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MOLECULAR DOCKING DOES NOT PREDICT THERAPEUTIC VIABILITY: A CASE STUDY OF ARISTOLOCHIC ACIDS IN UROTHELIAL CANCER TARGETS

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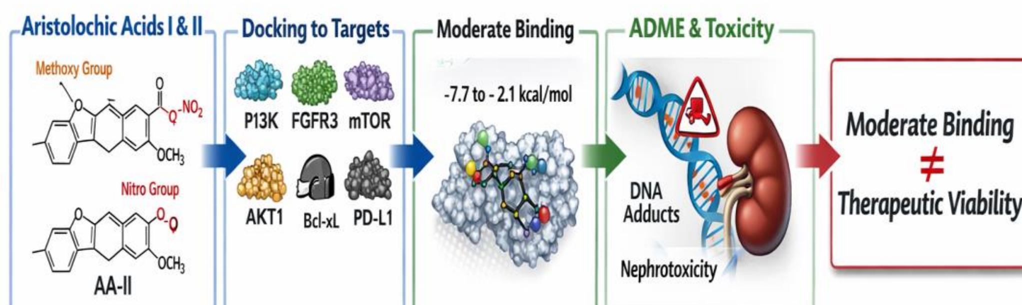
ABSTRACT

Aristolochic acids I (AA-I) and II (AA-II), bioactive constituents of *Aristolochia indica*, exhibit cytotoxic activity but are also well-established nephrotoxins and human carcinogens. This study evaluated their molecular interaction profiles with key cancer-associated proteins and assessed their therapeutic relevance using an integrated computational framework. Molecular docking was performed using the Glide Extra Precision (XP) protocol against multiple oncogenic targets, including PD-L1, FGFR3, PI3Kα, AKT1, mTOR, and Bcl-xL. Comparative analysis was conducted for all targets for which paired docking outputs were available. In parallel, in silico ADME and toxicological assessments were performed to contextualize docking outcomes. Both compounds demonstrated moderate to weak binding affinities across most targets, with the strongest interaction observed for AA-I against PI3Kα (−7.757 kcal/mol). However, the predicted binding modes lacked key structural features required for selective inhibition, including canonical hinge-binding interactions in kinase targets and appropriate interface complementarity for protein–protein interaction disruption. ADME evaluation indicated unfavorable pharmacokinetic properties, including low predicted gastrointestinal absorption, high plasma protein binding, and preferential renal accumulation. Importantly, both compounds undergo metabolic activation to form DNA-reactive intermediates, leading to persistent DNA adduct formation and mutational signatures associated with urothelial carcinogenesis.

Overall, these findings demonstrate that moderate docking scores do not translate into therapeutic viability. The biological activity of aristolochic acids is dominated by genotoxicity rather than selective protein inhibition, rendering them unsuitable as anticancer agents in their native form.

GRAPHICAL ABSTRACT



KEYWORDS: Aristolochic acids; molecular docking; urothelial carcinoma; PI3K/AKT/mTOR; ADME; genotoxicity; in silico analysis; toxicology.

1. INTRODUCTION

Cancer is a multifactorial disease characterized by genetic and epigenetic alterations that drive uncontrolled cell proliferation, resistance to apoptosis, and metastatic progression.[2] Despite advances in targeted therapy and immunotherapy, treatment outcomes remain limited by drug resistance, tumor heterogeneity, and dose-limiting toxicities.[1,2] Natural products remain an important source of bioactive molecules in anticancer drug discovery because of their structural diversity and capacity to interact with complex biological targets.[3,4] Among such compounds, aristolochic acids I (AA-I) and II (AA-II), derived from *Aristolochia indica*, have attracted attention due to their pronounced cytotoxic effects observed in experimental systems. However, their pharmacological profile is highly controversial because they are also recognized as potent nephrotoxins and established human carcinogens.[10,13] AA-I and AA-II are nitrophenanthrene carboxylic derivatives that differ structurally by the presence of a methoxy group in AA-I, rendering it relatively more lipophilic than AA-II. Both compounds possess a rigid, planar aromatic core with nitro and carboxyl functional groups that support hydrophobic interactions and π–π stacking within protein binding pockets. At the same time, the nitro moiety contributes directly to metabolic activation and DNA adduct formation, which underlies both their observed cytotoxicity and their well-

established genotoxic and carcinogenic properties.[13,15] Mechanistically, aristolochic acids undergo metabolic activation to form highly reactive intermediates that covalently bind to DNA, generating persistent aristolactam–DNA adducts and characteristic mutational signatures.[14,15] These lesions are strongly linked to urothelial carcinogenesis, particularly upper tract urothelial carcinoma.[10,11] In addition, aristolochic acids exhibit a high degree of renal accumulation, especially in proximal tubular epithelial cells, leading to progressive interstitial fibrosis and irreversible kidney damage.[12] This combination of genotoxicity and organ-specific toxicity critically limits their therapeutic applicability and necessitates cautious interpretation of any apparent bioactivity.

Urothelial carcinoma is a malignancy arising from the epithelial lining of the urinary tract and is strongly associated with exposure to carcinogens that induce DNA damage.[16,17] At the molecular level, urothelial carcinoma is driven by dysregulation of pathways involved in cell proliferation, survival, immune escape, and apoptosis. Alterations in the PI3K/AKT/mTOR pathway promote uncontrolled growth and resistance to apoptosis, FGFR3 mutations are frequently observed in early-stage disease, anti-apoptotic proteins such as Bcl-xL support tumor cell survival, and immune checkpoint proteins such as PD-L1 facilitate immune evasion.[18,19] To evaluate the interaction profile of aristolochic acids in a urothelial carcinoma-relevant context, six protein targets were selected based on their established roles in tumor progression, survival, immune evasion, and apoptosis regulation. PD-L1 was included because immune checkpoint signaling is a major mechanism of immune escape in advanced urothelial carcinoma. FGFR3 was selected because activating alterations in this receptor are among the most characteristic molecular events in urothelial carcinoma, particularly in non-muscle-invasive disease. PI3Kα, AKT1, and mTOR were chosen as components of the PI3K/AKT/mTOR signaling axis, which is frequently dysregulated in urothelial carcinoma.[18,19] Bcl-xL was included as a representative anti-apoptotic protein because it contributes directly to tumor cell survival and treatment resistance by suppressing mitochondrial apoptosis.

TP53 remains mechanistically important in aristolochic acid-associated urothelial carcinogenesis because AA-induced DNA adducts generate characteristic mutational signatures.[14] However, TP53 was not included in the docking panel because of the absence of a suitable small-molecule binding pocket. HER2 was also considered biologically relevant due to pathway overlap with proliferative signaling networks, but it was not included in the

final docking panel to avoid redundancy with the PI3K/AKT/mTOR-centered target set. A major limitation of docking-based studies is the frequent disconnect between predicted binding affinity and actual biological activity.[5,7] Therefore, the present study aimed to investigate the interaction profile of aristolochic acids I and II with selected cancer-associated protein targets using molecular docking, while critically integrating pharmacokinetic and toxicological considerations to evaluate their therapeutic viability.

2. MATERIALS AND METHODS

2.1. Protein Preparation

Crystal structures of target proteins were obtained from the Protein Data Bank [8]. The selected structures were PD-L1 (5J89), FGFR3 (4K33), PI3Kα (4L23), AKT1 (3OCB), mTOR (4DRN), and Bcl-xL (2ACO). Protein preparation was performed using the Protein Preparation Wizard in the Schrödinger Suite. Preparation steps included removal of co-crystallized ligands and non-essential water molecules, addition of hydrogen atoms, assignment of bond orders, and optimization of protonation states at physiological pH (~7.4). Energy minimization was performed using the OPLS3e force field, with heavy atoms restrained to an RMSD of approximately 0.3 Å to preserve the crystallographic backbone. Where available, multiple conformational states were evaluated to partially account for protein flexibility.

2.2. Ligand Preparation

Aristolochic Acid I (AA-I) and Aristolochic Acid II (AA-II) were prepared using the LigPrep module. Structures were converted into optimized three-dimensional conformations with correct stereochemistry and bond orders. Ionization states were generated at physiological pH (7.4 ± 0.5), and energy minimization was performed using the OPLS3e force field.

2.3. Receptor Grid Generation

Receptor grids were generated using the Glide Grid Generation module. The grid center was defined based on the centroid of the co-crystallized ligand or known active-site residues. The inner grid box was set to 10 Å and the outer grid box to 20 Å.

2.4. Docking Protocol

Docking simulations were performed using Glide in Extra Precision (XP) mode to improve pose discrimination and reduce false-positive predictions.[9] AA-I and AA-II were docked independently into each receptor grid. Multiple ligand poses were generated and ranked using

the Glide XP GScore, where more negative values indicate stronger predicted binding affinity. Post-docking minimization was applied to refine ligand geometry within the binding pocket.

2.5. Post-Docking Analysis

Docked complexes were analyzed using PyMOL and LigPlot+ to evaluate binding orientations and interaction profiles. Hydrogen bonding, hydrophobic interactions, and π–π interactions were examined. Docking results were interpreted with consideration of methodological limitations, including rigid receptor approximation and lack of explicit solvent modelling.[5,7]

3. RESULTS AND DISCUSSION

3.1. Docking Analysis

Molecular docking of aristolochic acids I (AA-I) and II (AA-II) was performed against six urothelial carcinoma-associated protein targets: PI3Kα (4L23), FGFR3 (4K33), mTOR (4DRN), AKT1 (3OCB), Bcl-xL (2ACO), and PD-L1 (5J89). Both compounds exhibited moderate to weak binding affinities across all targets, with docking scores ranging from approximately −7.757 to −2.190 kcal/mol. No target demonstrated strong or selective binding indicative of high-affinity inhibition.[5,7]

3.2. Docking Scores

Protein (PDB ID)	Target	AA-I Docking Score	AA-II Docking Score
2ACO	Bcl-xL	-7.507	-6.775
3OCB	AKT1	-2.954	-2.995
4DRN	mTOR	-5.178	-5.364
4K33	FGFR3	-5.979	-6.803
4L23	PI3Kα	-7.757	-6.530
5J89	PD-L1	-2.190	Not available*

*AA-II docking output for PD-L1 was not available in the exported dataset.

3.3. Target-Specific Interpretation

3.3.1. PI3Kα (4L23)

AA-I demonstrated the strongest interaction observed in the study (−7.757 kcal/mol), followed by AA-II (−6.530 kcal/mol). The ligand occupied the ATP-binding pocket; however, aristolochic acids lack canonical hinge-binding motifs required for stable ATP-competitive kinase inhibition. Thus, the interaction is more consistent with geometric accommodation within the pocket than with functionally meaningful kinase inhibition. For

comparison, clinically relevant PI3K inhibitors typically exhibit docking scores in the range of −9 to −11 kcal/mol, indicating that the binding affinity of aristolochic acids is comparatively weak.[18]

3.3.2. FGFR3 (4K33)

AA-II showed relatively improved binding toward FGFR3 (−6.803 kcal/mol) compared with AA-I (−5.979 kcal/mol). Although this suggests somewhat better compatibility with the FGFR3 kinase domain, the predicted affinities remain modest relative to clinically validated FGFR inhibitors. Functional inhibition therefore cannot be inferred from the docking scores alone.

3.3.3. mTOR (4DRN) and AKT1 (3OCB)

Both ligands showed weak binding toward mTOR (AA-I: −5.178 kcal/mol; AA-II: −5.364 kcal/mol) and very weak binding toward AKT1 (AA-I: −2.954 kcal/mol; AA-II: −2.995 kcal/mol). The absence of strong interaction across multiple nodes of the PI3K/AKT/mTOR pathway argues against coordinated pathway inhibition.[18,19]

3.3.4. Bcl-xL (2ACO)

AA-I demonstrated moderate binding within the hydrophobic groove of Bcl-xL (−7.507 kcal/mol), whereas AA-II showed somewhat weaker interaction (−6.775 kcal/mol). However, effective inhibition of Bcl-xL requires precise BH3-mimetic interactions, which are not supported by the rigid polyaromatic structure of aristolochic acids.

3.3.5. PD-L1 (5J89)

AA-I exhibited poor predicted binding toward PD-L1 (−2.190 kcal/mol), indicating minimal compatibility with the protein–protein interaction interface. The corresponding AA-II docking value was not available in the exported dataset; therefore, no comparative interpretation was made for this target. The low AA-I score is consistent with the structural requirement for larger, more flexible scaffolds for effective PD-L1 inhibition.

3.4. Comparative Analysis of AA-I and AA-II

AA-I generally showed slightly stronger binding than AA-II, likely due to increased lipophilicity and hydrophobic surface area. However, the differences between the two compounds were modest, approximately 0.5–1.2 kcal/mol across targets, and fall within the uncertainty range of docking scoring functions. Accordingly, the present results do not

support any meaningful selectivity or potency difference between AA-I and AA-II. The progressive reduction in binding affinity across targets further indicates a lack of coordinated multi-target engagement and supports the interpretation of non-selective binding behavior.

3.5. Structural and Mechanistic Interpretation

Aristolochic acids are rigid, planar nitrophenanthrene derivatives that favor non-specific hydrophobic interactions within protein binding pockets. The observed docking profile is consistent with lack of hinge-binding features in kinase targets, limited hydrogen-bonding diversity, and poor compatibility with broad protein–protein interfaces. Thus, the predicted binding behavior is best interpreted as non-specific accommodation rather than targeted inhibition.

3.6. ADME and Toxicological Evaluation

Because docking scores alone do not establish therapeutic utility, in silico ADME and toxicological evaluation was used to contextualize the interaction profile of aristolochic acids I and II. Both compounds show physicochemical features associated with poor drug behavior, including elevated polarity, limited passive membrane permeability, low predicted gastrointestinal absorption, high plasma protein binding, and poor blood–brain barrier penetration.[20,21] A central limitation of both aristolochic acids is the presence of a nitro-aromatic toxicophore and their metabolic activation through nitroreduction, which generates reactive intermediates that form covalent aristolactam–DNA adducts.[14,15] Thus, their dominant biological effect is genotoxicity rather than selective inhibition of oncogenic protein targets.[10–13] Comparatively, AA-I is slightly more lipophilic and has been more extensively associated with strong DNA adduct formation, whereas AA-II is somewhat more polar but retains pronounced genotoxic, nephrotoxic, and carcinogenic liabilities. Therefore, neither compound is suitable as an anticancer scaffold in its native form.

3.7. Pathway-Level Interpretation

At the pathway level, the observed interactions do not support coordinated multi-target modulation. For the PI3K–AKT–mTOR axis, the relatively stronger interaction of AA-I with PI3Kα is offset by weak engagement of AKT1 and mTOR, arguing against coherent pathway inhibition. For FGFR3, the moderate affinity of AA-II remains insufficient to support meaningful kinase inhibition. Similarly, the Bcl-xL interaction does not indicate BH3-mimetic activity, and the poor PD-L1 score reflects structural incompatibility with protein–protein interaction interfaces.

3.8. Mechanistic Implications

The overall interaction profile reflects molecular promiscuity typical of planar polyaromatic compounds rather than rational multi-target engagement. More importantly, the biological behavior of aristolochic acids is dominated by covalent DNA interaction following metabolic activation, which overrides any predicted non-covalent protein-binding interactions.[14,15]

3.9. Key Insight

The present findings demonstrate that moderate docking affinity does not equate to therapeutic potential, particularly for compounds with inherent toxicological liabilities.

4. LIMITATIONS

Molecular docking predicts static ligand–protein interactions and does not account for protein flexibility, solvent effects, binding kinetics, or intracellular pharmacokinetics. Consequently, docking scores represent approximate estimates of binding affinity rather than definitive evidence of biological activity.[5,7] An additional limitation of this study is the intrinsic toxicological profile of aristolochic acids. Their metabolic activation to DNA-reactive intermediates results in persistent DNA adduct formation and mutagenesis, fundamentally limiting their relevance as drug-like molecules regardless of predicted binding interactions[14,15] Interpretation of these results must therefore remain within a toxicological rather than therapeutic framework.

5. FUTURE PERSPECTIVES

Future investigations should focus on structural modification of the aristolochic acid scaffold to eliminate genotoxic functional groups, particularly the nitro moiety responsible for metabolic activation. However, such modifications are likely to alter electronic structure and binding behavior, necessitating systematic structure–activity relationship studies. Advanced computational approaches, including molecular dynamics simulations and free energy calculations such as MM-GBSA, may provide improved insight into binding stability and energetics.[5,7] Ultimately, experimental validation using structurally modified, non-genotoxic analogues will be essential to determine whether selective target engagement can be achieved independently of DNA-reactive toxicity.

6. CONCLUSIONS

The present study evaluated the molecular interaction profiles of aristolochic acid I (AA-I) and aristolochic acid II (AA-II) against six cancer-associated protein targets using molecular

docking. Both compounds demonstrated moderate to weak binding across all targets, with AA-I showing relatively stronger interactions with PI3Kα and Bcl-xL, and AA-II exhibiting slightly improved affinity toward FGFR3. However, these differences were minor and fell within the uncertainty range of docking scoring functions, indicating no meaningful selectivity or potency.

The observed interaction patterns are consistent with non-specific accommodation within hydrophobic binding pockets rather than selective inhibition. Critically, the biological activity of aristolochic acids is dominated by their well-established genotoxic mechanism, which overrides any predicted protein-binding interactions[10,13] Therefore, despite measurable in silico binding, aristolochic acids cannot be considered viable anticancer agents in their native form.

This study demonstrates the critical disconnect between docking-derived binding affinity and true pharmacological suitability, emphasizing the need to integrate molecular docking with pharmacokinetic and toxicological evaluation in early-stage drug discovery.

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