

Glyphosate Interaction with eEF1 α 1 Indicates Altered Protein Synthesis: Evidence for Reduced Spermatogenesis and Cytostatic Effect

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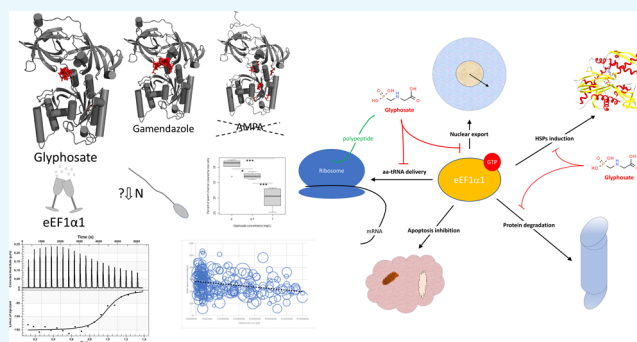


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ABSTRACT: The broad-spectrum herbicide, glyphosate, is considered safe for animals because it selectively affects the shikimate pathway that is specific to plants and microorganisms. We sought a previously unknown mechanism to explain the concerns that glyphosate exposure can negatively affect animals, including humans. Computer modeling showed a probable interaction between glyphosate and eukaryotic translation elongation factor 1 subunit alpha 1 (eEF1 α 1), which was confirmed by microcalorimetry. Only restricted, nondisrupted spermatogenesis in rats was observed after chronic glyphosate treatments (0.7 and 7 mg/L). Cytostatic and antiproliferative effects of glyphosate in GC-1 and SUP-B15 cells were indicated. Meta-analysis of public health data suggested a possible effect of glyphosate use on sperm count. The *in silico*, *in vitro*, and *in vivo* experimental results as well as the metastatistics indicate side effects of chronic glyphosate exposure. Together, these findings indicate that glyphosate delays protein synthesis through an interaction with eEF1 α 1, thereby suppressing spermatogenesis and cell growth.



1. INTRODUCTION

Glyphosate, *N*-(phosphonomethyl)glycine, is the most frequently used herbicide globally, and its increasing popularity for agricultural and nonagricultural use has been documented.¹ The glyphosate active substance acts as a broad-spectrum nonselective herbicide that specifically and exclusively inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). EPSPS, which is involved in the shikimate pathway, is present in plants and microorganisms but not in animals; thus, glyphosate has been considered to be safe for animals.² However, in the last decade, concerns that glyphosate can impact animals, including humans, as nontargeted organisms have increased.³ There is a controversy on whether glyphosate is a carcinogen to humans. In March 2015, the World Health Organization International Agency for Research on Cancer (IARC) classified glyphosate as a probable group 2A carcinogen to humans, but this classification was based on limited evidence of cancer in humans.⁴ In 2017, the European Chemicals Agency (ECHA) Committee for Risk Assessment (RAC)⁵ and the United States Environmental Protection Agency (EPA)⁶ claimed that glyphosate is not likely to be carcinogenic. Overall, authorities principally involved in the regulation of pesticides have conducted an intensified assessment of glyphosate safety, but the different outputs have resulted in a controversy.^{7–9} Indeed, additional research is

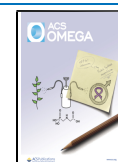
required to ascertain whether previously unknown mechanisms exist in traditional toxicological studies.¹⁰ An indirect adverse effect of glyphosate may be involved in affecting the gut microbiome, which contains EPSPS, and the possibility of dysbiosis.^{11,12} It is also possible that glyphosate exposure may affect the biochemistry of nontargets in animals and humans by unexpected side mechanisms through interactions with molecules derived from their own genome.

Some studies have hypothesized that glyphosate affects amino acid metabolism/protein synthesis in mammals, but there is disunity in studies regarding the supposed cancer effect. It is important to consider not only glyphosate as the parent compound but also its relevant metabolites. In animals and humans, similar to the case in microorganisms, glyphosate is metabolized not only to aminomethylphosphonic acid (AMPA) but also to other metabolites, such as glyoxylate.¹³ Although Ford et al.¹⁴ showed that glyphosate is metabolized

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in mouse liver to glyoxylate, they used high doses (200 mg/kg) that were intraperitoneally administered once a day for 7 days. Thus, the reactive metabolite may affect cysteines in proteins and suppress fatty acid oxidation only at an excessive exposure.¹⁴ Further, it has been suggested that glyphosate is a substitute for glycine in protein polypeptide chains,¹⁵ but another study has negated this assumption.¹⁶ Molecular modeling has shown that glyphosate binding to glycyl-tRNA synthetase is unlikely,¹⁶ but the same study did not indicate any significant changes in human breast cancer cells via proteomic analysis, which was similar to another previous study by Mesnage et al.,¹⁷ who did not observe changes in MDA-MB-231 cell growth characteristics after treatment with 100 mg/L glyphosate.¹⁶ However, Mesnage et al.¹⁷ observed that ≥ 10 mg/L glyphosate treatment promotes proliferation in MCF-7 cells because these cells are estrogen receptor (ER)-positive. Importantly, the MDA-MB-231 cell line used by Antoniou et al.¹⁶ is ER-negative (triple negative) and insensitive to antiestrogen treatments.^{18,19} Furthermore, a previous study hypothesized that glyphosate and AMPA, as glycine analogues, inhibit serine hydroxymethyltransferase (SHMT), which catalyzes serine to glycine and *vice versa*.²⁰ Indeed, Li et al.²⁰ provided evidence that glyphosate and AMPA inhibit proliferation and promote apoptosis in certain cancer cell lines but not in two immortalized normal cell lines, and they indicated that hormone sensitivity of the cells is the likely factor for this phenomenon.²⁰ The link between the hormone/estrogen sensitivity of certain cancer cell lines toward glyphosate/AMPA treatment may be the involved mechanism.^{17,20}

Studies have indicated that glyphosate and AMPA affect the cell cycle in certain cell lines. Li et al.²⁰ indicated that the effect is cell cycle-dependent, suggesting that glyphosate/AMPA are more effective against rapidly proliferating cells. In addition, the glyphosate metabolite, AMPA, has been shown to arrest cancer cells at the G1/G0 phase.²⁰ Lin et al.²¹ observed cell cycle-specific eukaryotic translation elongation factor 1 subunit alpha 1 (eEF1 α 1) expression in breast carcinomas, and they demonstrated that eEF1 α 1 mRNA levels are high in G1 and low in proliferating cells. However, the eEF1 α 1 transcript level is underexpressed, while the eEF1 α 1 protein level is overexpressed in ductal breast carcinomas, including ER-positive tumors. These researchers also suggested a link between estrogen signaling and the eEF1 α 1 mRNA level because estrogen promotes proliferation.²¹ Moreover, depletion of eEF1 α 1 impairs cell vitality and cell growth but arrests cells in the G1/G0 phase.²² Indeed, eEF1 α 1 influenced HCC cell proliferation via regulation of the G1 phase.²³ Thus, there may be a potential link between glyphosate/AMPA treatment and impairment of the cell cycle, and the target may be eEF1 α 1.

We do not reject the hypothesis that glyphosate alters protein synthesis in nontargets.¹⁵ In agreement with Antoniou et al.,¹⁶ we did not consider that glyphosate binds the active site of aminoacyl-tRNA synthetase, which is responsible for the covalent amino acid linking to tRNA.²⁴ Instead, we sought to investigate whether glyphosate treatment alters the different phases of protein synthesis. As a target for our study, we selected the stage in which the aminoacyl-tRNA is delivered to the ribosome, an event mediated by eEF1 α ,^{25–27} which is present in two isoforms, and both isoforms are oncogenes.²⁸ Importantly, while eEF1 α 1 has been established to be proapoptotic, an inverse antiapoptotic effect has been suggested for eEF1 α 2.²⁹ Another difference between the two

isoforms is the different expression in tissues and their replacement during cellular differentiation, i.e., eEF1 α 1 expressed in embryonic and postnatal development is later replaced by eEF1 α 2 expressed in long-lasting terminally differentiated cells.^{25,30,31} Considering the observed misexpression of eEF1 α 1 in tumor cells²¹ and the proapoptotic effects of glyphosate and AMPA on cancer cell lines,²⁰ we hypothesize that glyphosate and AMPA are associated with the proapoptotic eEF1 α 1 functions.²⁹

Previous studies have reported that the contraceptive drug, gamendazole, interacts with eEF1 α 1.^{32,33} Thus, if glyphosate treatment suppresses eEF1 α 1 similarly to gamendazole,^{32,33} then an analogous role of glyphosate treatment in spermatogenesis may be suggested. Therefore, we additionally hypothesized that there may be a link between glyphosate use and the increased problems in human fertility and testicular cancer over the last few decades.^{34,35} Several studies have investigated the adverse effect of glyphosate on cancer and reproduction, but the results of the studies are controversial. Some of these studies used high nonrealistic doses and/or formulated glyphosate.^{10,36} However, a low glyphosate effect has been observed on rat male reproductive organs,³⁷ and a meta-analysis has indicated a potential effect of glyphosate on sperm counts in rodents.³⁸ Thus, it is necessary to further investigate the adverse potential of glyphosate, especially from chronic exposure.¹⁰ The realistic contents of glyphosate and AMPA in the environment can be considered up to hundreds of micrograms or a few milligrams parts per million (ppm). Incidentally, the U.S. Environmental Protection Agency has set a drinking water maximum contaminant level (MCL) of 0.7 mg/L for glyphosate.^{39,40}

In this study, we sought to determine whether glyphosate and/or AMPA impact the functions of eEF1 α 1. In addition, we investigated the potential involvement of glyphosate treatment in spermatogenesis and its cytostatic effects, which may be affected by the interaction of glyphosate with eEF1 α 1.

2. EXPERIMENTAL SECTION

2.1. Molecular Modeling.

The structure comparison of glyphosate and AMPA 3D was performed using the QSAR Toolbox.⁴¹ The eEF1 α 1 3D protein structure was obtained as described previously.³² Briefly, human eEF1 α 1 was identified in UniProt (Accession No. P68104), and the BLAST tool⁴² on this server was used to identify template structures. The 1F60, 1G7C, 1IJE, 1IJF, 2B7B, 2B7C, and 4C0S crystallographic template structures available from the RCSB PDB were selected for comparative modeling using MODELLER.^{43–45} The template structures were from different yeasts and rabbits. Yeast eEF1 α 1 showed 80.7% identity with human eEF1 α 1, and rabbit eEF1 α 1 had an identity of 92.6%. The first step in comparative modeling was the 3D alignment of the templates and target sequences performed using the SALIGN 3D module of MODELLER.^{43–45} This automodel module generated 100 protein structures. The generated structures were verified using VERIFY3D⁴⁶ and PROCHECK,⁴⁷ and the best 10 models were selected for docking experiments as targeted protein structures. Scripts from AutoDockTools⁴⁸ were used to create input files for the ligands and the protein for submission to AutoDock Vina.⁴⁸ Each optimized ligand structure was docked 10 times in each protein model using both standard B3LYP (Becke, 3-parameter, Lee–Yang–Parr) and RHF (restricted Hartree–Fock) methods,⁴⁹ which resulted in 100 docked structures for each compound, from which only the best pose

was selected for further investigation. With regard to gamendazole, the 10 best docking results were used to make the statistical sampling more reliable. The structures of glyphosate and AMPA were obtained from the ChemSpider database.^{50,51} The molecular docking experiment was performed using AutoDock Vina software version 1.1.2.⁴⁸

2.2. Isothermal Titration Calorimetry (ITC) Experiments. The interaction of glyphosate with elongation factor eEF1 α 1 (ProSpec, Rehovot, Israel) was studied using ITC. ITC measures the changes in heat during an interaction and provides thermodynamic information about the binding affinity of a ligand to a protein. ITC is a straightforward method for determining the binding affinity constant (K) and binding stoichiometry (n), and the enthalpy of binding (ΔH) that occurs over the course of a reaction in solution and the entropy changes (ΔS) are calculated from the following equation: $\Delta G = -RT \ln K_a = \Delta H - T\Delta S$.

The ITC experiment was performed at 25 °C with a Nano ITC Low Volume instrument (TA Instruments, New Castle, DE). During all measurements, 20 injections of 4 μ M ligand (2.5 μ L each) were titrated into 250 μ L of protein (1 μ M) with time intervals of 300 s and a stirring speed of 250 rpm. All ITC experiments were conducted with degassed 100 mM phosphate buffer solutions (pH 7.4). Control experiments included the titration of each complex solution into buffer. Corrected data refer to the experimental data after subtraction of the compounds from the buffer control data. The resulting thermograms were analyzed using the "Independent" model within NanoAnalyze software (TA Instruments, New Castle, DE).

2.3. Animal Experiment. The animal experiment was performed on the premises of the Institute of Pharmacology and Pharmacy (Building No. 22, Room 222) following the approved Rules of Conduct. The animal experiment was approved by the Ethics Committee, Ministry of Education, Youth and Sports of the Czech Republic, Czechia, and was in accordance with the Czech Animal Protection Act No. 246/1992 Coll. - VFU (18020).

Sixty Wistar rats aged approximately 10 weeks and weighing approximately 225 g were used in the experiment. The rats were kept in a controlled temperature environment (25 °C) on a normal photoperiod (12 h light, 12 h dark) and provided regular daily surveillance and health care. The animals were divided into three groups with 10 males and 10 females in each group. The first group was untreated throughout the experimental period and received water *ad libitum* without glyphosate (control group). The remaining two groups received water *ad libitum* with 0.7 or 7 mg/L glyphosate (Cat No. 89432, TraceCERT, Supelco, Sigma-Aldrich, St. Louis, MO) for 100 days.⁵² All animals received standard nutrition. Water consumption was monitored in each experimental group every day. At the end of the experimental period, the rats were starved overnight and then painlessly sacrificed. Tissue samples were then collected and used for histological examination. To verify that the source of drinking water did not influence our experiment, we confirmed that the drinking water did not contain pesticides or metabolite residues. This analysis included validation methods for 301 pesticide residue compounds performed as a service in an accredited laboratory of ALS Czech Republic (Part of ALS Limited). Thus, we verified that the water source did not significantly influence our results because it did not contain any pyrethroids, quaternary ammonium salts, glyphosate, or

AMPA. In addition, two compounds were detected in trace amounts from a wide range of screening, namely, 0.087 μ g/L chloridazon-desphenyl (experimental uncertainty analysis $\sim \pm 35\%$) and 0.069 μ g/L alachlor ESA (experimental uncertainty analysis $\sim \pm 35\%$), which were present in levels that were approximately 10 000- and 100 000-fold lower than that of the tested glyphosate, respectively.

2.4. Histology of Rat Testicles. Rat testicles from the above-described experiment were fixed in 10% neutral buffered formalin. Each testicle was embedded in paraffin wax and cross-sectioned at a thickness of 4 μ m. Sections were made through the center of the testicle, and the tissue was stained with hematoxylin and eosin. In one cross-section per animal, the tubules were evaluated for the presence of spermatogonia, spermatocytes, and spermatids. One testicle was rated for its spermatogenic potential (spermatogenic index) on a scale of 1–6 from 10 circular sections of tubules, each from a different testicular region that was homogeneous throughout with respect to cell association and spermiogenesis. The spermatogenic index was based on the appearance of spermatogenic cells throughout the testicle and included the number of cell layers, the types of cells, and the presence of late spermatids in the seminiferous tubules. The index and criteria were as follows: (1) only spermatogonia present; (2) spermatogonia and spermatocytes present; (3) spermatogonia, spermatocytes, and round (early) spermatids present with <5 late spermatids per tubule; (4) spermatogonia, spermatocytes, and round spermatids present with up to 25 late spermatids per tubule; (5) all cell types present and 50–75 late spermatids per tubule; and (6) all cell types present and >100 late spermatids per tubule.

The criteria (Table S1) for the assessment of the spermatogenic index was based on the testicular morphology from Whitsett et al.⁵³ The area covered by ripe spermatid cells in tubules was determined using Fiji, a standard open-source platform for biological image analysis.⁵⁴

2.5. Inhibition of Cancer Cells. **2.5.1. Cell Culture.** The spermatogonia GC-1 cell line (ATCC CRL-2053)⁵⁵ derived from BALB/c mouse testes and the human B-lymphoblastic leukemia cell line SUP-B15 (ATCC CRL-1929)⁵⁶ were obtained from the American Type Cell Culture Collection (ATCC). The GC-1 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich), while the SUP-B15 cell line was grown in RPMI-1640 complete medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco, Dublin, Ireland) and 1% penicillin–streptomycin (Sigma-Aldrich). Cells were incubated in 5% CO₂ at 37 °C.

2.5.2. Cytotoxicity Assays. GC-1 cells were seeded in 96-well plates (2500 cells per well), and SUP-B15 cells were seeded in 24-well plates (2.5 $\times 10^5$ cells per well). In our experiments, SUP-B15 cells grew better in flat bottom, low adherent 24-well plates than in 96-well plates. On the next day, cells were treated for 24, 48, or 72 h with glyphosate (Cat No. 89432, TraceCERT, Supelco, Sigma-Aldrich) using four different concentrations (25 mM, 4.17 mM, 694 μ M, and 116 μ M). Further, glyphosate cytotoxicity was evaluated using two different assays.

A Cytotoxicity Detection Kit^{PLUS} (Roche, Basel, Switzerland) was used to evaluate the cytotoxicity based on lactate dehydrogenase (LDH) activity. The absorbance of the supernatants was measured with an Infinite 200 PRO reader (Tecan, Mannedorf, Switzerland). The reference wavelength was set at 680 nm, and the samples were measured at 490 nm.

Each LDH activity measurement involved wells with growth medium without cells as a background control, a positive control with maximum LDH release (obtained by the addition of 5 μ L of lysis solution from the kit at the end of 30 min of incubation at 37 °C), and nontreated cells.

The second cytotoxicity analysis was performed using a WST-1 Cell Proliferation Assay Kit (Roche). This assay is used for cell viability, cell proliferation, and cytotoxicity analysis by measuring the level of formazan, which is a cleavage product of the WST-1 tetrazolium salt. Absorbance was measured in the proliferating cells at 450 nm using an Infinite 200 PRO reader (Tecan). The reference absorbance was measured at 630 nm. The results were normalized by comparing each value to the negative controls.

2.6. Public Health Statistics. Because we did not find any publicly available statistical data on the total sperm count, we performed a meta-analysis of published data. For this purpose, we closely followed a previously described procedure.⁵⁷ The glyphosate use data were obtained from the U.S. Department of the Interior National Water-Quality Assessment (NAWQA) Project⁵⁸ and from other published articles.^{1,59} Data on cancer incidence were obtained from the U.S. Department of Health and Human Services, National Institutes of Health, and National Cancer Institute.⁶⁰ For all statistical computing, we used the R suite.⁶¹

2.7. Role of the Funding Source. The funder had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all data in the studies and had the final responsibility for the decision to submit these data for publication.

3. RESULTS AND DISCUSSION

The results of our *in silico* experiment in Figure 1 show that glyphosate interacted with eEF1 α 1 in a similar manner as previously identified for the eEF1 α 1 inhibitor, gamendazole;^{32,33} however, the interaction energy was less favorable than that of gamendazole (Table 1). We also verified whether the AMPA metabolite interacts with eEF1 α 1, but the interaction sites of AMPA with eEF1 α 1 were different (Figure 1), and the interaction energy for AMPA was less favorable than that of glyphosate (Table 1). Because our results indicated that it is not likely that AMPA, in contrast to glyphosate, affects the reaction center in eEF1 α 1, we used only glyphosate for the microcalometric experiment. Importantly, the microcalorimetric estimation of the dissociation constant (Figure 2) confirmed the *in silico* analyses. The resulting dissociation constant value of 6.5×10^{-9} kJ/mol indicated the existence of a stable complex between glyphosate and eEF1 α 1. Thus, our results indicated that glyphosate may affect the delivery of aminoacyl-tRNAs to ribosomes.^{25–27} However, the eEF1 α 1 function altered by glyphosate may have various possible consequences affecting cell signal transduction, nuclear export, proliferation/apoptosis, cell vitality, and heat shock protein (HSP) response.^{21,22,28,62–67} Thus, based on our finding that eEF1 α 1 interacts with glyphosate, the proposed effects that glyphosate may exert may be due to this interaction (Figure 3). For instance, a change of the eEF1 α 1 conformation due to interaction with glyphosate affects the phosphorylation at Ser300 induced by the type I transforming growth factor β receptor (T β R-1), resulting in inhibition of cell proliferation.⁶² This is an example of a potential glyphosate effect on eukaryotic translation through affecting the aminoacyl-tRNA interaction site of eEF1 α 1.⁶² Thus, despite the unlikely direct

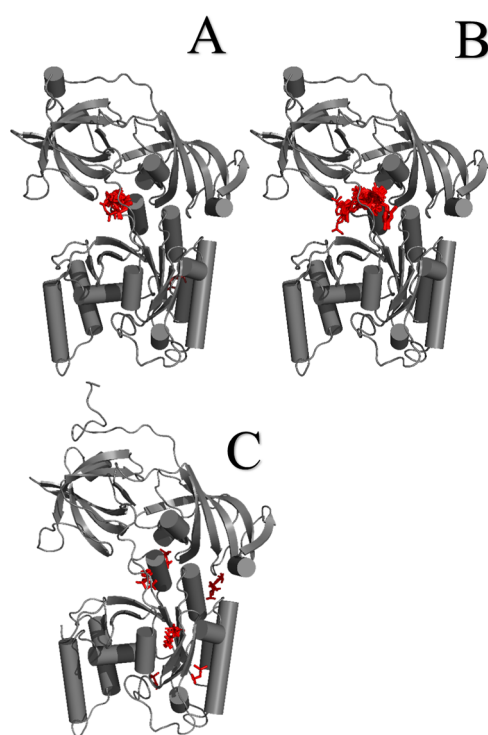


Figure 1. 3D Structure of eEF1 α 1 obtained by computer modeling together with docked glyphosate (A), a male contraceptive gamendazole (B), and AMPA (C). These visualizations show that the glyphosate interaction site with eEF1 α 1 is the same as that for gamendazole; however, the interaction sites of AMPA with eEF1 α 1 are different.

Table 1. Comparison of the Interaction Energy of Gamendazole, Glyphosate, and AMPA with eEF1 α 1^a

method	compound	docking energy (kcal/mol)
B3LYP	gamendazole	−9.1
	glyphosate	−5.3
	AMPA	−4.1
RHF	gamendazole	−9.1
	glyphosate	−5.3
	AMPA	−4.1

^aThese results were obtained by docking the ligand structures computed by the B3LYP (Becke, 3-parameter, Lee–Yang–Parr) and RHF (restricted Hartree–Fock) methods⁴⁹ into the protein structure obtained by homology modeling. The negative docking energy indicates the possible interaction of the three tested compounds with eEF1 α 1.

binding of glyphosate to aminoacyl-tRNA synthetase,¹⁶ our results indicated that glyphosate affects protein synthesis through the aminoacyl-tRNA delivery to ribosomes.

Because tRNA is composed of ribonucleosides,^{68,69} we investigated whether glyphosate and AMPA mimic ribonucleotides. Although an exhaustive quantitative structure–activity relationship (QSAR) search⁴¹ has shown high similarity of both glyphosate and AMPA with the four ribonucleotide monophosphates (Table 2), we consider this effect unlikely because otherwise the effect of glyphosate and AMPA would be obvious and destructive at low concentrations.

Our results showed the cytostatic effect of glyphosate using two cell lines, namely, GC-1 and SUP-B15, which are both ER-positive.^{70–73} The use of LDH (Figure 4) and the WST-1

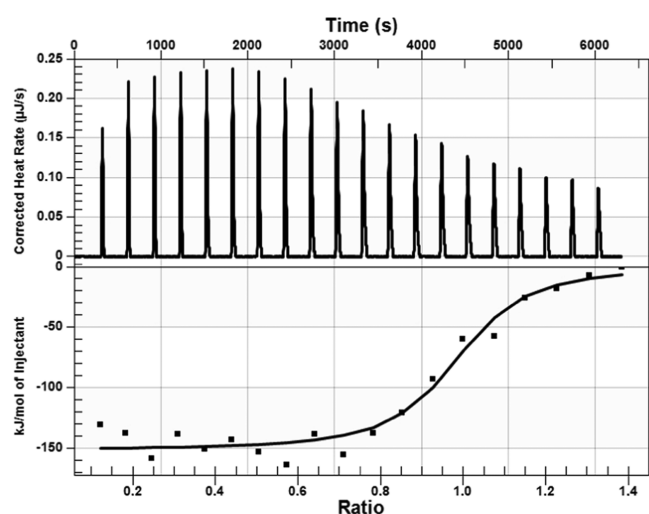


Figure 2. Microcalorimetric estimation results of the dissociation constant of the glyphosate and eEF1 α 1 interaction. The dissociation constant of $K_d = 6.494 \times 10^{-9}$ ($\Delta H = -150.8$ kJ/mol, $\Delta S = -349.0$ J/(mol·K)) indicates a strong interaction of glyphosate with eEF1 α 1.

assay (Figure S2) provided evidence for cytostatic and antiproliferative effects of glyphosate in both tested cell lines. These *in vitro* studies demonstrated that glyphosate treatment decreased the number of viable cells. Thus, our results support the previously observed cytostatic effect on ER-positive cancer cell lines.^{17,20} We were unable to elucidate the exact mechanism to explain the observed higher susceptibility of ER-positive cancer cells to glyphosate; however, our results suggested an association between estrogen signaling and the eEF1 α 1 mRNA level.²¹ Future studies will be performed to identify whether glyphosate treatment alters eEF1 α 1 levels at the transcript or protein level in certain cells and whether the expression is cell cycle-dependent.

Table 2. Results of the Exhaustive Quantitative Structure–Activity Relationship (QSAR) Search^a

compound	nucleotide	QSAR similarity (Yule, PubChem features)
glyphosate	AMP	74.359%
	CMP	76.923%
	GMP	87.179%
	UMP	82.051%
AMPA	AMP	88.235%
	CMP	88.235%
	GMP	88.235%
	UMP	88.235%

^aThese results indicate high similarity of glyphosate and AMPA to all four nucleotides, but it is not likely that they substitute glyphosate or AMPA.

According to our results, glyphosate interaction with eEF1 α 1 was similar to that of gamendazole (Figure 1), thereby prompting us to investigate whether glyphosate exhibits an antispermatogenic effect.^{32,33} To verify whether glyphosate treatment affects spermatogenesis *in vivo*, we determined the total amount of sperm cells in the tubules of male rats. We did not observe any unripe sperm cells in the tubules, which was similar to a previous study³⁷ but contradictory to another study reporting abnormal sperm cells and degenerative testicular lesions.⁷⁴ We identified a significant decrease in the space covered by ripe sperm cells (ANOVA; $p = 1.65 \times 10^{-6}$), suggesting possible antispermatogenic effects of glyphosate (Figures 5 and S1), which agrees with the decreased sperm count observed in previous studies.^{37,74} In addition to the observed effect on spermatogenesis, rats in glyphosate-treated groups were smaller at the end of the 100-day experiment, but only the female rats were significantly smaller ($p = 0.005$) after the 7 mg/L treatment (Figure S3). The decrease in body weight has been previously observed in chronic/subchronic glyphosate exposure in mice.⁷⁵

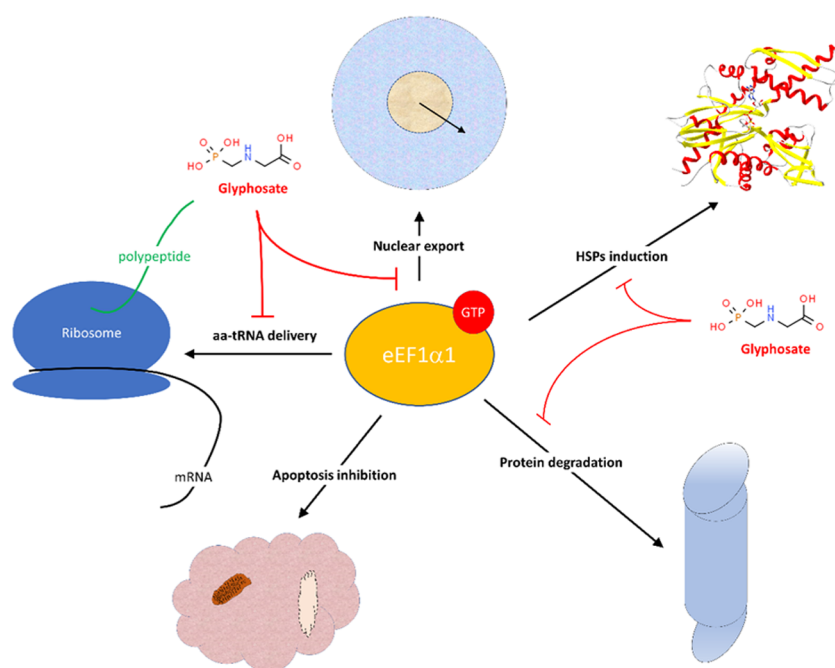


Figure 3. Proposed consequences of glyphosate interaction with eEF1 α 1 due to known eEF1 α 1 function. The modulated function of eEF1 α 1 affects the aminoacyl-tRNA delivery to ribosome, HSP response, and apoptosis/proliferation.

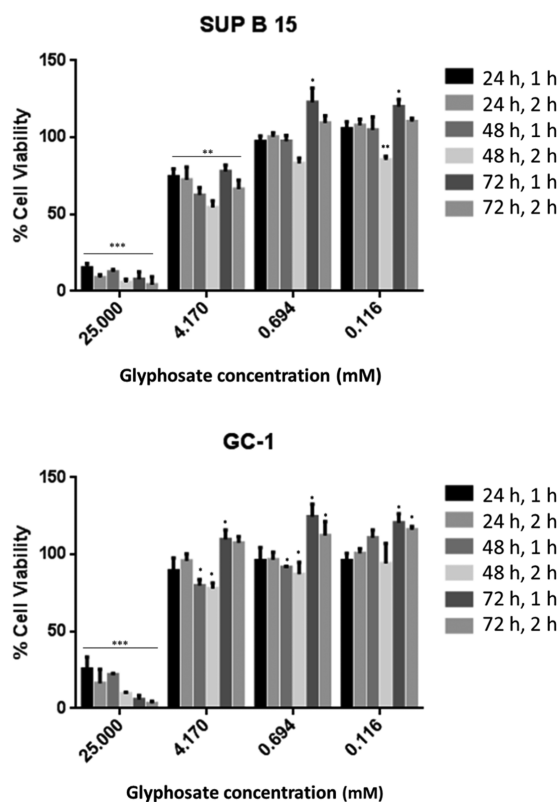


Figure 4. Vitality graphs determined by lactate dehydrogenase assay using SUP-B15 and GC-1 cell lines. p -values = $0 < (***) < 0.001 < (**)$ < $0.01 < (*) < 0.05$. The controls were set as 100% vitality. For the proliferation assays, only the results compared to the controls are shown (100% proliferation). These results clearly indicate the cytostatic effect of glyphosate, which is attributed to eEF1 α inhibition.

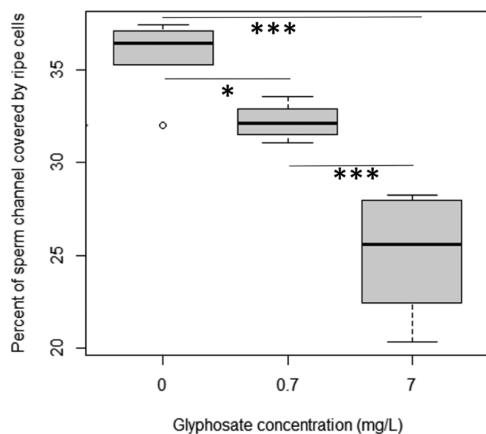


Figure 5. Percentage of sperm channel area covered by ripe sperm cells ($p = 1.65 \times 10^{-6}$). Oral glyphosate treatment of rats results in decreased spermatogenesis. Both 0.7 and 7 mg/L glyphosate treatments for 100 days in drinking water significantly ($p < 0.05$) decreased the sperm channel coverage by ripe cells. The negative effect of glyphosate on spermatogenesis increased with increasing glyphosate concentration.

The decrease of body weight due to glyphosate interaction with eEF1 α is likely due to the effect on protein synthesis.

Because our results indicated spermatogenesis reduction in rats and a cytostatic effect on cells (see above) and a previous meta-analysis of published studies has indicated a potential

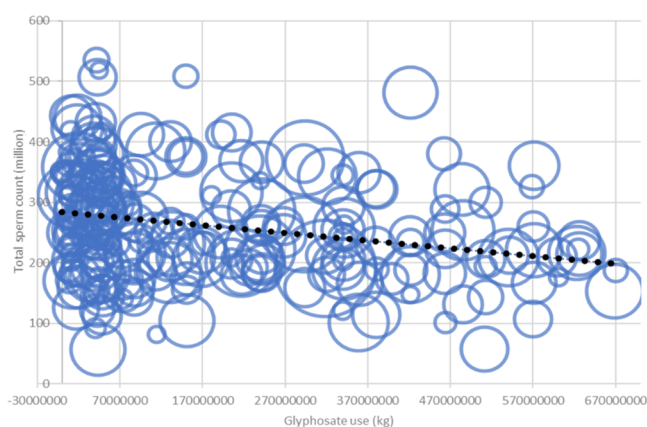


Figure 6. Meta-analysis results of average sperm count related to total glyphosate use in the USA (Spearman correlation $R = -0.2781609$ and $p = 1.511 \times 10^{-5}$).

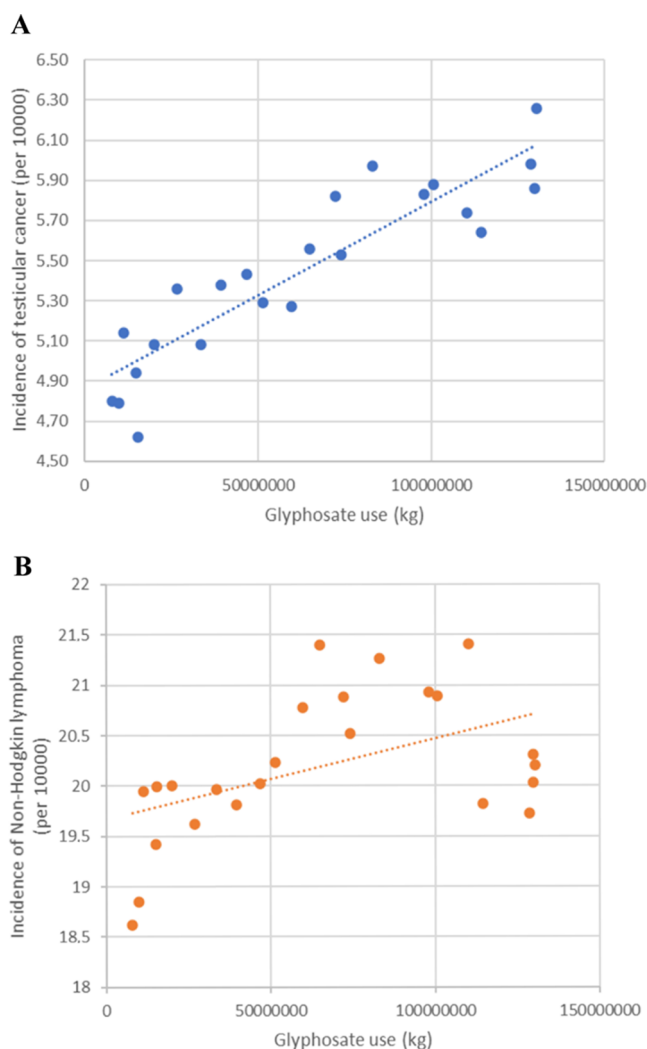


Figure 7. Graphs of selected cancer incidences plotted against glyphosate use in the USA. The resulting Spearman correlations were (A) $R = 0.9149815$ and $p = 3.885 \times 10^{-10}$ for testicular cancer and (B) $R = 0.5582609$ and $p = 0.005253$ for non-Hodgkin lymphoma.

effect of glyphosate on sperm counts in rodents,³⁸ we investigated whether this effect occurs at a population level in humans. Our meta-analysis using the published yearly

glyphosate use^{1,58} showed a small negative correlation for glyphosate use. Although the resulting Spearman correlation coefficient ($R = -0.2781609$) was low, the correlation was still significant ($p = 1.511 \times 10^{-3}$). Thus, the results in Figure 6 do not rule out the possible negative impact of glyphosate on spermatogenesis, which may be attributed to the glyphosate interaction with eEF1 α 1.

Because studies have implicated an association between aberrant eEF1 α 1 and cancer,^{21,23,76–78} we analyzed several cancer incidences and their correlations with glyphosate use. The most striking observed correlation was the incidence of testicular cancer (Figure 7A) with a Spearman correlation of $R = 0.9149815$ and $p = 3.885 \times 10^{-10}$. We then compared this result with the previously identified correlation with non-Hodgkin lymphoma (Figure 7B),^{79–81} which resulted in a Spearman correlation of $R = 0.5582609$ and $p = 0.005253$. Based on these results, glyphosate may impact tumorigenesis.

Even though the negative correlation coefficient is relatively low, it is still significant ($p < 0.01$). Together with the other results in this study, these data indicate that the effect of glyphosate on human spermatogenesis due to alteration of eEF1 α 1 function cannot be ruled out.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.1c00449>.

Details of the criteria for the assessment of the spermatogenic index; representative microphotographs of rat testes tissue; the average weight gain differences in female and male rats among glyphosate treatments; and the proliferation of GC-1 and SUP-B15 cells analyzed by WST-1 assay (PDF)

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Author Contributions

B.S. and T.E. were involved in the conception of the study. B.S. was involved in the study design. G.T., E.A., and B.S. performed the modeling experiments. B.S. was the study statistician. M.M. and M.H. were responsible for the microcalorimetric estimations. J.C., J.F., and M.S. performed the animal experiment and subsequent histological analysis. G.B. and M.A. were involved in the cell culture analysis. B.S. and T.E. were involved in interpreting the data. T.E. and B.S. wrote the main manuscript. All authors have read and approved the final manuscript.

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Notes

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