Substructure-activity relationship studies on antibody recognition for phenylurea compounds using competitive immunoassay and computational chemistry

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Based on the structural features of fluometuron, an immunizing hapten was synthesized and conjugated to bovine serum albumin as an immunogen to prepare a polyclonal antibody. However, the resultant antibody indicated cross-reactivity with 6 structurally similar phenylurea herbicides, with binding activities (expressed by IC₅₀ values) ranging from 1.67 µg/L to 42.71 µg/L. All 6 phenylurea herbicides contain a common moiety and three different substitutes. To understand how these three different chemical groups affect the antibody-phenylurea recognition activity, quantum chemistry, using density function theory (DFT) at the B3LYP/6-311++G(d,p) level of theory, was employed to optimize all phenylurea structures, followed by determination of the 3D conformations of these molecules, pharmacophore analysis, and molecular electrostatic potential (ESP) analysis. The molecular modeling results confirmed that the geometry configuration, pharmacophore features and electron distribution in the substituents were related to the antibody binding activity. Spearman correlation analysis further elucidated that the geometrical and electrostatic properties on the van der Waals (vdW) surface of the substituents played a critical role in the antibody-phenylurea recognition process.

Phenylurea herbicides constitute an important group of herbicides extensively utilized for herbaceous and perennial weed control in non-crop areas and for the preemergent treatment of fruit crops. Globally, phenylurea herbicides have been detected in surface water, groundwater, soil and sediment in areas where their extensive use occurred. Phenylurea herbicides are relatively long-lived in the environment, and their introduction to the food chain via the environment is deemed a serious risk to human health. Therefore, it is evident that environmental risk and food safety problems need to be mitigated.

Currently, immunoassays, such as enzyme-linked immunosorbent assay (ELISA), have been widely used in practice for the fast monitoring of pesticide residues in environmental or food samples. However, individual small molecules always belong to one class of compounds, for instance, the phenylurea herbicide family, and most of the resultant antibodies elicited by one specific hapten carrier usually cross-react with other structural analogues. This is particularly true if they share an identical or very similar epitope with the hapten used. The mechanism of the cross-reaction between antibodies and different analogues is difficult to elucidate. Although many studies have suggested an antibody recognition mechanism based on X-ray graphs of the antibody with a hapten, crystallization of the title antibody-hapten complex (particularly in polyclonal antibodies) has become a new challenge due to stringent experimental conditions.

Since the 1990s, computational chemistry, such as quantum chemistry and molecular mechanics methods, has been used for preliminary guidance on immunoassay development. Computational chemistry not only provided a powerful method to explain the cross-reactivity but also became a useful tool to understand the
hapten-antibody recognition mechanism in the absence of antibody physicochemical information. In particular, 2D-QSAR or 3D-QSAR methods, such as CoMSIA and CoMFA Contour, have been used in many studies to investigate the binding activities of polyclonal\textsuperscript{14,18,19} and monoclonal\textsuperscript{20,21} antibodies against structurally similar compounds. These methods, however, are only suitable for broad-specific antibodies that can recognize a large number of targets and are not suitable for antibodies that only recognize several compounds\textsuperscript{22}. In this study, a specific hapten was synthesized and conjugated to bovine serum albumin, as the immunogen, to produce a new anti-fluometuron polyclonal antibody. However, when we used the resultant antibody to develop a competitive ELISA, we encountered a general issue, as in most other immunoassays: the specific antibody could also recognize five other analogues with different activities. To explore how this antibody, elicited by one specific immunogen, could cross-react with other targets, the half maximal inhibitory concentration (IC\textsubscript{50}) values, presented as the binding activity of the antibody, were calculated and analyzed. Subsequently, quantum chemistry and molecular mechanics methods were employed to explain how the substituents of the six phenylurea compounds caused the differences in binding activity. Lastly, the molecular surfaces were quantitatively analyzed to correlate the binding activities and the molecular surface descriptors of substituents in the phenylurea compounds.

Results

Antibody binding activity analysis. According to the two-dimensional structures of the phenylurea molecules (Fig. 1), the 6 title compounds were divided into three groups. The first group consisted of fluometuron and fenuron, which have different substituents at the R\textsubscript{1} position. The second group contained neburon and diuron, which have different substituents at the R\textsubscript{2} position. The third group included, chlorbromuron and linuron, which have different substituents at the R\textsubscript{3} position.

We performed a direct competitive ELISA to evaluate the binding activity of the resultant antibody. IC\textsubscript{50} and cross-reactivity (CRs) values were calculated to represent the binding activity and specificity of the antibody. These values are shown in Table 1.

![Figure 1](https://www.nature.com/scientificreports/)  
**Figure 1.** Common structure of the phenylurea molecules in this study. R\textsubscript{1}, R\textsubscript{2} and R\textsubscript{3} represent different chemical groups in the different molecules, and R\textsubscript{4} represents the methyl group. a-j represent the number of atoms in the molecule.

| Analytes      | R\textsubscript{1} | R\textsubscript{2} | R\textsubscript{3} | IC\textsubscript{50} (µg/L) | CRs (%) |
|---------------|---------------------|---------------------|------------------------|-----------------------------|---------|
| Fluometuron   | -CF\textsubscript{3} | -H                  | -CH\textsubscript{3}   | 1.67                        | 100.00  |
| Neburon       | -Cl                 | -Cl                 | -CH\textsubscript{2}CH\textsubscript{2}CH\textsubscript{3} | 2.41                        | 69.40   |
| Diuron        | -Cl                 | -Cl                 | -CH\textsubscript{3}   | 8.35                        | 19.99   |
| Chlorbromuron | -Cl                 | -Br                 | -OCH\textsubscript{3}  | 9.77                        | 17.10   |
| Fenuron       | -H                  | -H                  | -CH\textsubscript{3}   | 11.61                       | 14.38   |
| Linuron       | -Cl                 | -Cl                 | -OCH\textsubscript{3}  | 42.71                       | 3.91    |

Table 1. Structures, IC\textsubscript{50} and CRs of the title phenylurea compounds. The antibody (with a better titer and cross-reactivity, 80000 U and 83.1%, respectively) obtained from number II rabbit was used to establish the ELISA method.
increased the antibody-chlorbromuron binding activity dramatically. In addition, the comparison of the binding activity to linuron (IC<sub>50</sub> = 42.71 µg/L) and diuron (IC<sub>50</sub> = 8.35 µg/L) suggested that the different groups at the R₃ position, where a methoxy group replaced the methyl group, could cause an obvious decline in antibody activity for linuron.

The ELISA results demonstrated that the differences in the hapten-antibody binding activity may be caused by the different substitutes in the otherwise structurally similar analytes. However, we cannot explain how different substitutes affect the binding ability just by examining the two-dimensional structural formulas. In the hapten-antibody recognition process, the 3D configuration of the hapten molecule should match that of the active pocket of the antibody, according to the geometric configuration matching mechanism<sup>13,17,23</sup>. Cross-reactions will occur if molecules have the same or similar geometric configuration as the hapten. To understand the recognition mechanism, the 3D configuration of phenylurea molecules were optimized (Fig. 2) and studied by employing quantum chemistry and molecular mechanics methods.

**Conformational analysis and phenylurea molecules alignment.** As seen from the optimized structures in Fig. 2, all molecules have similar bond lengths, bond angles and dihedral angles from atom a to atom j. Thus, they are well superposed based on their common structures, and the difference in the geometric configuration is mainly located in the different substitutes. Moreover, we can see that molecules in each group have a high degree of alignment based on their common structures (Fig. 3). In the first group, the fluometuron molecule has a very high fitting degree with fenuron based on their common structure (atoms a-j, R₂ and R₃), showing an alignment RMS value of 0.0190. Even so, the -CF₃ group at the R₁ position in fluometuron caused the carbon atoms on the phenyl ring to slightly bend out of the phenyl ring plane. This change contributes to an almost 7-fold gap in the IC₅₀ value determined from the geometric configuration. We speculate that the antibody raised by fluometuron contained a -CF₃ complementary cavity, and this cavity could spatially recognize certain bulky groups, such as -CF₃.

For neburon, the existence of the bulky butyl group at the R₃ position also dramatically changed the general conformational structure that was exposed to the antibody, especially the dihedral angle of C₁₀⁻C₁⁻C₁₂⁻C₁₃ (−164.4°). This value is obviously smaller than those of the other 5 molecules (are all more than 174.0°), and the steric hindrance of the butyl group caused the whole neburon molecule be poorly aligned with the diuron molecule (the alignment RMS value was only 0.9346). Furthermore, neburon shows a better binding ability than diuron. For the third group, the alkoxy group at the R₃ position in the chlorbromuron and linuron molecules also affects the spatial conformation of the methyl group at the R₄ position. Chlorbromuron has a high degree of alignment with linuron, and the alignment RMS value is 0.0106. However, the replacement of the chlorine atom by a bromine atom at the R₂ position resulted in the bond angles of C₂⁻C₁₁⁻C₁₃ (122.2°) and C₂⁻C₁₁⁻C₁₃ (121.9°) in chlorbromuron being larger than those in linuron. This scenario contributes to an almost 4-fold activity gap in the binding activity. The geometric configuration analysis shows that certain bulky chemical group at the R₁ and R₃ positions play a positive role in the antibody-phenylurea binding process. Moreover, the antibody binding sites can also accurately recognize variations of the common structures of these molecules, resulting in differences in binding activity.
Pharmacophore model analysis. Fig. 4 shows the pharmacophore models of the phenylurea molecules. The pharmacophore is an ensemble of steric and electronic features of the molecule, such as hydrophobic centroids, hydrogen-bond acceptors or donors and aromatic rings, which are necessary for molecular recognition of a ligand by a biological macromolecule. The pharmacophore model of each phenylurea molecule had an aromatic ring feature in the phenyl ring area, a hydrophobic centroid at the R4 position and a hydrogen-bond acceptor at the carbonyl group position. The -CF3 group at the R1 position in fluometuron serves as a hydrophobic centroid. Simultaneously, the chemical features of neburon indicate that it cannot align well with diuron, though...
their only difference is located at the R3 group; the hydrophobicity of the butyl group may influence the steric configuration of the whole neburon molecule, resulting in a higher activity (IC50 = 2.41 µg/L) than that of diuron.

The chlorbromuron molecule has the same pharmacophore features as linuron but with a bromine atom, and all chlorine atoms serve as hydrophobic centroids. However, we cannot offer a reason for the different activities based simply on pharmacophore features. Except for the R3 group, linuron has the same structure as neburon, but the oxygen atom in the R3 group in the linuron molecule serves as a hydrogen-bond donor. This led to a decrease in activity, as suggested by the pharmacophore features.

There are some limits in exploring the activity differences according to the pharmacophore analysis. For example, the bromine atom in chlorbromuron and the chlorine atom in linuron serve as hydrophobic centroids, yet we cannot distinguish the differences between them. Furthermore, the hydrogen-bonding and hydrophobic interactions were essentially caused by the electron distribution on the molecular surface. In this way, ESP is also an important and indispensable parameter in the study of the hapten-antibody recognition mechanism.

Molecular electrostatic potential analysis. The ESP has a unique role in the prediction and analysis of molecular recognition and is often helpful in demonstrating non-covalent molecular interaction properties. By employing the ESP surface, we can determine the spatial regions in the molecular structure at which the ESP is negative or positive. This helps visualize charged regions of a molecule and is qualitatively useful in the analysis of electrostatic interactions between the antibody and the title compound.

Figure 5. The structures optimized by DFT and the ESP of the phenylurea molecules on the 0.001 a.u. contours of the electronic density of the molecules. The negative ESP regions are indicated in red, and the positive regions in blue. Potential is coded in the following order: red < orange < yellow < green < blue. The red and white spheres represent the minima and maxima of the ESP on the vdW surface.
electron-withdrawing groups, under the influence of the electron-attracting effect created by groups R<sub>1</sub> and R<sub>2</sub>, the electron distribution over the phenyl ring also changed.

There are no electron-withdrawing groups associated with the phenyl ring in fenuron, so more electrons are around its phenyl ring than around those of the other five phenylurea molecules. In addition, the minimum values in this area are −19.06 and −19.36 kcal/mol. For fluometuron, the -CF<sub>3</sub> group, an electron-withdrawing group, attract some electrons around the phenyl ring, resulting in a moderate electron density and three minimum points in front of the R<sub>1</sub> group, and this -CF<sub>3</sub> group also led a decrease in the electron density and minimum ESP value around the phenyl ring. We further speculated that the electron-withdrawing group at the R<sub>1</sub> position could change the electron distribution around the phenyl ring and the R<sub>1</sub> region, and this was reflected in the higher selectivity for the antibody by electrostatic interaction.

Molecules in the second group have almost identical surface charges, except for in the region of the R<sub>3</sub> group. As the electron-withdrawing effect is caused by chlorine atoms, there is a high electron density area and two ESP minima between the R<sub>1</sub> and R<sub>2</sub> groups. However, the four-carbon chain in neburon changes the electron distribution around the R<sub>3</sub> position, resulting in a lower electron density and several lower ESP maxima compared with diuron (electron density of +22.95 and the ESP maxima of +27.74 kcal/mol and +27.96 kcal/mol). This indicates that the alkyl chain could scatter the electron distribution of the R<sub>3</sub> position and increase the antibody-phenylurea binding activity.

The alkoxy group in linuron reduced the binding activity, possibly because the oxygen atom attracted electrons around it and shared the electrons around the carbonyl group, resulting in a moderate electron density and a minima (−12.57 kcal/mol) around the alkoxy group. Compared with linuron, chlorbromuron also has an alkoxy group at the R<sub>3</sub> position. However, the chlorine atom at the R<sub>2</sub> position in linuron has a stronger electron-withdrawing ability than the bromine atom at R<sub>2</sub> in chlorbromuron. Thus, chlorbromuron has a lower electron density and smaller minima in this area, and the change in the charge distribution may result in the difference in activity.

**Quantitative analysis of the molecular surface.** Weak interactions in biochemical systems can be well predicted and explained by analyzing the ESP descriptors on the molecular surface<sup>30,31</sup>. The quantitative analysis of the molecular surfaces in this study is summarized in Table S1. The Spearman correlation analysis between the molecular fragment descriptors and antibody activity (IC<sub>50</sub> values) is summarized in Table 2. We can see that the parameters – including S<sup>+</sup>, P, A, Va<sup>+</sup> and B of the R<sub>1</sub> group; B of the R<sub>2</sub> group; and S<sup>−</sup>, P, I, Va, Va<sup>−</sup> and B of the R<sub>3</sub> group – have a positive relationship with the antibody activity, while S and S<sup>−</sup> of the R<sub>1</sub> group have a negative relationship with antibody activity. Among these parameters, B of the R<sub>1</sub> group has a key relationship with the antibody activity coefficient, which is significant at the 0.01 level (2-tailed). S<sup>+</sup>, S<sup>−</sup>, P, A and Va<sup>+</sup> of the R<sub>1</sub> group also have a crucial relationship with the antibody activity coefficient. All are significant at the 0.05 level. The results showed that the antibody binding activity is linked to geometrical and electrostatic descriptors on the surfaces of the phenylurea molecules, particularly the substituent groups. Specifically, the R<sub>1</sub> and R<sub>2</sub> groups played a key role in the antibody-phenylurea binding process.

**Discussion**

In summary, ELISA analysis confirmed that the obtained antibody could recognize six structurally similar analogs. By analyzing the geometrical conformation and physicochemical properties of the optimized structures, we speculated that the antibody binding pocket could recognize slight differences in the geometric conformation of the molecules. In addition, the negative electrostatic potential of the hydrophobic -CF<sub>3</sub> group at the R<sub>3</sub> position and the hydrophobic butyl group at the R<sub>2</sub> position also contributed to the molecule-antibody binding activity. Meanwhile, the alkoxy group, which served as hydrogen-bond donor, at the R<sub>3</sub> position decreased the binding activity. Through Spearman correlation analysis between the molecular descriptors and antibody activity, we found that S<sup>−</sup>, S<sup>−</sup>, P, A, Va<sup>−</sup> and B of the R<sub>3</sub> group had key relationships with the antibody activity. The results indicated that the geometrical and electrostatic properties on the vDW surface of the R<sub>3</sub> groups, especially the R<sub>1</sub> and R<sub>3</sub> groups, played a key role in the antibody-phenylurea binding activity. This investigation demonstrated that we could explore the antibody recognition mechanism using computational chemistry techniques, despite lacking structural information of the antibody. This study also draws attention to the need to design new haptns to produce specific or broad-spectrum antibodies in the future.

| Group | Method          | S    | S<sup>+</sup> | S<sup>−</sup> | P    | I    | A    | Va   | Va<sup>+</sup> | Va<sup>−</sup> | B     |
|-------|-----------------|------|--------------|--------------|------|------|------|------|--------------|--------------|-------|
| R<sub>1</sub> | Spearman Correlation | −0.667 | 0.943** | −0.943** | 0.943** | 0.314 | 0.943** | 0.314 | 0.943** | −0.2 | 0.841* |
|       | Sig. (2-tailed)  | 0.148 | 0.005        | 0.005        | 0.005 | 0.544 | 0.005 | 0.544 | 0.005        | 0.704 | 0.036 |
| R<sub>2</sub> | Spearman Correlation | 0.143 | −0.029       | 0.086        | 0.314 | 0.174 | −0.029 | −0.143 | −0.086       | 0.086 | 0.600 |
|       | Sig. (2-tailed)  | 0.787 | 0.9572       | 0.872        | 0.544 | 0.742 | 0.957 | 0.787 | 0.872        | 0.872 | 0.208 |
| R<sub>3</sub> | Spearman Correlation | 0.257 | −0.086       | 0.671        | 0.872 | 0.714 | −0.257 | 0.754 | 0.257        | 0.621 | 0.621 |
|       | Sig. (2-tailed)  | 0.623 | 0.872        | 0.215        | 0.0539 | 0.111 | 0.623 | 0.084 | 0.623        | 0.054 | 0.188 |

Table 2. Spearman correlation analysis for molecule fragments ESP descriptors and antibody binding ability (expressed by IC<sub>50</sub> values). **Correlation is significant at 0.01 level (2-tailed). *Correlation is significant at 0.05 level (2-tailed).**
Methods

Antibody preparation. In this study, a hapten with a three-carbon spacer arm was synthesized, and then, an immunogen- and enzyme-labeled antigen was obtained by conjugating the hapten to BSA and HRP via the active ester method. The hapten-BSA conjugates were used as the immunogen to immunize two 3-month-old male New Zealand white rabbits (numbers I and II). After the initial immunization and four additional immunizations, the rabbits were sacrificed, and the sera were obtained by centrifugation. Aliquots of the sera were stored at 4 °C in 50% saturated ammonium sulfate. Subsequently, the antibodies were purified by Protein A-Sepharose 4B immunoaffinity chromatography.

Direct competitive ELISA protocol. The resulting antibodies were evaluated by a conventional direct competitive ELISA method. First, 0.25 µg/well of antibody was coated on a polystyrene 96-well plate using 100 µL/well of coating buffer (0.05 mol/L NaHCO₃, pH 9.6). After overnight incubation at 4 °C, the excess binding sites were blocked by 200 µL of a blocking solution at 37 °C for 1 h. Then, a mixture of the standard solution and hapten-HRP solution were simultaneously added to each well. After 1 h of competing reaction at 37 °C, substrates A and B were added sequentially for coloration. Finally, the enzymatic reaction was stopped by adding 2 mol/L H₂SO₄. The absorbance was determined at a primary wavelength of 450 nm, with a reference wavelength of 650 nm, using a microplate reader. After each step, 250 µL of PBST (10 mM PBS, pH 7.4, 0.05% Tween-20) was used to wash the plate. IC₅₀ and cross-reactivity (CR) values were calculated to evaluate the activity of the resulting antibody.

Calculation of the antibody binding activity. Inhibition ratios and IC₅₀ values were calculated. The inhibition ratio was defined by the following equation:

\[
\text{Inhibition ratio} = \frac{A_{\text{Control}} - A_{\text{Blank}}}{A_{\text{Inhibit}} - A_{\text{Blank}}} \times 100\%
\]

For this equation, \(A_{\text{Control}}\) represents the absorbance value of the control group and \(A_{\text{Inhibit}}\) and \(A_{\text{Blank}}\) represent the absorbance values of the inhibited group and blank group, respectively. The IC₅₀ (µg/L) value was the analyte concentration when the inhibition ratio was 50%.

Geometry optimization and molecular alignment. All structures were built in Gaussian 09 ES64L-G09RevE.01 (Gaussian, Wallingford, CT, USA) according to the phenylurea configurations in the Pubchem database. Then, DFT calculations with the B3LYP functional and 6-311++G(d,p) basis set were performed to optimize the molecules using the Gaussian 09 package. There were no imaginary frequencies for all structures, ensuring the local minimum was found. The Molecule Overlay dialog in Discovery Studio 2016 was employed in this study. This protocol generated ten pharmacophore models for each molecule, and we chose the best one for analysis.

Molecular electrostatic potential analysis. The Gaussian 09 and Gaussian View 5 packages were employed to conduct the molecular electrostatic potential (ESP) analysis of the title compounds. Minima and maxima of the ESP were generated in the Multiwfn software package and mapped onto the vdW surface.

Quantitative analysis of the molecular surface. By defining the specific chemical groups (\(R_1\), \(R_2\), and \(R_3\)) in the Multiwfn package, descriptors were calculated. Spearman correlation via SPSS software served to detect relationships between molecular descriptors of the molecular fragments of phenylurea and the recognition ability (IC₅₀) of a polyclonal antibody.

References
1. Wang, D. et al. Phenylurea herbicide sorption to biochars and agricultural soil. J Environ Sci Health B 50, 544–551 (2015).
2. Barchanska, H. et al. Atrazine, triketone herbicides, and their degradation products in sediment, soil and surface water samples in Poland. Environ Sci Pollut Res Int 24, 644–658 (2017).
3. Fingler, S. et al. Herbicide micropollutants in surface, ground and drinking waters within and near the area of Zagreb, Croatia. Environ Sci Pollut Res Int 24, 11017–11030 (2017).
4. Guo, J. et al. Occurrence of Atrazine and Related Compounds in Sediments of Upper Great Lakes. Environ Sci Technol 50, 7335–7343 (2016).
5. Wu, J. et al. Triphenylamine-based hypercrosslinked organic polymer as adsorbent for the extraction of phenylurea herbicides. J Chromatogr A 1520, 48–57 (2017).
6. Dong, X., Liang, S., Shi, Z. & Sun, H. Development of multi-residue analysis of herbicides in cereal grain by ultra-performance liquid chromatography-electrospray ionization-mass spectrometry. Food Chem 192, 432–440 (2016).
7. Sharma, P., Sablok, K., Bhalla, V. & Suri, C. R. A novel disposable electrochemical immunosensor for phenyl urea herbicide diuron. Biosens Bioelectron 26, 4209–4212 (2011).
8. Bhalla, V., Sharma, P., Pandey, S. K. & Suri, C. R. Impedimetric label-free immunodetection of phenylurea class of herbicides. Sensors and Actuators B: Chemical 171–172, 1231–1237 (2012).
9. Yuan, M. et al. Immunoassay for phenylurea herbicides: application of molecular modeling and quantitative structure-activity relationship analysis on an antigen-antibody interaction study. Anal Chem 83, 4767–4774 (2011).
10. Cui, Y. et al. Hapten synthesis and monoclonal antibody-based immunoassay development for the analysis of thidiazuron. Journal of Plant Growth Regulation 35, 357–365 (2015).
22. Zhang, Y. F., Ma, Y., Gao, Z. X. & Dai, S. G. Predicting the cross-reactivities of polycyclic aromatic hydrocarbons in ELISA by institutional affiliations.

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Author Contributions

F.Z. conducted the experiments, results analysis and manuscript writing; B.L. and G.L. conducted the instruction of experiments; Y.Z., J.W. and S.W. reviewed the manuscript.

Additional Information

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