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STRUCTURAL FEATURES OF CARROT α-TUBULIN PREDETERMINING THE NATURAL RESISTANCE TO DINITROANILINE HERBICIDES

Aim. To explain the natural resistance of *Daucus carota* L. to dinitroaniline herbicides. To clarify features of the carrot α-tubulin that may affect formation of the ligand-protein complex based on the structural and electrostatic analysis of the ligand-binding site. ***Methods***. Reconstruction of the spatial structure of α-tubulin from *D. carota* and *Toxoplasma gondii* using profile (Swiss-Model) and denovo (AlphaFold2) modeling. Molecular dynamics (MD) simulations of the built 3D-models in Gromacs. Analysis of the molecular electrostatics with PDB2PQR/APBS tools. Visualization and analysis of molecular structures in PyMOL. ***Results***. It has been shown that along with the typical positive charge of the dinitroaniline-binding pocket, all isotypes of carrot α-tubulin demonstrate negatively charged regions that may cause conflicts with the nitro-groups of the ligands. Also, the MD-stable negatively charged "bridge" between Cys316 and the aryl-fragment of Phe255 was observed in all α-tubulin isotypes. In our opinion, it not only competes with the cyclic fragment of dinitroanilines, but overall prevent the opening of the site pocket in carrot α-tubulin. ***Conclusions.*** It was clarified that natural resistance of *D. carota* to dinitroaniline herbicides may be associated with steric and electrostatic conflicts observed in in all α-tubulin isotypes. In our opinion, it prevents interaction with dinitroaniline compounds at the stage of primary site recognition on the early stages of protein-ligand complex formation.

Key words: α-tubulin, dinitroanilines, herbicides, resistance, trifluralin, microtubules.

Dinitroaniline herbicides (DH) belong to compounds whose primary mechanism of action is associated with depolymerization of microtubules (MT), and commonly known as root or mitotic disrupter herbicides [1, 2]. In general, they used as pre-emergence, surface-applied herbicides that block the anisotropic growth of plant cells [2]. DH are widely used as pre-planting and pre-emergence herbicides to control annual grasses and broadleaf weeds in a variety of crops, orchards, vineyards, lawns and around ornamental plants by selectively influencing the physiological growth processes [2]. 2,6-Dinitroanilines compounds can also be used as an alternative to colchicine to induce polyploidy in plants [3, 4]. Despite being identified as pollutants, derivatives of 2,6-dinitroanilines (e.g., trifluralin, ethalfluralin, pendimethalin, oryzalin, etc.) have low aqueous solubility, are not volatile and these compounds have low mammalian toxicity and carcinogenic potential [5].

2,6-Dinitroaniline compounds are known inhibitors of MT organization in protists and higher plants. Based on this, carrot (*Daucus carota* L.) is a notable example of natural resistance to DH. Immunofluorescence and electron microscopy showed that carrot root microtubules are unaffected by dinitroaniline treatment [6]. Dinitroaniline herbicide pendimethalin was mentioned as one of the herbicides used on carrot, however, this herbicide has actually been tested as for seed treatment at the dormant stage, and these tests confirmed that in the case of combination of pendimethalin with oxyfluorfen (diphenyl-ether herbicide) there were no visual effects on carrot seedlings [7]. At the same time, oxyfluorfen, used for broad-spectrum pre- and post-emergence control of annual broadleaf and grass weeds, caused necrotic spots on carrot leaves that were visible at 6 and 28 days after application but were no longer visible 42 days after application [7]. Differences in mechanisms of action explain the observed effect of oxyfluorfen and the lack of it in the case of pendimethalin [7]. Therefore, to date, there is no reliable information that any of the 2,6-dinitroanilines have a herbicidal effect on carrot.

The fact that dinitroanilines interact specifically with α-tubulin was demonstrated more than 20 years ago [8]. However, in the case of dinitroaniline compounds, the specification of binding site has been a subject of in-depth studies for a long time [1, 8-12]. It suggests that the resistance of carrot to DH is most likely associated with the structure and amino acid composition of the binding site, which demonstrate the ligand-induced nature of the pocket and exist in active and apo-form [12]. As a result of previous studies, certain variations in the residues forming the site pocket (a. a. within 6 Å from the ligand cluster) were revealed. At the same time, we did not find significant steric deviations between the structures of α-tubulin isotypes, when comparing the coordinates of Cα-atoms (RMSD<1) [13]. However, it should be noted that the electrostatic component of the protein-ligand interaction can also have a significant impact on primary site recognition and complex formation [12]. Therefore, the objective of current research was the analysis of electrostatic state of *D. carota* α-tubulin, and clarification of possible differences in the electrostatics and structure of the target site. We hope that the results of this study will contribute to clarification of cell molecular mechfnisms of the natural resistance of *D. carota* to 2,6-dinitroanilines.

Materials and methods

Due to the absence of experimental structures of carrot α-tubulin in the RCSB Protein Data Bank (www.rcsb.org), the objects of structural research were sequences and structural models of *D. carota* α-tubulin isotypes specified based on our preliminary revision of genomic and proteomic information [13]: UniProtKB (www.uniport.org), GenBank (www.ncbi.nlm.nih.gov/genbank/), as well as specialized genomic projects USDA ARS Carrot Genome Project and European Nucleotide Archive (https://plants.ensembl.org). Based on gene loci information (chromosome, nucleotide coordinates and direction), 8 α-tubulins were identified: TBA1 (UniProtKB: A0A162B2L8), TBA2/TBA (Q9FT36/A0A3G2K878); TBA3 (A0A162AKE6); TBA4 (A0A165YBU4), TBA5 (A0A164Y0E4), TBA6 (A0A161XEE0), TBA7 (A0A164VQB6), TBA8 (A0A164U182) [13].

Visualization, analysis, alignment of structures and site specification were processed in PyMOL V.2.5.4 (Schrödinger, LLC – www.pymol.org). The contribution of electrostatics was analyzed using the PDB2PQR/APBS server (https://server.poissonboltzmann.org/pdb2pqr) and PyMOL plugin APBS Tool2.1 [14] with pKa parameters: pH=7.0 and CHARMM ff parameterization of report file. Additional analysis of interaction was performed with BIOVIA Discovery Studio 2021 Client (https://biovia-discovery-studio-2021-client.software.informer.com/).

The analysis of the dinitroaniline-binding site region was based on the binding model, proposed for *Toxoplasma gondii* Nicolle & Manceaux [12]. Due to the ligand-induced mechanism of site formation, it was necessary to study all *T. gondii* and carrot α-tubulin isotypes in open and closed conformations. The closed (inactive) forms were constructed with AlphaFold2 (https://alphafold.ebi.ac.uk), while open conformations were constructed with Swiss-Model server (https://swissmodel.expasy.org) based on previously modeled homologous complexes as the templates. Open conformations of α-tubulins were also compared with the RCSB Protein Data Bank structure 5fnv (DOI: 10.2210/pdb5FNV/pdb) of *Gallus gallus* Linnaeus (α-tubulin-pironetin complex), which was used for prediction of the dinitroaniline site in *T. gondii* [12].

Docking of ligands was processed with CCDC GOLD Suite (CSD-Discovery Suite 2023.2 CSD Release, License Agreement N. 021554/2023/1) [28]. In total, 10 control complexes with each ligand were sampled from the stabile regions of the MD trajectory (Gromacs, >50 ns) [15]. Due to the existence of closed and open conformations of target site, the template modelling protocols varied. In order to minimize local tensions in the 3D-models and for more natural conformational states, all carrot α-tubulin models were optimized in Gromacs (MD settings: Charmm36ff, Berendsen barostat, solvent model tip3p and temperature of 310 K) [16].

Results and discussion

Earlier, based on model of *T. gondii* α-tubulin, it was revealed that the key factor in the primary recognition of the target site by dinitroanilines is the electrostatic interaction of their negatively charged nitro groups with positively charged regions of the primary protosite [12]. The presence of a similar positively charged region on the surface of plant α-tubulin site affected by dinitroanilines was confirmed by the analysis of Van der Waals interactions and the charges of the site pocket surface determined using the APBS service. However, as mentioned earlier, carrot is one of the unique plants that exhibits natural resistance to DH compounds [6]. To determine molecular mechanisms that may prevent their molecules to be placed in binding site, we performed

a similar analysis of all carrot α-tubulins. This study was performed by structural modelling and electrostatic analysis. The electrostatic profiles of the complex of trifluralin with *T. gondii* α-tubulin were used as a control.

The objects of 3D modelling and structural and biological studies were the isotypes of carrot α-tubulin reviled under the previous revision of genomic and proteomic information [13]. Since no structures and annotated sequences of *D. carota* α-tubulins are currently available, our revision included unannotated UniProtKB, related nucleotide sequences of GenBank, data of genomic projects – USDA ARS Carrot Genome Resources and European Nucleotide Archive. Based on the locus coordinates (chromosome, nucleotide coordinates and ORF direction), 8 unique α-tubulin isotypes were identified: TBA1 (UniProtKB: A0A162B2L8), TBA2/TBA (Q9FT36/A0A3G2K878); TBA3 (A0A162AKE6); TBA4 (A0A165YBU4), TBA5 (A0A164Y0E4), TBA6 (A0A161XEE0), TBA7 (A0A164VQB6), TBA8 (A0A164U182) [19]. In this study, specified α-tubulin isotypes have become the subject of an ongoing structural and biological study.

Today, it is clear that dinitroaniline complexes with α-tubulin are a very mobile and dynamic system, which is a brightly illustrate an example of ligand-protein adaptation [12, 13]. Therefore, based on results of molecular dynamics (GROMACS + Charmm36ff/TPR3) of solvated trifluralin complex with α-tubulin of *T. gondii*, we obtained 100 frames from the stabilized part of the trajectory. Sampling complexes from the different MD frames, we tested surface electrostatics of the site pocket of the stable *Toxoplasma* α-tubulin complex formed under the influence of interaction with trifluralin (Fig. 1).

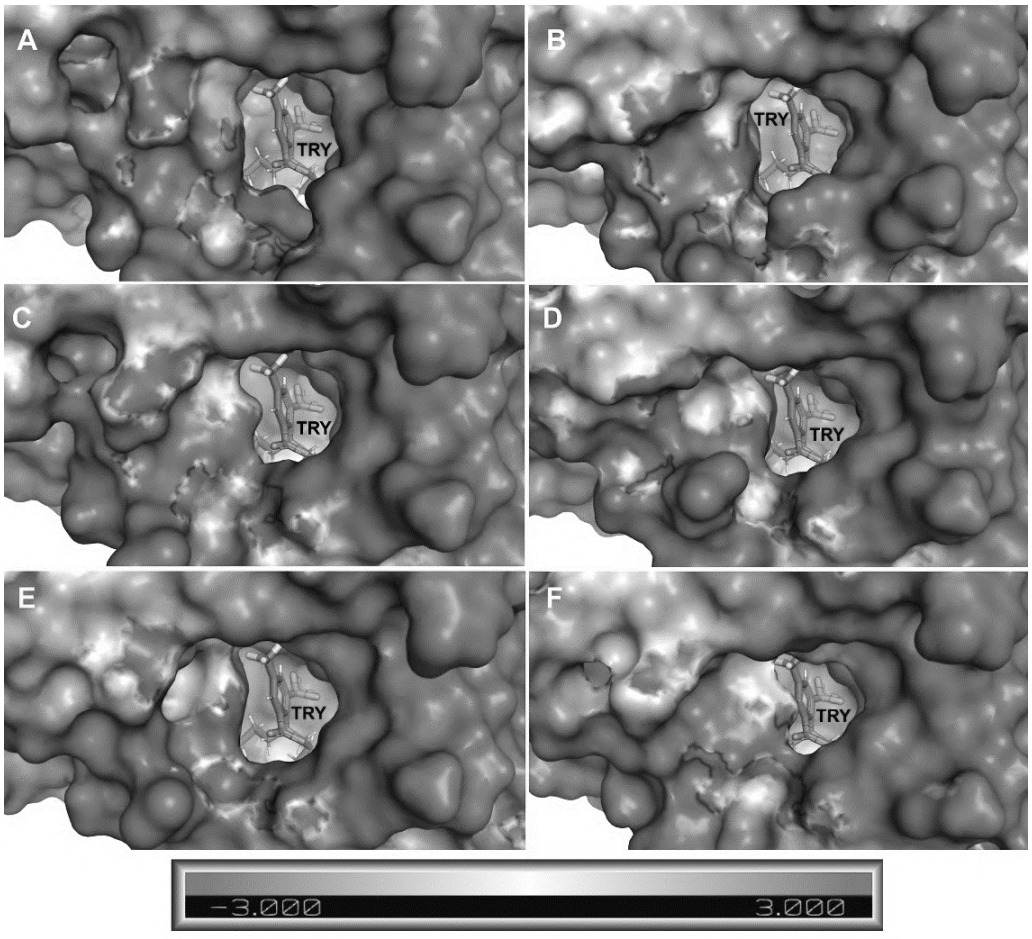

Fig. 1. Electrostatic state of the dinitroaniline-binding site pocket formed as a result of ligand-induced adaptation. The model is based on the results [18], using *T. gondii* α-tubulin as an example. A-F The electrostatic surface of the pocket of the *T. gondii* trifluralin α-tubulin control complex site on different frames of the stable molecular dynamics site of the complex, which was constructed by molecular docking in the CCDC GOLD program.

In the case of carrot α-tubulin, after 60 ns of MD, the electrostatic and structural state of the site pockets of all isotypes was analyzed (temporal analysis of MD frames), and the factors that are most likely to interfere with ligand binding were identified (Fig. 2). Thus, we identified two reasons that, in our opinion, are the key and make ligand-bind impossible. The first is the interaction of amino acid residues Cys316 and Phe255. According to the results of our modelling, these amino acids form a kind of "bridge" that physically prevent entry of the ligand into the site and pocket formation (Fig. 2 A (1), B). Another probable factor is the presence of atypical negatively charged areas in the site pocket (Fig. 2 A, 2). We believe that this causes an electrostatic conflict with the negatively charged nitro groups of dinitroaniline (trifluralin). All this is complicated by the impossibility of canonical stacking of cyclic fragments of dinitroanilines and Phe255 (Fig. 2 B).

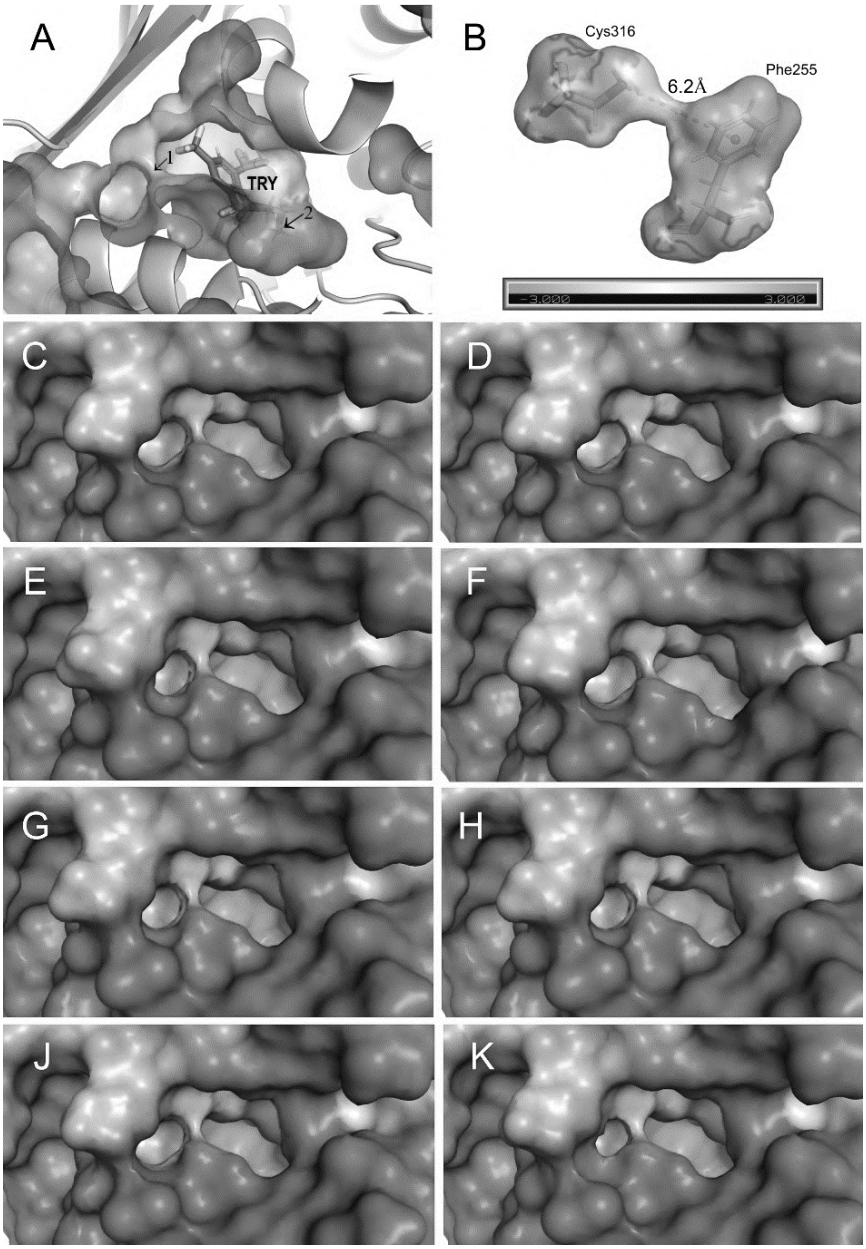

Fig. 2. Electrostatic surface of *D. carota* α-tubulin models built based on the template with open site and undergoing protein-ligand site pocket deformation provoked by fast MD optimization: A – an example of a site pocket deformation with a transferred control ligand. B – electrostatic interaction between residues Cys316 and Phe255. C-K – electrostatic surface of the site in the case of carrot α-tubulin isotypes 1-8, C-K (TBA1 – TBA8).

It is important to note that, despite the previously found variations in the amino acid composition of the target DH site in the α-tubulin isotypes of *D. carota*, the described structural conflicts are inherent to all isotypes (Fig. 2 C-K). Most likely, it is the electrostatic interaction between residues Cys316 and Phe255 and negatively charged areas in the cavity of the potential dinitroaniline binding site pocket of α-tubulin isotypes that are the key reasons of this resistance of *D. carota*. We believe that this prevents, if not the initial recognition of the protosite, then definitely complicates the process of ligand-induced pocket formation, creating obstacles for the deepening of the ligand, and formation its canonical poses in the site.

Conclusions

The analysis of the electrostatic and structural states of the potential dinitroaniline binding site region allowed us to make assumption on native resistance of *Daucus carota* L. to this class of compounds. First of all, we associate it with uncommon interaction between a. a. residues of Cys316 and Phe255, as well as the presence of a negatively charged arias in the site cavity. We believe this results in structural conflict obstructing ligand insertion into the site pocket, and also causes electrostatic conflict with negatively charged nitro-groups of dinitroanilines and disrupts canonical π-π stacking of ligands aryl group and Phe255 in all α-tubulin isotypes.

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СТРУКТУРНІ ОСОБЛИВОСТІ α-ТУБУЛІНУ МОРКВИ, ЩО ЗУМОВЛЮЮТЬ НАТИВНУ СТІЙКІСТЬ ДО ГЕРБІЦИДІВ ДИНІТРОАНІЛІНОВОГО РЯДУ

Мета. Пояснити природну стійкість моркви (*Daucus carota* L.) до гербіцидів динітроанілінового ряду. З'ясувати особливості молекул α-тубуліну моркви, які можуть впливати на формування ліганд-білкового комплексу, на підставі структурного та електростатичного аналізу сайту зв'язування. ***Методи.*** Реконструкція просторової структури α-тубуліну *D. carota* і *Toxoplasma gondii* за допомогою профільного (Swiss-Model) і de-novo (AlphaFold2) моделювання. Молекулярна динаміка (МД) побудованих структурних моделей у Gromacs. Аналіз електростатичного стану молекул за допомогою інструментів PDB2PQR / APBS. Візуалізація та аналіз молекулярних структур за допомогою програми PyMOL. ***Результати.*** Показано що поряд із характерним позитивним зарядом карману сайту зв'язування сполук динітроанілінового ряду, у всіх раніше визначених ізотипів α-тубуліну моркви існують негативно заряджені ділянки, здатні конфліктувати з окси-групами лігандів. Також, у всіх ізотипів було визначено формування негативно зарядженого «містку» між Cys316 та арильним фрагментом Phe255, який, на нашу думку, не лише конкурує з циклічним фрагментом лігандів, але й в цілому створює перепони для розкриття кишені сайту. ***Висновки.*** З'ясовано, що природна стійкість *D. carota* до дії динітроанілінових гербіцидів може бути пов'язана зі стеричними та електростатичними конфліктами, які виявляються у всіх ізотипів α-тубуліну. На нашу думку, це перешкоджає взаємодії із сполуками динітроанілінового ряду ще на етапі первинного впізнавання сайту, тобто найперших етапах потенційного утворення комплексу.

Ключові слова: α-тубулін, динітроанілінові сполуки, гербіциди, резистентність, трифлуралін, мікротрубочки.