A NEWLY IDENTIFIED SPECIFIC BIOLOGICAL ACTIVITY OF GLYPHOSATE – INHIBITION OF RGD-BINDING INTEGRINS

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Abstract

In this study we investigated the inhibitory effect of the widely used broad-spectrum herbicide active ingredient glyphosate and its related analogues on αVβ3 integrin binding to the shortest oligopeptide recognizing motif of integrins, the arginine-glycine-aspartic acid (RGD) sequence. Integrin binding characteristics were assessed in a modified enzyme linked immunosorbent assay (ELISA) and by a label-free optical biosensor technique. At 22 mM, glyphosate reached full inhibition of αVβ3, and the inhibitory activity of its main metabolite, aminomethylphosphonic acid (AMPA) was also above 95%, while another environmentally relevant metabolite, sarcosine exerted only a weaker effect, approximately 35% inhibition. In turn, the half maximal inhibitory concentration (IC_{50}) of glyphosate and AMPA were reported to be 2.7±0.5 mM and 1.3±0.2 mM, respectively. The inhibitory effects of the other related compounds investigated (acetylglycine, glycine and iminodiacetic acid) at the same concentration, 22 mM were below 50%. Inhibitory effects on cell adhesion to RGD-modified surfaces by whole cells containing several types of RGD-binding integrins including αVβ3 were detected using the biosensor technique, where the integrin antagonist activity of glyphosate was also demonstrated.

Introduction

After its discovery as a potential herbicide active ingredient in 1971, glyphosate has been considered as a non-harmful compound to human health. As its popularity and usage intensity sharply increased on the market, the active ingredient and its metabolites emerged with increasing occurrence in environmental matrices as pollutants. Glyphosate residues have been detected in surface and subsurface water, soil, and also in food and biological samples. This fact paired with the contradictory toxicological classification by various international organizations (e.g., classified as probably carcinogenic to humans by the International Agency for Research on Cancer, but this statement has been refuted by the European Food Safety Authority and other risk assessment agencies) indicates uncertainties regarding the safety of this regulated product and an increasing rate of human exposure [1; 2]. Some of the revealed toxicity effects were proven to be attributable to the previously commonly used formulating agent, polyethoxylated tallowamine (POEA), yet certain effects, as the integrin antagonist activity reported in this study, clearly belong to the active ingredient itself. The basis of our modified receptor-binding enzyme-linked immunosorbent assay (ELISA) method was the connection between the arginine-glycine-aspartic acid (RGD) sequence and the αVβ3 integrin. Integrins, as cellular bidirectional signaling transmembrane receptors, have important roles in comunication between cells but are also involved in communication of the cells and their external environment [3]. Thus, integrins can bind different extracellular matrix components [4] which is provides a means for the cell to sense its surrounding medium [5]. Through specific

binding to their target ligands from the extracellular matrix integrins mediate crucial cellular physiological processes, such as cell adsorption and spreading on surfaces, migration and differentiation, and in a broader sense survival, proliferation, motility and differentiation [5, 6]. In addition, they also may contribute to pathological processes as well, as inhibitory effects on them may disturb normal cellular functions.
As RGD is the shortest recognizing motif for numerous integrins with different functions [7, 8], the inhibition of their binding can have a more extensive group of effects, including alterations in processes in several diseases comprising some types of cancer [9, 10]. Our study included characterization of cellular adhesion processes based on an Epic BenchTop (BT) optical biosensor investigation, which indicated the potential inhibitory effect of glyphosate on RGD-depended integrins [6], and compared the results with the extent of the αVβ3 integrin antagonist effect revealed in the ELISA and biosensor cell-free tests. To assess chemical moieties necessarily present in the chemical structure of the integrin antagonist, the inhibitory potential of structural derivatives of glyphosate has also been assessed within the study. All the chosen potential inhibitors are metabolites and amino acid analogs of the main target compound.

Experimental

ELISAs were carried out in high-capacity 96-well microplates (Nunc, Roskilde, Denmark, #442404) coated with 250 µg/mL PLL-g-PEG-RGD in the form of poly(L-lysine)-graft-poly(ethylene glycol) terminated with the sequence GGGGYGRGDSP (SuSos, Dübendorf, Switzerland) in 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES, pH = 7.4) for 1 h and blocked after a washing step using phosphate buffer saline containing Tween with 20 mM TrisB, pH = 7.5. After washing, competition steps were performed by adding 50 µL of the inhibitor first (e.g., glyphosate in dilution series in a concentration range of 137.5 µM – 22 mM), and then 50 µL integrin (2 µg/mL) for 1 hour incubation. A subsequent washing step was followed by a 1-hour incubation of the primary antibody (mouse anti-human CD51/61, 2 µg/mL). After washing again, the second antibody labeled with a reporter enzyme horseradish peroxidase (anti-mouse IgG-HRP, 100 µL/well, 1 µg/mL) was incubated for 1 hour. After the last washing step, 100 µL/well of the substrate (hydrogen peroxide, 1.2 mM) and a chromophore (3,3′,5,5′-tetramethylbenzidine, 1.2 mM) were added together in 0.5 M citrate buffer (pH = 5.0). Incubations were performed at room temperature. The enzymatic reactions were stopped at the approppriate color intensity by adding 50 µL/well of 4 N sulfuric acid. Colorimetric signals were detected using a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA) in endpoint mode at 450 nm.
Cell adhesion inhibition assays were performed in Epic BT instrument (Corning Incorporated, Corning, NY, USA) using 384-well Corning Epic assay microplate coated with PLL-g-PEG-RGD. The MC3T3-E1 cells were harvested and solutions containing glyphosate at varying concentrations were added to the cell suspension, resulting in a final cell density of 8000 cells/well. Then 30 µl of cell suspensions were seeded into the wells and biosensor responses were recorded for 1 h. Competitive biosensor integrin binding assays were performed in 384-well Corning Epic assay microplates coated with 250 µg/ml PLL-g-PEG-RGD. Solutions of echistatin, glyphosate, and tirofiban were added to the biosensor wells (20 µl/well). Upon stable baselines had been established for all wells, 20 µl of αVβ3 integrin solution was added to a final concentration of 4 µg/ml and biosensor responses were recorded for 1 h. Snake venom disintegrin echistatin was chosen as a positive control, while tirofiban, with no blocking effect on αVβ3 integrin, was applied as a negative control.
Comounds tested for integrin antagonist activity included, besides glyphosate, included its common or less frequent metabolites or contaminating substances. The chemical structures of

glyphosate and two of its major metabolites are depicted on Figure 1. Glyphosate was applied in the form of its zwitterionic free acid or its isoprolylammonium salt.

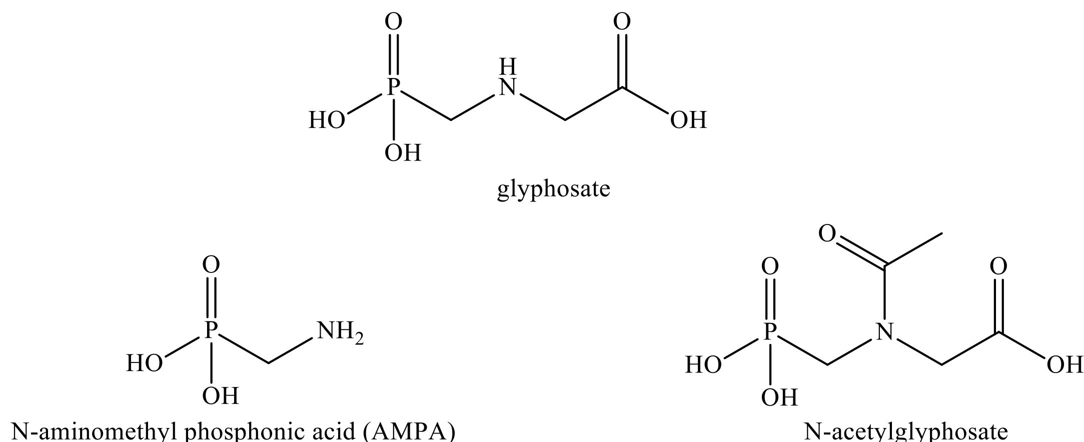

Figure 1. The chemical structure of the target compound glyphosate, and two of its metabolites, aminomethylphosphonic acid (AMPA) formed by oxidative degradation N-acetylglyphosate formed by N-acylation.

Results and discussion

ELISAs were performed to determine the inhibitory effect of glyphosate and related compounds on αVβ3 integrin binding to RGD sequence. Background levels were measured in coated and blocked wells with buffer. The main inhibitor herbicide caused total inhibition at 22 mM, but the half of that concentration (11 mM) still resulted in substantial inhibition as high as around 90% (Figure 2, left). To obtain comparable results with other compounds related to the target molecule, glyphosate, all the other compounds were investigated also up to this molar concentration, 22 mM. Surprisingly the main metabolite aminomethylphosphonic acid (AMPA) reached almost the same level of inhibition with a signal decrease above 95%, but other related compounds tested had only weaker effects with inhibition rates detected between 20% and 50%, and the activity of the weakest inhibitor, iminodiacetic acid did not reach 25%. The other important metabolite, sarcosine also caused only 35% inhibition. The potent inhibitory activity of AMPA was even more demonstrated by the half maximal inhibitory concentrations (IC_{50}s), in which AMPA showed an even slightly stronger inhibition than glyphosate: the IC_{50} values of glyphosate and AMPA were reported to be 2.7±0.5 mM and 1.3±0.2 mM, respectively.
Glyphosate is decomposed mainly by two mechanisms: a redox pathway in which aminomethylphosphonic acid (AMPA) and glyoxylic acid are formed via the C–N bond cleavage in the aminoacetic acid part of the molecule (AMPA pathway) catalyzed by the enzyme glyphosate oxidoreductase and other dehydrogenases, and a dephosphonylative pathway in which N-methylaminoacetic acid (sarcosine) and phosphate are formed via the C–P bond cleavage in the aminophosponic acid part of the molecule (sarcosine pathway) catalyzed by the enzyme C–P-lyase [1; 11]. In addition, glyphosate can also be inactivated by an N-acylation pathway in which N-acetylglyphosate is formed catalyzed by the enzyme glyphosate acetyltransferase [1; 12].
The effect of the integrin antagonist activity of glyphosate was demonstrated by determining its effect on cell adhesion of osteoblastic MC3T3-E1 cells to RGD-displaying polymer surfaces measured by label-free optical biosensing (Figure 2, right). For this purpose, the evanescent field-based surface sensitive resonant waveguide grating biosensor Epic BenchTop (Epic BT),

proven as a useful method for real-time, high-throughput, and label-free detection of cell adhesion, spreading and signaling events based on measuring of dynamic mass redistribution within a 150 nm range on the sensor surface, has been used [13-15].

The Epic cell assay was capable to assess changes in the kinetics of cell adhesion on the sensor surface. Sigmoid-shaped kinetic response is known to be typical phenomenon in receptor-mediated cell adhesion events [15-17]. In the presence of an inhibitor, cell adhesion is decreasing resulting in reduction in the maximal biosensor signal and indicating considerable effects of glyphosate on the cell adhesion process. The binding between RGD-specific integrins in the intact MC3T3-E1 cells and soluble glyphosate has been demonstrated to occur through a competition for binding to the RGD-motifs with an estimated value of the K_d of 0.352 mM and of the IC_{50} of 20.6±0.3 mM [6]. In addition, adhesion of the MC3T3-E1 cells to the RGD-modified surface was completely blocked by preincubation with 74.5 mM glyphosate. The somewhat lower integrin antagonist activity of glyphosate in the Epic BT biosensor tests than in the ELISAs can be explained by the fact that the living whole cells contain various integrins in addition to αVβ3, therefore cell adhesion facilitated by integrins other than αVβ3 can take place even when all αVβ3 integrins are fully inhibited. In competitive αVβ3 integrin binding assay in biosensor format the 11 mM glyphosate resulted in inhibition level of about 50%.

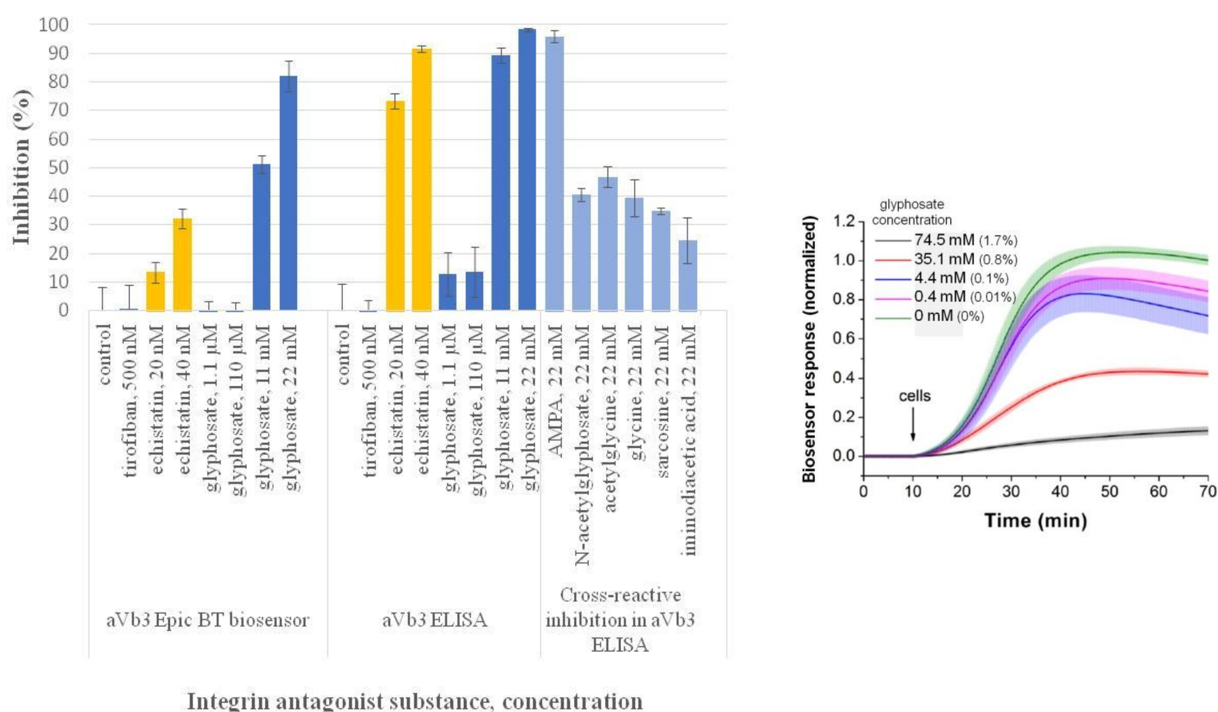

Figure 2. *Left*: Inhibitory potential of glyphosate and its structurally related analogues on αVβ3 integrin determined in a biosensor (Epic BT) and an immunoassay (ELISA) format. *Right*: Inhibition of MC3T3-E1 cell adhesion to an RGD-displaying sensor surface by glyphosate at various concentrations. A monotonous concentration-dependent inhibitory activity of glyphosate on αVβ3 integrin is observed in the concentration range of 0.4–74.5 mM (0.01–1.7%).

Conclusion

Our study has evidenced the inhibitory effect of glyphosate on αVβ3 integrin. It has also been revealed that the main metabolite AMPA, far more persistent than glyphosate itself, exerted inhibition of αVβ3 nearly as potently as glyphosate. This may emerge as a health concern not only from the aspect of environmental relevance but also when human exposure is considered.

Possible health consequences, due to the physiological processes affected by inhibition or inactivation of the functions of αVβ3 integrin may influence our approach towards safety assessment of glyphosate.

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