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# DISORDERS IN DINITROANILINE SITE OPENING AS THE FACTOR, PREDETERMINING HERBICIDE RESISTANCE OF WILD CARROT

***Aim.*** To investigate the structural basis of carrot's natural resistance to dinitroaniline herbicides based on analysis of binding site volume and sequence variations in α-tubulin isotypes. ***Methods***. AI-assisted molecular modeling (Protenix, based on AlphaFold3) for protein-ligand complexes reconstruction. Binding site volume and shape analysis applying CavitOmiX. Bioinformatics comparisons including sequence alignment (ClustalX), phylogenetic analysis (MEGA11) and 3D-visualization (PyMOL, Discovery Studio). ***Results***. Structural and computational analysis of 8 α-tubulin isotypes from *Daucus carota* L. revealed significant structural and functional heterogeneity in dinitroaniline-binding regions (DBR). The binding site analysis, using Protenix demonstrated variability among carrot α-tubulin isotypes. Although TBA1, TBA5 and TBA8 showed a partially shaped binding pockets of reduced volume, the remaining isotypes (TBA2-TBA4, TBA6-TBA7) revealed its complete disruption. It suggests mechanism where some isotypes retain reduced binding potential, while others completely lack of it. ***Conclusions.*** In the frame of studies of the native resistance of carrot plants to dinitroaniline herbicides, current study indicates critical collapse in binding site pocket formation. Considering the ligand-induced nature of the dinitroaniline binding site, the disruption of the site pocket formation mechanism may be one of the main reasons for such natural resistance, making interaction with the ligand initially impossible.

*Keywords*: α-tubulin, dinitroaniline, resistance, oryzalin, herbicides, binding site volume.

The wild carrot (*Daucus carota* L.) is the most well-known plant with natural resistance to dinitroaniline herbicides (DH). The nature of this resistance is still missing, but the most probable reason, is the features of dinitroaniline binding site in α-tubulin isotypes [1]. Dinitroaniline binding site been the subject of discussions for a long time [1–8]. It suggests that resistance of carrot to DH is most likely associated with the spatial structure of the site pocket and its amino acid (a.a.) composition. Based on current data, DH binding site demonstrate ligand-induced nature of the pocket and occur in active and apo-form [2]. Our previous studies revealed certain variations in a.a. residues forming the site pocket (a.a. in 6 Å distance from the reference ligands) in carrot α-tubulins. At the same time, we did not find significant steric deviations between positions of Cα-atoms (RMSD<1) in α-tubulin isotypes [9].

At the same time, it should be noted that electrostatic factor of the protein-ligand interaction can also have a significant impact on primary site recognition and complex formation [2]. Our previous genomic and proteomic analysis of *D. carota* has revealed at least 8 isotypes of α-tubulin differ in a.a. sequences and gene loci. The site of potential dinitroaniline-binding-like (DBL) region was also variable. It revealed isotype-similar differences in a.a. composition, probably associated with native resistance to DH [10].

At the same time, the analysis of the electrostatics and structural states of the potential dinitroaniline binding sites, recognize unusual interaction between a.a. residues of Cys316 and Phe255, and the presence of unusual negatively charged area in the site cavity. We believe it effects in structural conflicts hindering 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 [10]. Based on these features, we made the assumption that natural resistance of wild carrot to DH is most likely associated with abnormalities, arresting site pocket opening, making ligand penetration initially

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impossible. At the very least, it should impact the shape, volume and properties of the target site pocket. Also, such variations may complicate reconstruction of protein-ligand complex with artificial intelligence methods. To test this hypothesis, as the part of an ongoing study, we performed modeling of protein-ligand complexes with Protenix Server (https://protenix-server.com/add-prediction). Designed based on AlfaFold3 algorithms, Protenix reconstruct multipart molecular complexes. To detect site anomalies in potential DH-binding sites, the volume and the shape of potential binding pockets of carrot α-tubulin isotypes were explored with Catalophore™ cast method.

**Materials and methods**

The objects of structural research were sequences and structural models of *D. carota* (NCBI: txid4039) α-tubulin isotypes specified based on our preliminary revision of genomic and proteomic information: TBA1 (UniProtKB: A0A162B2L8), TBA2 / TBA (Q9FT36 / A0A3G2K878); TBA3 (A0A162AKE6); TBA4 (A0A165YBU4), TBA5 (A0A164Y0E4), TBA6 (A0A161XEE0), TBA7 (A0A164VQB6), TBA8 (A0A164U182) [9]. Complete amino acid sequences of *D. carota* α-tubulin molecules were fetched from the UniProtKB (www.uniport.org). The analysis of the dinitroaniline-binding site region, were based on the binding model, proposed by Aguayo-Ortiz R., & L. Dominiguez (2022) for *Toxoplasma gondii* [2].

RCSB Protein Data Bank (www.rcsb.org) was used for structural information and templates for structural modeling. The regions of potential binding sites were specified according to a cluster of reference ligands, fetched from 20 frames of stable MD region. Clasterisation of binding sites pockets was processed based on the grope of amino acids, specified according to a 4 Å distance from the reference [11]. Sequence alignments were processed in ClustalX v. 2.1 (www.clustal.org). Visualization and analysis of the NJ-tree were performed in MEGA12 (https://www.megasoftware.net/).

For structural modeling of proteins and protein-ligand complexes, we processed with Protenix Server (https://protenix-server.com/), assembled on AlfaFold3 algorithms (https://deepmind.google/; https://alphafoldserver.com/). The models of protein-ligand complexes were selected based on the best scores of AI-reconstructions.

Visualization and analysis of molecular structures were performed using PyMOL v.3.1.3 software (Schrödinger LLC, www.pymol.org). The CavitOmiX plugin (https://innophore.com/cavitomix/), installed in PyMOL was used for analysis of shapes and volumes of the binding sites. Data processing and visualization was performed in MS Excel.

**Results and Discussion**

To predict toxoplasma and wild carrot α-tubulin-oryzalin complexes, the methods of structural reconstructions with artificial-intelligence were applied. In particular, the 3D-structures of protein-ligand complexes, we reconstructed with Protenix Server. Initially, the efficiency of this tool was tested using sets of PoseBusters V2 (https://catalog.ngc.nvidia.com/), the test based on PDB structures with low homology, and the set of CASP15 for RNA (https://predictioncenter.org/casp15/). At the time of application, the tool was trained using structures from the RCSB Protein Data Bank, as well as AI-structures previously predicted with AlphaFold2 (https://alphafold.ebi.ac.uk/) and OpenFold (https://openfold.io/). Especially the originators of Protenix pay attention to the tasks of complex predictions of protein-ligand interactions (https://protenix-server.com/). The tests demonstrate that even in the absence of similar structures, the trained AI model is able to make predictions with a high level of reliability. Thanks to the inbuilt ranking, the program successfully selects the top hypotheses of the complex and rank them according to the model quality scores (pTM, ipTM, etc.) (ByteDance AML AI4Science Team, et al, 2025).

Reconstructing α-tubulin-oryzalin complexes, we built models for *T. gondii* (TBA-ORY) as well as complexes for 8 α-tubulin isotypes (TBA1-8) from *D. carota*, selected based on our previous results [9]. The accuracy of AI-prediction was confirmed based on *T. gondii* α-tubulin-oryzalin complex, comparing results of AI-model with final frames of previously processed 200 ns of molecular dynamics simulations in Gromacs. It was shown that, in opposite to control, AI-reconstructions for carrot α-tubulins demonstrate missing of protein-ligand complexes for TBA2, TBA3, TBA4, TBA6 and TBA7, or complexes with abnormal site deformations (TBA1, TBA5 and TBA8).

In order to estimate the defects in formation of binding site pocket of wild carrot α-tubulin isotypes, its shapes and volumes were analyzed with CavitOmiX plugin, filling the pockets with pseudoatom-based 3D point-clouds of catalophores (Fig. 1). Since there is an equal distance between discrete points, the number of catalophores correlate with the volume of the site. At the same time, the protein-ligand complex, constructed for *T. gondii*, was used as control. It was found that in the case of *T. gondii*, the site has twice volume pocket in opposite to the best value for carrot (TBA5). Significantly worse indicators were in the cases of TBA1 and TBA8, while in other isotypes TBA2, TBA3, TBA4, TBA6 and TBA7, the site was closed (Fig. 1, Fig. 2).

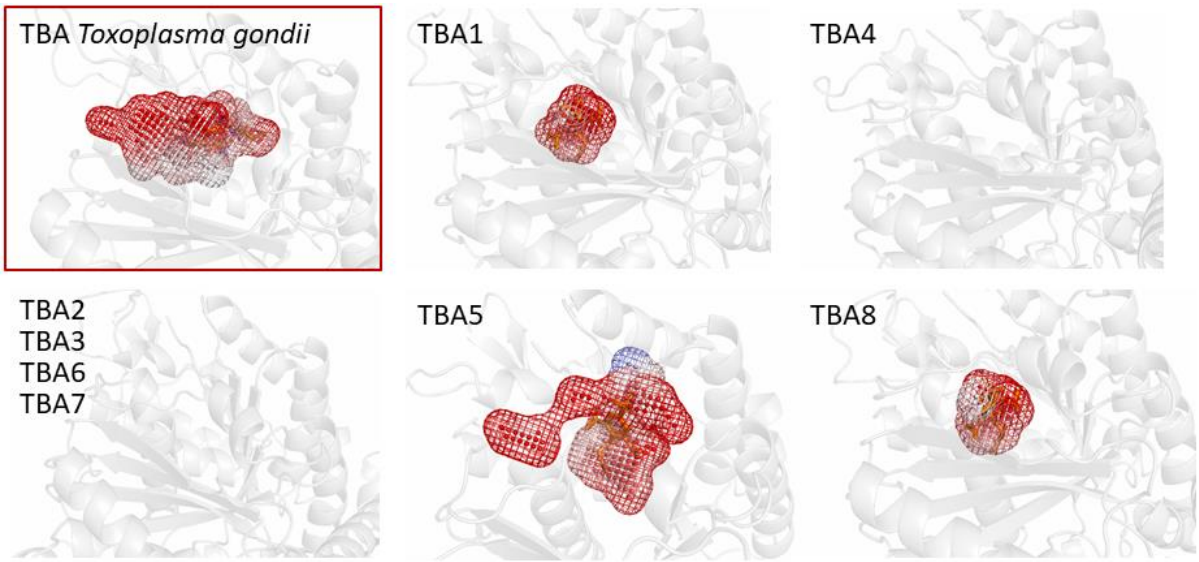


Fig. 1. The CavitOmiX (Catalophore™) analysis identify anomalies in pockets casts of potential dinitroaniline herbicides binding sites of wild carrot α-tubulin isotypes TBA1, 5 and 8. The formation of pockets in TBA2, 3, 4, 6 and 7 was missed. The structure of *T. gondii* α-tubulin (TBA) in complex with oryzalin (ORY) was used as control of AI-modelling and pocket casting.

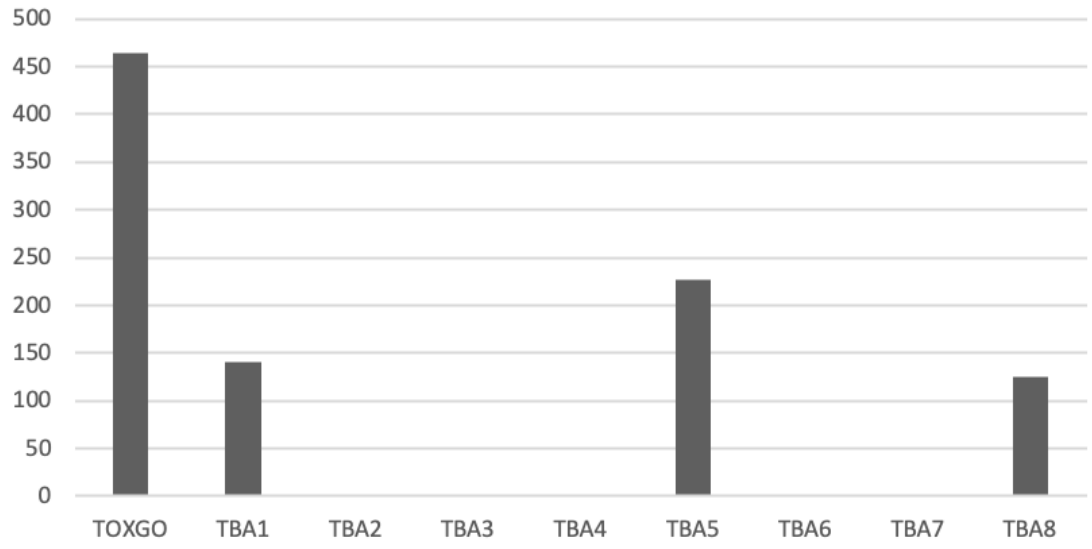


Fig. 2. Differences in volume of potential dinitroaniline herbicides binding site, based on the number of CavitOmiX catalophores in 3D point-clouds filling out the cavity of the site.

Trying to understand the molecular nature of differences in predicted capabilities to form protein-ligand complexes among carrot α-tubulin isotypes, we analyzed sequence variations within the binding site regions. The sequence alignment, revealed that the binding pocket of carrot isotypes mainly possess inner conservativism, excepting some notable a.a. variations in «pseudo» herbicide-binding group: TBA1, TBA5 and TBA8 (Fig. 3A). The phylogenetic clustering has also provided evidence of site differentiation among carrot isotypes. The clustering of binding sites, demonstrate groping of isotypes with partially opened pockets (TBA1, TBA5, TBA8) and closed «non-binding» isotypes (TBA2-TBA4, TBA6-TBA7) (Fig. 3B).

This finding suggests that some isotypes potentially retain minor binding capacity while others completely lack it. Such reduced ability to form correct site pocket is completely reliable with the structural features of the site that we identified earlier. In particular, the stable electrostatic interaction between residues Cys316 and Phe255 [9]. In our opinion, this interaction, it not only competes with the cyclic part of dinitroanilines, but also prevent the opening of the site pocket in all isotypes of carrot α-tubulin.

## Conclusions

It was shown that, in opposite to control (α-tubulin from *T. gondii*), AI-reconstructions of wild carrot α-tubulin isotypes in complexes with oryzalin, demonstrate missing of complexes formation (α-tubulin: TBA2, TBA3, TBA4, TBA6 and TBA7), or abnormal protein-ligand complexes with site deformations (TBA1, TBA5 and TBA8). At the same time, even if a model of the complex was formed, the binding site pocket was considerably reduced in the volume. As a consequence, we believe, that native resistance of *D. carota* to dinitroaniline herbicides is most likely associated with inability of last ones to interact with all existing isotypes of α-tubulin, due to the initial inability of the site's pocket to open.

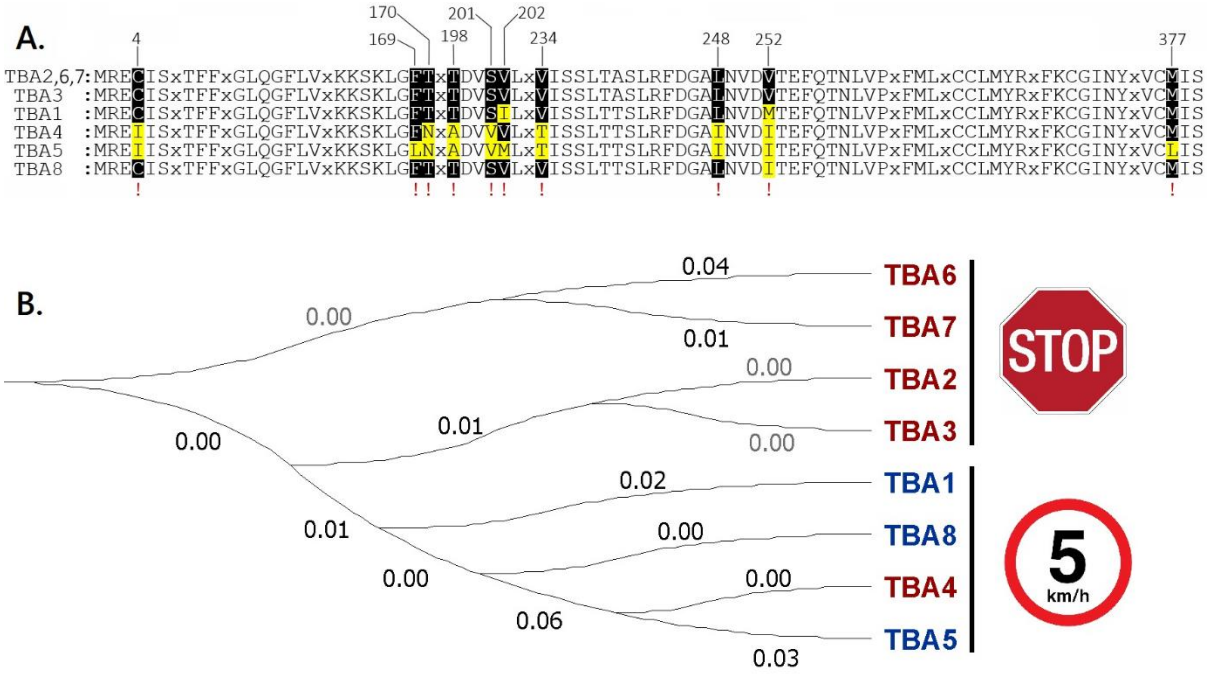


Fig. 3. Amino acid variability in dinitroaniline binding region of *D. carota* α-tubulin isotypes. Wild carrot α-tubulin isotypes that are unable to form the site pocket are presented in red. Tubulin isotypes that formed abnormal site pockets with signs of deformation are represented in blue.

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## ПОРУШЕННЯ РОЗКРИТТЯ САЙТУ ЗВ'ЯЗУВАННЯ ДИНІТРОАНІЛІНІВ ЯК ФАКТОР СТІЙКОСТІ РОСЛИН ДИКОЇ МОРКВИ ДО ГЕРБІЦИДІВ

***Мета.*** Дослідити структурну природу нативної стійкості рослин моркви до дії динітроанілінових гербіцидів на основі аналізу варіацій об'єму кишень сайтів зв'язування та амінокислотного складу послідовностей ізотипів α-тубуліну. ***Методи.*** Реконструкція ліганднх-білок комплексів за допомогою методів штучного інтелекту (Protenix, на основі AlphaFold3). Аналіз об'ємів та форм сайтів ліганд-білкової взаємодії із застосуванням плагіну CavitOmiX. Вирівнювання послідовностей (ClustalX), філогенетичний аналіз (MEGA11) та візуалізація просторових структур молекул (PyMOL, Discovery Studio). ***Результати.*** Аналіз 8 ізотипів α-тубуліну *Daucus carota* L. виявив значну структурну та функціональну гетерогенність ділянок потенційних сайтів зв'язування сполук динітроанілінового ряду (DBR). Реконструкція структур комплексів за допомогою сервісу Protenix показала варіабельність кишень цільового сайту на рівні ізотипів α-тубуліну моркви. Незважаючи на те, що ізотипи TBA1, TBA5 і TBA8 показали часткове розкриття кишень сайту, їх об'єм виявляється зменшеним, а решта ізотипів (TBA2-TBA4, TBA6-TBA7) взагалі виявились нездатними формувати комплекси з оризаліном. Це дозволяє припустити що, саме це є однією з причин того, що молекули α-тубуліну моркви або значно зменшили потенціал взаємодії з динітроанілінами, або повністю його втратили. ***Висновки.*** У рамках досліджень природної стійкості рослин моркви до динітроанілінів, результати актуального дослідження вказують на суттєві порушення у механізмах формування кишені цільового сайту. Враховуючи ліганд-індуковану природу зазначеного сайту, такі порушення виключають можливість взаємодії ще на стадії формування кишені сайту, що є однією з найбільш вірогідних причин нативної стійкості рослин *D. carota* до похідних динітроанілінового ряду.

*Ключові слова*: α-тубулін, динітроаніліни, резистентність, оризалін, гербіциди, об'єм сайту зв'язування.