JOURNAL OF
EMERGING INVESTIGATORS

Article

Toxicity of Aminomethylphosphonic Acid via the Wnt Signaling Pathway

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SUMMARY

Glyphosate is one of the most ubiquitous herbicides in the world due to its effective plant removal. However, even though glyphosate and its main metabolite, aminomethylphosphonic acid (AMPA), contaminate the environment and pose threats to humans, their mechanism of toxicity is not well-elucidated. One possible mechanism of action is through the canonical Wnt pathway. This pathway regulates many cellular processes, such as cell fate determination and organogenesis, but its dysfunction can result in cancer. The goal of this study was to assess the toxicity of AMPA and elucidate its effects on Wnt signaling by using planarian regeneration models and molecular docking simulations. To evaluate the toxicity of AMPA, planaria were exposed to environmental concentrations of AMPA and they generally exhibited significantly reduced locomotion, delayed regeneration, and smaller blastema areas. AMPA displayed comparable toxicity to glyphosate and was harmful at low concentrations. To probe the mechanism of action of AMPA and glyphosate, we conducted in silico molecular docking in PyRx to predict its binding to canonical Wnt targets. Both AMPA and glyphosate had moderate simulated binding affinities for proteins in the Wnt pathway, with glyphosate exhibiting stronger affinities than those of AMPA. According to our molecular docking results, AMPA may be capable of binding to Wnt targets. However, it is unclear whether AMPA inhibited or activated Wnt proteins, so it cannot be ascertained that AMPA is definitively carcinogenic. Regardless, AMPA was toxic to planaria, and herbicide application should be more closely regulated to avoid environmental deposition of glyphosate and AMPA.

INTRODUCTION

Glyphosate is one of the most ubiquitous herbicides in the world because of its ability to efficiently and inexpensively kill more than 150 different types of weed species (1). The most popular glyphosate-based herbicide (GBH) is Roundup, which contains the active ingredient glyphosate (2). Since Roundup's formulation in 1974, over 1.6 billion kilograms of glyphosate have been applied to crops in the United States (3).

Glyphosate, also known as N-(phosphonomethyl) glycine, is a nonselective herbicide that kills most plants it comes into contact with (4). Glyphosate is applied on oats, wheat, corn, soybeans, and other common crops. However, inefficient application of glyphosate is a primary reason for its excessive accumulation in the environment, which may have negative effects (4, 5). Regulatory agencies have not reached a conclusive consensus regarding the toxicity of glyphosate: while the International Agency for Research on Cancer (IARC) labeled glyphosate as a possible carcinogen in 2015, the Environmental Protection Agency (EPA) claimed that glyphosate was unlikely to be carcinogenic in 2016 (6). Nevertheless, the widespread use of glyphosate can result in runoff getting into water supplies, accumulating in the environment, and contaminating groundwater due to its hydrogen bonding capabilities and water solubility (5, 7).

In water or soil, glyphosate is broken down mainly by microorganisms for use as a phosphorus source (2,8). The primary product of glyphosate degradation is aminomethylphosphonic acid (AMPA), which is formed by the C-N bond cleavage of glyphosate (7). Other products such as sarcosine may be formed, but AMPA is the most common and maintains the toxic aspects of glyphosate (9).

Glyphosate has a low acute oral toxicity; however, its chronic toxicity at low concentrations is more concerning (10). While rodents may tolerate glyphosate uptake of up to 20000 mg/kg body weight in a single day, rats exposed to glyphosate at concentrations between 5 and 490 mg/kg every 2 days for 75 days had irreversible damage to hepatocytes (epithelial liver cells), which led to exacerbated oxidative stress and apoptosis (11, 12). Glyphosate's effects are not limited to rats: humans can be exposed to and harmed by glyphosate by consuming glyphosate-contaminated food products, especially cereals like rice and wheat (13). Out of 107 human patients with acute oral glyphosate exposure, 47.1% had hypotension, 38.6% developed cognitive decline, and 30% suffered from respiratory failure (14). In SH-SY5Y human neuroblastoma cells, concentrations of 5 mM of glyphosate increased both malondialdehyde, a marker for oxidative stress, and caspase-3, a marker for apoptosis (15). Oxidative stress is characterized by an excess of free radicals, which can exacerbate lipid peroxidation, increase cell damage, and accelerate aging (16). Apoptosis is necessary for maintaining the immune system and cell balance, however, excessive apoptosis leads to uncontrolled cell death (17). Both oxidative stress and apoptosis are also associated with neurodegenerative disorders such as Alzheimer's

and Parkinson's disease (17). It is important to consider that glyphosate's acute toxicity (5 mM) in this study is not particularly high, with other carcinogens typically exhibiting toxicity at much lower orders of magnitude (on the scale of μM or nM) in SH-SY5Y cells. However, the chronic health effects of glyphosate may not be negligible. and glyphosate exposure has implications in severe, debilitating diseases.

AMPA is the primary metabolite of glyphosate and has similar structure and toxicity (18). AMPA, however, is more environmentally persistent than glyphosate: it is 3-6 times more resistant to degradation and has a longer kinetic half-life (240 days while that of glyphosate is 197 days) (5, 8, 13). Similarly to glyphosate, AMPA can be hazardous even at low concentrations: 0.07 μg/L of AMPA significantly increased embryonic mortality (from 2% control mortality to 6%) and development duration (up to 0.4 days longer) of spined toads. Since natural AMPA concentrations in water range from 0.07 to 3.57 μg/L, the mortality results demonstrate that typical environmental concentrations of AMPA can be toxic and interfere with cellular growth and development (19). Since there are significantly fewer studies that evaluate the toxicity of AMPA than that of glyphosate despite the potential harm of AMPA, it is valuable to more conclusively assess the toxicity of AMPA.

The Wnt signaling pathway, which is controlled by Wnt proteins, regulates a variety of developmental and biological processes—mainly, cell proliferation and neurogenesis—in many vertebrates and invertebrates, such as humans, frogs, and Drosophila (20). Hyperactivity of Wnt signaling can result in excessive cellular proliferation, leading to neurological diseases and cancer (21). The canonical Wnt signaling pathway relies on β-catenin to activate transcription of Wnt target genes (22). In the "off state", the destruction complex phosphorylates β-catenin and prevents transcription in the absence of a Wnt ligand (23). Once a Wnt ligand binds to the Frizzled-8 (Fz) receptor and Low-Density Lipoprotein Receptor-Related Protein 6 (LRP6) coreceptor, the destruction complex is inactivated, allowing β-catenin to bind to the T-cell factor/lymphoid enhancer factor and activate transcription (22, 23). The dysregulation of these targets, mainly Fz and LRP6, have deleterious effects: overexpression of Frizzled-8 was associated with lung cancer, and LRP6 overexpression promoted tumor cell proliferation *in vivo* (24, 25). Thus, it is essential to identify inhibitors of specific targets in the Wnt pathway for treatment of diseases that affect Wnt signaling. One method of finding inhibitors is molecular docking, a computational procedure used to predict the binding of different drugs, known as ligands, to the Wnt proteins, also known as the receptors (23). The docking calculates the strength of the bonding and provides insights into whether specific molecules are likely to interact effectively with the Wnt pathway (23, 26).

Glyphosate and AMPA can interact with the Wnt signaling pathway and have significant effects on neuronal and biological development in organisms such as rats (27). Glyphosate exposure in rats resulted in the inhibition of the noncanonical Wnt5a-CaMKII signaling pathway, which hinders the development of neural circuits and induces developmental neurotoxicity (27). Glyphosate significantly downregulated the mRNA expression of Camkiia and Camkiib—also known as the calcium/calmodulin protein kinase II α and β pathways—which are essential in neuronal development and cellular proliferation (5). In SH-SY5Y human neuroblastoma cells, which are used to model neurodegenerative disorders, glyphosate upregulated *WNT3A, WNT5A,* and *WNT7A*, three crucial developmental genes: *WNT3A* regulates cell proliferation and differentiation, *WNT5A* activates the β-catenin signaling to regulate cell polarity and migration, and *WNT7A* regulates anterior-posterior polarity (15). In contrast, AMPA significantly downregulated expression of the CaMKII pathway and *WNT3A*, but did not significantly alter *WNT5A* or *WNT7A* expression (5). While AMPA and glyphosate have not been tested in direct binding studies with canonical Wnt targets, they are predicted to have high binding affinities to Wnt proteins due to their extensive hydrogen bonding forces (28).

Planaria (*Dugesia tigrina*) belong to the class of flatworms and are a good model organism for this study because they exhibit bilateral symmetry, a central nervous system (CNS), and similar brain development to humans, as they have the same neuronal subpopulations and neurotransmitters (30). Additionally, they have remarkable regeneration abilities controlled by a variety of factors and signaling processes.

Molecular Target	Binding Affinity of AMPA (kcal/mol)	Binding Affinity of Glyphosate (kcal/mol)
Dvl	-4.7	-6.0
LEF1	-4.6	-5.1
LRP6	-4.4	-5.7
CK1	-4.4	-4.9
Frizzled-8	-4.3	-6.2
GSK3β	-4.1	-4.7
β-catenin	-4.0	-4.7
APC	-3.9	-4.1
Axin 1	-3.1	-4.0

Table 1: AMPA and glyphosate had strong binding affinities with Wnt macromolecules. Molecular docking was conducted in AutoDock Vina, and the highest binding affinity was recorded for each interaction. Values around -5 kcal/mol were considered strong interactions and are highlighted in orange (38). Dvl = Disheveled, LEF1 = Lymphoid Enhancing Factor 1, LRP6 = Low-density lipoprotein receptor-related protein 6, CK1 = casein kinase 1, GSK3β = glycogen synthase kinase 3 β, APC = Adenomatous polyposis coli.

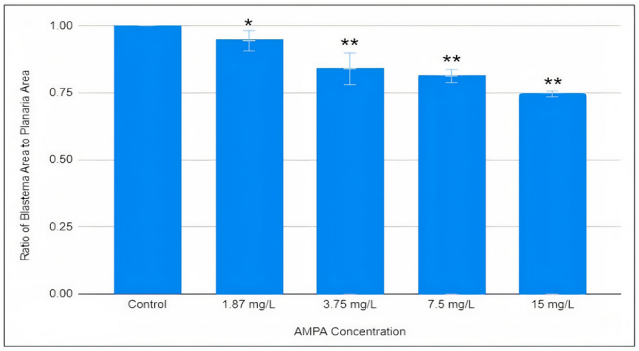

Figure 1: AMPA treatment significantly reduced blastema size. The graph shows the average scaled blastema area 7 days after amputation for each group (n=10); error bars show ± SEM. Prior to starting the assay, planaria were exposed to 0 (untreated control), 1.87, 3.75, 7.5, and 15 mg/L of AMPA for 15 minutes daily and decapitated after 5 days. The number of pixels in the blastema was measured with ImageJ, and the area was calculated as a ratio of the blastema size to the size of the whole planarian to normalize for differences in worm size. Ratios were scaled relative to control. Dunnett's test, *p < 0.05, **p < 0.01.

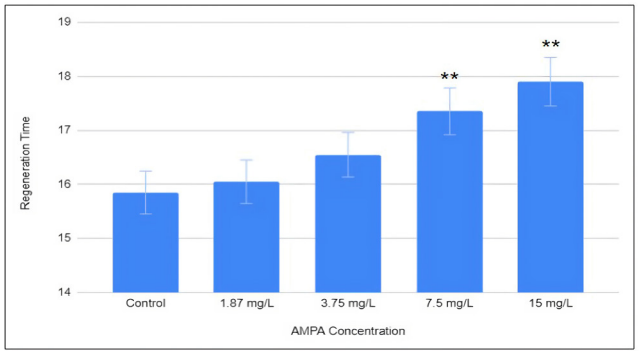

Figure 2: AMPA treatment increased the average regeneration time of planaria. The graph shows the regeneration time after amputation. The error bars show ± SEM. Prior to starting the assay, planaria were exposed to 0 (untreated control), 1.87, 3.75, 7.5, and 15 mg/L of AMPA for 15 minutes daily (n=10 per group) and cut after 5 days. The number of days from cutting until complete regeneration was recorded. Regeneration of a planarian was considered complete once the head, photoreceptors, and auricles were fully formed. Dunnett's test, *p < 0.05, **p < 0.01.

Shortly after injury, planaria are able to regenerate most of their body. The blastema, a proliferating pool of non-differentiated cells called neoblasts, is responsible for regenerating missing structures (31). More importantly, planaria are a simple but effective model to study the canonical Wnt pathway because their regeneration from the blastema is modulated by Wnt signaling (31-33). Many canonical Wnt targets play important roles in planaria development. Glycogen synthase kinase-3 (GSK3) is an essential protein that regulates the Wnt pathway and neuronal development; inhibition of GSK3s in planaria resulted in abnormal CNS and peripheral nervous system (PNS) regeneration (34). Disheveled (Dvl), an important Wnt phosphoprotein involved in cellular differentiation, was also found to play an important role in neural connectivity in planaria (35).

Glyphosate can also alter planarian regeneration (36). Typically, planaria regenerate with one head and one tail; however, aberrant Wnt signaling can result in abnormal regeneration of two heads or two tails (32, 36). Planaria exposed to glyphosate also regenerated at a significantly slower rate (a delay of 9 hours) when exposed to 15 mg/L glyphosate; the planaria that regenerated had many malformations with misshapen auricles and extra photoreceptors (36).

The goal of this study was to evaluate the toxic effects and mechanism of action of AMPA by using planarian regeneration as a model of the Wnt-signaling pathway and molecular docking simulations to model chemical interactions between AMPA and proteins of the Wnt pathway. We hypothesized that AMPA would hinder planarian regeneration and bind well to Wnt proteins in the molecular docking simulations. Planaria were exposed to varying concentrations of AMPA for 5 days, and the blastema size, regeneration time, and locomotion of the planaria were measured afterwards. We found that AMPA significantly impaired regeneration, hindered blastema growth, and decreased planarian locomotion, indicating that AMPA is toxic to planaria. The results suggest that AMPA is potentially toxic at low concentrations and may pose threats to human health. Additionally, both glyphosate and AMPA had strong interactions with canonical Wnt targets. It is possible that AMPA may actually inhibit the activity of proteins in the Wnt pathway and prevent cancer progression, since the molecular docking does not give any indication of whether Wnt proteins were upregulated or downregulated. However, given that AMPA exhibited toxicity towards planaria, it may be more likely that AMPA hyperactivates the Wnt pathway, thus promoting cancer development.

RESULTS

AMPA Impairs Blastema Regeneration

Since planarian regeneration from a blastema is regulated by canonical Wnt targets, we measured the size of the blastema to evaluate Wnt signaling (31, 32, 33). We also found the lowest observed effect concentration (LOEC), which was the lowest AMPA concentration that resulted in a significant decrease in blastema size ($p<0.05$) compared to the untreated control group (36). Planaria were exposed to 0 (untreated control), 1.87, 3.75, 7.5, and 15 mg/L solutions of AMPA for 15 minutes daily over a 5-day period. After exposure, we transversely cut the planaria beneath the auricles into two fragments and assessed blastema size. Smaller blastema areas would indicate decreased cellular proliferation and impaired regeneration. We determined the number of pixels in the blastema area seven days after amputation with ImageJ (37). All AMPA concentrations (1.87, 3.75, 7.5, 15 mg/L) significantly hindered blastema regeneration (**Figure 1**, $p<0.05$). As the concentration of AMPA increased, a greater reduction in blastema area was observed: the 1.87 mg/L treatment decreased blastema area by an average of 5.6% ($p<0.05$), while the 15 mg/L treatment decreased blastema area on average by over 25% ($p<0.01$),

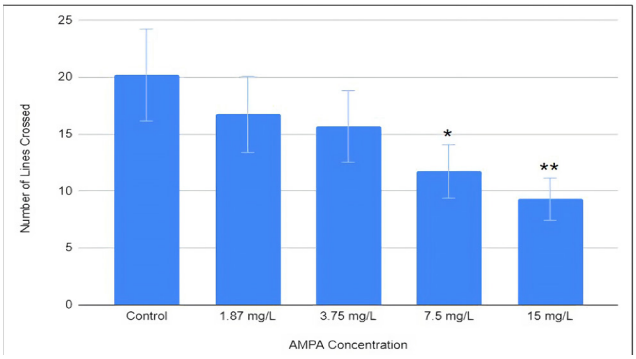

Figure 3: AMPA treatment significantly hindered planarian locomotion. The graph shows the average number of lines crossed by planaria. Error bars show ± SEM. Prior to starting the assay, planaria were exposed to 0 (untreated control), 1.87, 3.75, 7.5, and 15 mg/L of AMPA for 15 minutes daily (n=10 per group) and cut after 5 days. Planaria were placed over grid lines spaced 0.5 cm apart, and the number of lines crossed was counted over two minutes. Dunnett's test, *p < 0.05, **p < 0.01.

a nearly 5-fold decrease. The LOEC for the blastema assay was 1.87 mg/L of AMPA.

AMPA Delays Regeneration Time and Decreases Planarian Locomotion

We evaluated regeneration time to assess whether AMPA abnormally affected planarian regeneration. After decapitating the planaria, we recorded the number of days for a planarian (with or without AMPA treatment) to fully regenerate its head components, including photoreceptors and auricles. While the 1.87 and 3.75 mg/L treatments did not induce significant delays in regeneration ($p>0.05$), the 7.5 mg/L and 15 mg/L treatments significantly hindered regeneration, increasing the time for complete regeneration by 1.50 days ($p<0.01$) and 2.05 days ($p<0.01$) on average, respectively (**Figure 2**).

Healthy planaria are capable of a variety of movements, such as swimming and gliding, but cognitive dysfunction results in reduced locomotion (30). Thus, the neurological functioning and general health of the planaria were assessed with the planarian Locomotor Velocity (pLMV) assay. We placed planaria over 0.5 cm spaced grid lines and recorded the number of lines crossed over two minutes; a higher number of lines crossed indicated higher locomotion. The groups treated with 1.87 and 3.75 mg/L AMPA, while displaying an average decrease in the number of lines crossed, did not exhibit significantly decreased locomotion from untreated control ($p>0.05$) (**Figure 3**). The 7.5 and 15 mg/L concentrations did significantly hinder locomotion, with the planaria crossing an average of 11.7 ($p<0.05$) and 9.3 lines ($p<0.01$), respectively (**Figure 3**). The 15 mg/L treatment decreased locomotion of planaria by over 50% compared to untreated control planaria, which crossed an average of 20.1 lines.

In both the regeneration and pLMV assays, an increase in AMPA concentration generally corresponded to an increase in average regeneration time and decrease in locomotion; however, for each assay, only the 7.5 and 15 mg/L concentrations induced statistically significant differences from the untreated control. The LOEC for both assays was 7.5 mg/L.

AMPA and Glyphosate have Moderately Strong Binding Affinities with Wnt targets

We conducted molecular docking *in silico* to find the binding affinities of AMPA and glyphosate with molecular targets in the Wnt signaling pathway. Negative binding affinities indicate stronger interactions because free energy is lowered, resulting in a more stable bond (26). The objective was to determine whether the mechanism of toxicity of AMPA and glyphosate may have occurred via the Wnt pathway. We observed whether AMPA and glyphosate interacted with ten canonical Wnt targets: Dvl, LEF1, LRP6, CK1, Fz-8, GSK3β, β-catenin, APC, and Axin 1, which were chosen based on the important Wnt targets identified by Ng et al. (23). We conducted molecular docking in PyRx with AutoDock Vina to determine the binding affinities in kcal/mol. According to previous studies, established Wnt inhibitors Isorhamnetin, Fistein, Genistien, and Silibinin had strong simulated binding affinities of -4.98, -5.68 ,-5.44, and -5.32 kcal/mol, respectively, with Wnt proteins; thus, the strength of AMPA and glyphosate binding affinities were evaluated relative to these values (38). In general, AMPA had moderately strong simulated binding affinities with the ten canonical Wnt targets, ranging from -3 kcal/mol to nearly -5 kcal/mol. AMPA had the strongest interaction with Dvl with a binding affinity of -4.7 kcal/mol and had the weakest interaction with Axin 1 with a binding affinity of -3.1 kcal/mol (**Table 1**). However, simulated binding affinities of glyphosate with the Wnt targets of interest were generally stronger than those of AMPA, especially with the Wnt proteins Frizzled 8, LRP6, and Dvl with binding affinities of -6.2, -5.7, and -6.0 kcal/mol, respectively (**Table 1**). In a previous study, Fisetin, an established Wnt inhibitor, had a simulated binding affinity of -5.68 kcal/mol with Wnt proteins (38). Glyphosate had even higher binding affinities to Wnt proteins than Fisetin, highlighting the strong bonding between glyphosate and canonical Wnt targets.

DISCUSSION

The objective of this study was to investigate the toxic effects of AMPA in planaria and determine whether the inhibition of the Wnt pathway was a possible mechanism of toxicity. This study provided additional evidence regarding AMPA toxicity, and the molecular docking further corroborated that both AMPA and glyphosate may be capable of binding to Wnt targets. Planaria treated with 7.5 and 15 mg/L AMPA experienced increased toxicity compared to lower concentrations, as indicated by significantly reduced planarian locomotion, regeneration, and blastema area in a seemingly dose-dependent manner, although this was not statistically significant. These trends are similar to those of a previous study (36).

In this study, we found that AMPA had an LOEC of 1.87 mg/L for the blastema assay, which is the lowest concentration that significantly decreased planaria blastema size, and 7.5 mg/L for the pLMV and regeneration time assay, the lowest concentration that significantly reduced locomotion and delayed regeneration time. The results are consistent with previous studies, which found a glyphosate LOEC of 3.75 mg/L for the blastema assay and 7.5 mg/L for pLMV and regeneration time assays (36). The different LOECs may be due to the temporality of the assays—while the blastema assay was conducted 1 week post-amputation, the regeneration and pLMV assays were conducted 2-3 weeks after amputation. Regeneration is activated by different signaling pathways depending on time: the early regenerative response to a wound is Wnt-independent and activated by a signal transduction pathway upstream of Wnt, which involves Hedgehog signaling (32). Thus, it is possible that Hedgehog and Wnt signaling are differentially impacted by AMPA.

These findings demonstrate that AMPA is potentially more toxic than glyphosate due to its lower LOEC for the blastema assay (1.87 mg/L for AMPA versus 3.75 mg/L for glyphosate). This suggests that AMPA is more dangerous than glyphosate at lower concentrations, which is significant because AMPA is more prevalent than glyphosate in the environment due to rapid glyphosate metabolism (5, 8, 13). Additionally, the concentrations used in this study were based on environmental concentrations of glyphosate observed in a previous study (36). Thus, the data indicate AMPA's greater toxicity than glyphosate at similar concentrations. With regard to the planarian regeneration model, the AMPA reduced the blastema area and increased overall regeneration time, indicating that it negatively affected the regenerative ability of planaria. These results further bolster the conclusion that AMPA can exert its toxicity in planaria through abnormal regeneration. Exposure or intake of AMPA may pose health risks, and the levels of AMPA found in the environment may not have negligible health effects.

The molecular docking showed that both glyphosate and AMPA had moderately strong binding affinity to multiple canonical Wnt targets—especially Fz, Dvl, and LRP6—with simulated binding affinities generally ranging from -4 kcal/mol to -6 kcal/mol. Previous studies have shown that well-established Wnt inhibitors had simulated binding affinities of -4.98 to -5.68 kcal/mol with canonical Wnt targets (38). AMPA and glyphosate have similar binding affinities to Wnt pathway proteins as those of known Wnt inhibitors; the data imply that AMPA and glyphosate are capable of interacting effectively with canonical Wnt targets, potentially altering their activity.

There were some limitations in the planarian regeneration model. Only phenotypic assays were used, which do not provide information regarding the mechanisms of AMPA. Additionally, we did not quantitatively determine Wnt protein levels, so we could not confirm whether AMPA altered Wnt protein levels. Since multiple pathways are also involved in planarian regeneration and may interact with the Wnt pathway, such as hedgehog signaling, the mechanism of toxicity of AMPA cannot be attributed solely to Wnt signaling (32).

There were also limitations with regard to the molecular docking simulations. We did not conduct binding site analysis, which could have provided insight into the intermolecular forces, orientation, and location of the interaction between the proteins. Additionally, the molecular docking simulation only predicts the strength of interaction and does not provide any information about the up or downregulation of the Wnt targets; thus, we cannot ascertain whether AMPA and glyphosate would inhibit or hyperactivate the Wnt pathway. Future research can use RNA interference (RNAi) in planaria, which is a method of suppressing certain genes in order to more accurately assess gene expression and function (39). Additionally, Western blot can be used to accurately and quantitatively assess Wnt expression levels in planaria (40). The effect of AMPA can also be tested on other pathways implicated in planarian regeneration and Wnt signaling, such as the hedgehog pathway.

In this study, we exposed planaria to AMPA for five days. The short term effects of AMPA were deduced, but in order to more definitively establish the chronic toxicity of AMPA, future research should expose planaria to AMPA for longer periods of time. More trials should also be conducted in the future to ensure the reproducibility of these results.

Overall, AMPA displayed toxic effects toward planaria and may potentially pose threats to human health. AMPA interacts strongly with Wnt targets and may alter the Wnt pathway, which is a feasible mechanism of action. However, it is not definitive whether AMPA is carcinogenic or interferes with cancer, as the molecular docking does not determine whether AMPA inhibited or hyperactivated the Wnt pathway.

MATERIALS AND METHODS

Solutions

A 150 mg/L stock solution of AMPA (Sigma-Aldrich, St. Louis, MO) was prepared by dissolving AMPA in distilled water. 1.87, 3.75, 7.5, and 15 mg/L experimental solutions were obtained by dilution of the stock solution. The untreated control only contained distilled water with no AMPA.

Planarian Preparation

Dugesia tigrina (Sigma-Aldrich, St. Louis, MO) were cultured in a 10 cm petri dish in a dark environment at 25o C. Distilled water was added to the petri dishes and changed every two days. A pea-sized portion of fresh beef liver or hard-boiled egg was fed to the planaria once a week. To simulate environmental AMPA levels, 5 groups of planaria (n=10) were exposed to concentrations of 0 mg/L (untreated control group), 1.87 mg/L, 3.75 mg/L, 7.5 mg/L, and 15 mg/L of AMPA (36). Planaria in each group were transferred to a petri dish containing AMPA solution for 15 minutes and were then transferred back to a petri dish with fresh spring water. This was repeated each day for 5 days total. After exposure,

planaria were transversely cut beneath the auricles into two fragments and the pLMV and regeneration assays were conducted. The Lowest Observed Effect Concentration, or LOEC, was found for each individual assay by determining the lowest AMPA concentration that resulted in a significant difference from the untreated control group (36).

Planarian Locomotor Velocity (pLMV) Assay and Regeneration Assays

The pLMV assay was used to assess the locomotion of the planaria. After complete regeneration, each planarian was transferred to a new petri dish of fresh spring water placed over grid lines spaced 0.5 cm apart. The planaria were recorded for two minutes and the number of lines crossed was quantified.

To assess the effect of AMPA on regeneration, the regeneration time of the planaria and blastema area were analyzed. After amputation, as described in the previous section, the number of days it took for a planarian to fully regenerate was recorded. Full regeneration was defined by the complete formation of the head, photoreceptors, and visible auricles. 7 days after amputation, blastema areas were determined in ImageJ. A ratio of the blastema area to the area of the total planaria (in pixels) was calculated to account for differences in worm size (36).

Molecular Docking

Molecular docking was conducted to determine the binding affinities of AMPA and glyphosate with Wnt targets. AMPA (PubChem CID: 14017) and glyphosate (CID: 3496) ligands were obtained from PubChem and saved as 2D spatial data files (.sdf) before being converted to a 3D .pdb format with Online SMILES Translator. Canonical Wnt macromolecules Axin 1 (PDB ID: 4NM0), Adenomatous polyposis coli (3AU3), Disheveled (6ZC4), GSK3β (1Q3W), Frizzled-8 (6AHY), LRP6 (4DG6), β-catenin (4HM9), CK1 (1EH4), and LEF1 (3OUW) were obtained from RCSB Protein Data Bank and saved in 3D .pdb format. Docking was then performed in AutoDock Vina, and the binding affinity of the lowest energy configuration was recorded (26, 41).

Statistical Analysis

Statistical significance was determined by comparing each condition to the control via a one-way ANOVA followed by multiple comparisons with Dunnett's post hoc test (36). P-values less than 0.05 were considered significant.

ACKNOWLEDGMENTS

We would like to thank Kallista Zhuang for proofreading and reviewing the manuscript before submission.

Received: July 06, 2022
Accepted: August 23, 2022
Published: March 08, 2023

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