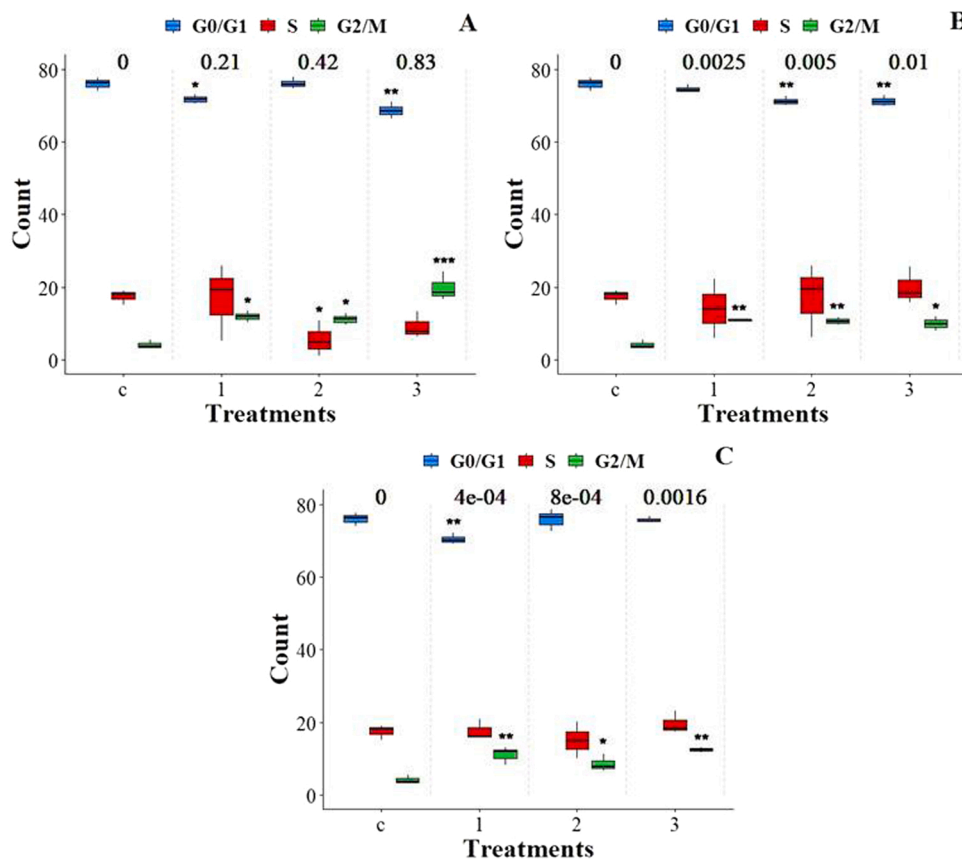


Fig. 7. Concentration-dependent effects on the cell cycle of MC3T3-E1 osteoblastic cells after 24 h of exposure to glyphosate IPA-salt (G IPA) (A), Roundup Classic (R) (B), and POE-15 (C) determined by Muse™ Cell Cycle Assay kit. Control treatment after 24 h, G IPA (0.21–0.83% Roundup Classic equivalent) after 24 h, Roundup (0.0025–0.001%) after 24 h, POE-15 (0.0004–0.0016% Roundup Classic equivalent) after 24 h. Data are shown as a mean±SD. Statistical analyses were performed with the R Statistical program v.4.0.0. (R Development Core Team, 2020). Asterisks indicate levels of significant differences from the control (*:p < 0.05, **:p < 0.01, ***: p < 0.001).

cells in the G2/M phase consistently increase after 24 h of exposure.

3.5. Comparative assessment of the genotoxicity of glyphosate with common genotoxic food contaminants

As seen from the above toxicity assessment, the formulant POE-15 was found to be more toxic than glyphosate in all toxicity tests carried out. In each test, the lowest toxic effect can be attributed to glyphosate and the highest to POE-15, while the lower toxicity of glyphosate seemingly milder the effect of POE-15 in the formulated product R. Curiously, however, the toxicity ratio between glyphosate and POE-15 was the lowest in the genotoxicity tests on MC3T3-E1 osteoblastic cells: here POE-15 was found to be “only” 13- and 73-fold more toxic than glyphosate. To assess the significance of this finding, we compared these genotoxicity values (determined by the Comet assay and the Muse™ DNA Damage kit) with corresponding values of known genotoxic agents used as additives in the food industry and reported as food contaminants. To assess genotoxicity, lowest genotoxic doses (LGDs), commonly applied genotoxicity [84] were used as the basis of the comparisons. The LGD values determined for glyphosate in the Comet assay in the present study was 3.2 times lower for the NE-4C cell line than that for the MC3T3-E1 cell line. This indicates that the NE-4C cell line is more sensitive to glyphosate than MC3T3-E1 cells for genotoxic effects (similarly to the other test types applied). Such genotoxicity values found in this study and reported in the scientific literature, including this study, are listed in Table 2.

The IARC monograph on glyphosate [37] contains all relevant toxicity studies regarding DNA damage, as well as effects on cell proliferation and apoptosis. Glyphosate-induced DNA alterations have been demonstrated in the scientific literature on various cell lines e.g., DNA strand breaks in human epithelial Hep-2 cells and induced chromosome aberrations in lymphocytes [50,73], as well as on GM38 fibroblasts and HT 1080 fibrosarcoma cells [90]. Our genotoxicity indices indicate that glyphosate appears to be more genotoxic on NE-4C cells than boric acid or only slightly less genotoxic than acrylamide on HepG2 cells, all detected in the Comet-assay. Nonetheless, benzoic acid, a commonly used preservative in foods such as fruit juices, jams, and pickles, is three-fold more genotoxic than glyphosate on the NE-4C cells. Glyphosate is slightly more genotoxic than benzoic acid on MC3T3-E1 cells.

Table 2

Comparison of genotoxic effects of glyphosate and given food additives and contaminants, determined in the Comet assay.

Substance	Cell line ^a	LGD ^b (mM)	Reference
bleomycin	HCM	0.0000706	[85]
deoxynivalenol	Caco-2	0.0005 ^c	[86]
brilliant blue	HSP	0.252	[87]
sunset yellow	HSP	0.442	[87]
citric acid	HSP	0.521	[87]
glyphosate	NE-4C	0.552 ^d	this study
benzoic acid	HSP	1.637	[87]
glyphosate	MC3T3-E1	1.779	this study
acrylamide	HepG2	2.5	[88]
boric acid	HepG2	24 ^e	[89]

^a Cell lines reported – HCM: human colonic mucosa cells; HSP: Human sperm cells; Caco-2: immortalized cell line of human colorectal adenocarcinoma cells; NE-4C: established from the cerebral vesicles of 9-day-old mouse embryos lacking the functional p53 genes; MC3T3-E1: osteoblast precursor cell line derived from *Mus musculus* (mouse) calvaria; HepG2: human liver cancer cell line.

^b The lowest genotoxic dose (LGD) value refers to the lowest dose at which the substance studied causes a positive response in the genotoxicity assay.

^c IC₁₀ reported value

^d Substantial DNA damage was observed also in the negative control as the p53 tumour suppressor gene is silenced in the NE-4 C cell line, therefore the cells cannot repair apparent DNA breaks

^e Genotoxicity level was measured at level of cytotoxicity (IC₅₀)

Glyphosate appears to be of similar genotoxicity on NE-4C cells as citric acid on human sperm cells. In this view, it seems ambiguous that citric acid is a food additive with no MRL set for food commodities, while glyphosate is classified by the IARC as Category 2 A. Acrylamide was found 4.5- and 1.5-fold more genotoxic than glyphosate on the NE-4C and MC3T3-E1 cell lines, respectively, while acrylamide is classified in the same IARC carcinogenicity category as glyphosate.

4. Conclusions

Several ascertainments can be concluded from the study presented. Firstly, we demonstrated that R and POE-15 are more toxic, than G IPA alone on both of the murine cell lines studied. Secondly, the two cell lines showed characteristic differences in all our experiments, as the NE-4C cells proved to be at least 2.5-fold more sensitive to the test substances. The IC₅₀ values determined indicated that the order of the inhibitory potency of the target compounds has unequivocally been POE-15 > Roundup Classic > > glyphosate IPA salt for both cell lines, and the neuroectodermal NE-4C cell line is more sensitive to G IPA, R and POE-15 than the osteoblastic MC3T3-E1 cell line. This in line with previous findings in the scientific literature. In cell proliferation test (MTT assay) glyphosate inhibited cell growth in eight human cell lines, but not in two immortalized normal prostate cell lines [91]. Several studies reported impacts of glyphosate-based formulations on apoptotic cell death in HepG2 cells, while glyphosate alone generally remained without effect or showed effects only at considerably higher concentrations. This demonstrates that the apoptotic effect of the formulations is higher than glyphosate alone [28,48].

In genotoxicity studies we measured the lowest effects on MC3T3-E1 cell for G IPA, compared other test substances, IC₅₀ value in Comet assays the differences between R and G IPA is 5.5-fold, and for DNA Damage kit is 9-fold. For the other measurements IC₅₀ value (viability, apoptosis) are more than 25-fold between R and G IPA. In agreement with previous findings, our studies also clearly demonstrated the explicit cytotoxicity of POE-15, exerted in other studies on cellular respiration and membrane integrity between 0.00155% and 0.0097% at Roundup Classic equivalent concentration. Our assessment indicated marked DNA damage measured by tail moment is cells exposed to G IPA, R, and POE-15 for 24 h, similarly to previous reports on other cell lines. Our results represent an additional genotoxic risk for human health and the ecosystem. The results observed in the present study clearly established that enhanced cytogenotoxic effects are exerted by Roundup Classic and POE-15. Our results also demonstrated the highest genotoxic effect by POE-15 both by the Muse DNA damage kit and by the Comet assay. The lowest IC₅₀ values were measured for the POE-15 in genotoxicity studies on NE-4C cell line, which also proves the high DNA-damaging effect of POE-15.

Finally, our assessment resulted in findings of effects of the target substances on the cell cycle distribution in the cell lines studied similar to those reported in the scientific literature. Our experiments in cell cycle assay on both neuroectodermal NE-4C and osteoblastic MC3T3-E1 cells indicated a high proportion of cells in the G0/G1 phase (compared to control) due to the adverse effects of the test substances, which inhibited cell progression to the S phase, so there are fewer cells in the S phase. Beyond extending our knowledge on the cytotoxicity of the target substances, the results of this study can have further significance in the currently on-going re-registration of glyphosate in the European Union.

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CRedit authorship contribution statement

Marianna Oláh: Investigation, Data curation, Formal analysis, Writing – original draft. **Enikő Farkas:** Data curation, Validation. **Inna Székács:** Conceptualization, Formal analysis, Visualization, Writing – review & editing. **Robert Horvath:** Writing – review & editing, Supervision, Funding acquisition. **András Székács:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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