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Predicting the possibility of two newly isolated phenetheren ring containing compounds from *Aristolochia manshuriensis* as CDK2 inhibitors

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Abstract:
Aristolochia manshuriensis has been used for centuries in Chinese medicinal system for their versatile medicinal uses. Recent studies have revealed two new aristolactames (compound A and B) with γ-lactame ring fused with the phenentherene ring as potent inhibitors of human Cycline Dependent Kinase2 (CDK2). Studies on aristolactames and related compounds claim for their CDK2 inhibition without delineating the involved mechanism and structural basis of interaction. Molecular structural model was used to we propose a structural basis of CDK2 inhibition. We showed that these compounds (A and B) can successfully dock into the inhibitor binding pockets of human CDK2. Predicted binding affinities are comparable to known inhibitors of CDK2. Results were in agreement with the earlier biochemical studies. Hence, suggest that studied compounds A and B can be a promising scaffold for rational design of novel and potential drugs against cancer.

Keywords: Aristolactame, Cycline Dependent Kinase2, Docking, AutoDock, Molecular docking Server

Background:
Aristolochia species have been used from ancient time during child birth and as a cure for snake bites **[1]**. Extracts Aristolochia plants are used in the traditional medicine of in many countries, such as China, Turkey, India and Argentina **[1-3].** In recent findings, compounds from Aristolochia manshuriensis has been reported as PIK1 **[4]** inhibitor and CDK2 (Cyclin-Dependent Kinase 2) inhibitors **[5]**. Aristolactams, a group of phenanthrene lactam alkaloids, is claimed to be responsible for most of the bioactivities *Aristolochia manshuriensis*. Structurally and biologically aristolactams are related to aporphines **[4]**. Phenanthrene lactams are regarded as the principal products of aristolochic acids detoxification metabolism **[6]**. Knowledge about the mode of action of aristolactams is somewhat nebulous; however, a recent opinion explains that cytochrome P-450 induced reduction pathway is involved in to aristolochic acid metabolism, while peroxidase involved in to the formation of a cyclic N-acylnitrenium ion embedded in an aristolactam unit with delocalized positive charge **[7]**. It is believed that this ionic species binds prefer to bind the exocyclic amino groups of purine nucleotides by the carbon atom at ortho position to the lactam nitrogen **[8]**. In the present study we considered on two aristolactames recently isolated from *Aristolochia manshuriensis*- a Chinese medicinal herb and considered as inhibito of CDK2. Molecular formulas of two compounds are $C_{16}H_{11}NO_4$ and $C22O_9H_{19}N$ (A and B respectively) **(Figure 1)**. Both compounds are composed of phenanthrene ring skeleton fused with a γ-lactame ring. Compound A has a hydroxy group (-OH) at C3 and C6 position and methoxy group (-OCH3) at C4 position **[9]**. This compound is closely related to Aristolactam AIIIa. Compound B is glycoside analog of compound A that is, a hexose sugar moiety has a glycosidic linkage at C8 position of phenanthrene ring. Dissimilar to compound A, position C3 and C4 of phenetherene ring is occupied by a dioxol (3, 4-dioxol) moiety in compound B. Compounds A and B were found to be

active in the CDK2 enzyme inhibition assay, with IC50values of 140 nM and >10 μM, respectively interestingly, compound B showed more selectivity against other kinases like CDK4, aurora-2 kinase and MAP-kinase **[9]**.

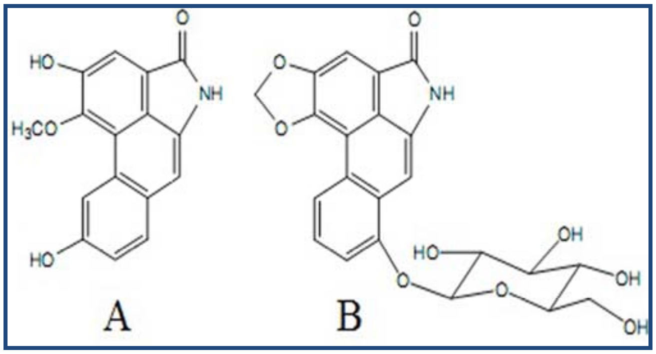

Figure 1: Proposed Structure of compound 1 and 2 isolated from *Aristolochia manshuriensis*.

CDK2 (cyclin-dependent kinase 2) is a member of cyclin-dependent kinase family of serine/threonine kinases, this is believed to be regulating cell-cycle progression. Since, CDK2/cyclin E complex phosphorylates and inactivate retinoblastoma tumor suppressor protein (RB), in turn transcription factor E2F get activated, hence during G1/S transition phase CDK2 is involved in to initiation of DNA synthesis **[10, 11]**. During S-phase CDK2/cyclin-A complex prevents unscheduled E2F activation and cell cycle passes this phase without interruption **[12]**.The exact role of CDK2 during the S phase of cell cycle is not well defined. However, due to its rate-limiting role the cell cycle, it has been regarded as a potential target for cancer therapy. This makes it an attractive target for antitumor drug discovery and drug design. Due to increasing attention of scientists toward CDK2 as a potential target for cancer therapy, a number of inhibiters had been studied. Few of the inhibitors are roscovitine, olomoucine, purvalanols, staurosporine, hymenialdisine, paullones and indirubin etc. All the inhibiters have same mode of action, competitive inhibitor of ATP as they bind kinase binding site of CDK2 **[13]**. Extensive X-ray crystallographic studies on complexes of the above listed inhibiters had elucidated the key features of interaction responsible for their affinity toward CDK2 protein. In this present *in silico* study we selected two newly isolated arisolactams from *Aristolochia manshuriensis*, which have been claimed to be a potent inhibitor of CDK2 and probably a lead for the development of anticancer drugs **[9]**. We studied and proposed structural basis of interaction of these two molecules with binding site of olomoucine on CDK2 protein (PDB ID 1W0X).

Methodology:

Softwares and data source

Symyx Draw 4.0 **[14]** and Dundee PRODRG Server **[15]** used in this study are freely available for academic use. Molecular docking server **[16]** was used on paid subscription. The pipeline software for server built on several world-leading applications in the field of molecular modeling. Autodock (http://autodock.scripps.edu) **[17]**, the most popular molecular docking program is used for molecular docking calculations. Chemaxon tools (www.chemaxon.com) are used for small molecule visualization and processing. MOPAC2009 (http://openmopac.net) and the revolutionary PM6 semi empirical method can be used to calculate small molecule geometries and electric properties. A detail about the working methodology can be retrieved from server from an URL http://www.dockingserver.com/web/gettingstarted/#featurs. **(Table 1, See supplementary material)** lists the software and servers used. PDB files of CDK2 protein was obtained from Protein Data Bank (http://www.pdb.org).

Protein Files Preparation

In our previous studies **[18]** we used Auto Dock Tools (ADT) to remove the added waters, add polar hydrogens and merge all non polar hydrogens. Then Kollman charges were added. Further .pdbqt, .pdbq .gfp etc files were prepared before start docking. But in present Study this all steps were performed by using molecular docking server. Briefly, PDB file for CDK2 protein (ID 1W0X) downloaded from Protein Data Bank was uploaded to server. At protein clean step charge calculation method was selected as Gasteiger. The ligand-inhibiter (OLO) was not, selected since we had to dock to the same inhibiter binding site. All water molecules were selected for cleaning. By completion of this step, protein clean, calculation of protein charges and solvation parameters as well as protein parameter file created. In the next step a Grid (a three-dimensional box) was created with a dimension of X=20 Angstrom, Y= 20 Angstrom, Z= 20 Angstrom, while center of mass was kept at a co-ordinate of X= 103.61, Y= 100.67, Z= 78.536. By complication of this step the protein was ready for the simulation/ Docking experiments.

Ligand file preparation

Molecular structures of the Compound A and B were taken from the published report **[9]** and drawn in "Symyx Draw 4.0" program. Files were saved as MOLfile (*.mol). MOLfiles were uploaded in to the Dundee PRODRG Server to retrieve PDB files **[15]**. Whereas, .pdb files for the known inhibitors of CDK2 inhibitors, namely olomoucine (OLO), 6-Cyclohexylmethyloxy-5-Nitroso-Pyrimidine-2, 4-Diamine (NW1) and 6-O Cyclohexylmethyl Guanine (CMG) were retrieved from protein data bank (PDB) server. Unlike our previous study we used the molecular docking server for the preparation of ligand before docking experiment. Briefly, ligands were uploaded singly to server. Charge calculation and geometric optimization methods were selected as Gasteiger and MMFF94 respectively; while pH was kept as 7.0. By the end of this process ligand files are ready for the docking.

Docking:

Docking was started by selecting a ligand (compound A, say) and protein (CDK2) from their respective folders. The number of individuals in the population (ga_pop_size) was kept 150, AutoDock counts for numbers of energy evaluations (ga_num_evals) were kept 25000000 and the number of generations (ga_num_generation) selected as 540000. And rest other settings kept as default setting. Finally simulation experiment started with keeping the numbers of run as 100. Since, AutoDock **[17]**, the most popular molecular docking program is used for molecular docking calculations at Molecular Docking Server and fidelity of Molecular Docking Server as a whole **[16]** and AutoDock separately, is tested several times in previous studies **[17-20]**. Hence we considered Molecular Docking Server as an appropriate docking tool for the present purpose. AstexViewer™ viewer (default viewer of docking server) and PyMol **[21]** viewer were used for the visualization of docking results.

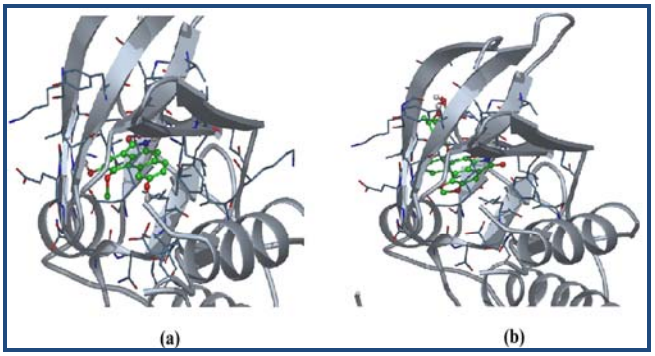

Figure 2: Visualization of the lowest energy conformation of compounds and CDK2 complex is shown in AstexViewer™ (an in built application of molecular docking server).

Results and Discussion:

Calculated binding energy for compounds A and B in the inhibitor binding site (IBS) were -7.38 and -9.70 kcal/mol respectively in the best pose **(Figure 2)**. Frequencies of occurrence out of total population were 100% and 73% as well as total surface of interaction between compounds and IBS were 689.78 and 913.12 respectively **(Table 2, See supplementary material)**. A comparison of different energies, interacting surfaces and frequencies of species etc. between compounds A and B as well as OLO, NW1 and CMG (known inhibitors) is listed in **(Table 2, see supplementary material).**

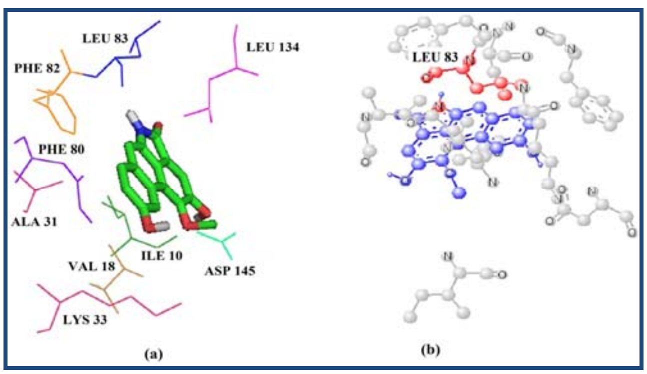

Figure 3: (a) An illustration of the amino acid residues involved in hydrophobic cage formation and stabilization of compound A inside the IBS of CDK2 (viewed with PyMol); **(b)** Illustration of hydrogen bond between nitrogen atom of γ-lactam of compound A and LEU 83 residue of IBS of CDK2.

Compound A structure can be split into two fragments, namely a phenanthrene ring skeleton (PRS) and a γ-lactame ring fused with PRS. A hydroxy group (-OH) at C3 and C6 position and methoxy group (-OCH3) at C4 position attached. Similarly compound B structure can be split in to three fragments. PRS and γ-lactame ring are there in same fashion as in compound A. A hexose sugar moiety has a glycosidic linkage at C8 position. Oxygen atoms bound at C3 and C4 positions are attached with a single carbon atom to form a dioxol moiety **(Figure 1) [10]**. As outlined in **(Figure 3a)**, compound A docked in to IBS of CDK2 outlined by ILE 10, VAL 18. ALA 31, LYS 33, PHE 80, PHE 82, LEU 83, LEU 134 and ASP 145. In this position nitrogen atom of γ-lactame ring appears remain hydrogen bonded with the oxygen atom of LEU 83(bond length = 2.74 γ Å) **(Figure 3b).** Polar interactions were made between hydroxyl group at C6 position and ASP 145. The hydrophobic cage for compound A comprised of ILE 10, VAL 18, ALA 31 and ILU 134. From our results we presumed that compound A is anchored in to the hydrophobic cage by a hydrogen bond between nitrogen atom of γ-lactame ring and oxygen atom of LEU 83, while the confirmation stability is bring about by other week interactions like π - π and cation-π, which involve PHE 80 and PHE 82 respectively.

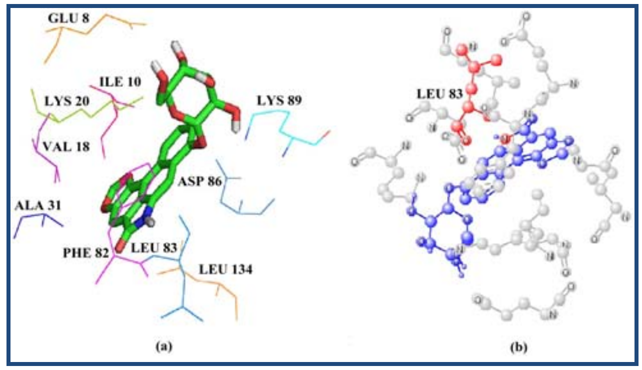

Figure 4: (a) An illustration of the amino acid residues involved in hydrophobic cage formation and stabilization of compound B inside the IBS of CDK2 (viewed with PyMol); **(b)** Illustration of hydrogen bond between nitrogen atom of γ-lactam of compound B and LEU 83 residue of IBS of CDK2.

Interestingly, docking results reveal that compound B inside IBS of CDK2 protein is outlined by GLU 8, ILE 10, VAL 18, LYS 20, ALA 31, PHE 82, LEU 83, ASP 86, LYS 89, and LEU 134 **(Figure 4a)**. And the hydrophobic pocket is comprised of ILE 10, VAL 18, PHE 82 and LEU 134. In a fashion similar as compound A, compound B is anchored by a hydrogen bond (bond length = 2.78 Å) between nitrogen atom of γ-lactame ring and oxygen atom of LEU 83 **(Figure 4b)**. GLU 8, LYS 20 and LYS 89 are involved in polar interactions as well as PHE 82 involve in cation-π interaction, which stabilize compound B conformation inside the IBS of CDK2 protein.

In the present study, we have made a detailed analysis of structure–activity relationship for compound A and B in the context of its inhibition of CDK2 activity. Both the compounds screened in the present study had the intact phenanthrene ring which is fused with γ-lactame ring **(Figure 1)**. Compounds having such moiety, most likely, offer a wide range of bioactivities including anti-inflammatory, antimycobacterial, and neuro-protective and antiplatelet activities **[22-24]**. Recently, such compounds had been shown to have anticancer activities **[5, 10]**. Hence, phenanthrene ring with a fused γ-lactame ring seemingly to be important for imparting CDK2 inhibitory activity.

Aristolochic acid which have phenetherene ring but no γ-lactame ring, has been reported for antitumor activity **[25]**. Sauristolactam and aristolactame AIIIa- derivatives of aristolochic acid which have γ-lactame ring fused with the phenenthere ring have been reported for their anti cancer and

antitumor activities in several cancer cell lines **[5, 26]**. Particularly, aristolactame AIIIa recognized recently as a ligand/inhibitor for polo-box domain of polo-like kinase 1. All these evidences indirectly suggest that the phenetherene ring is deemed to be an essential feature for aristolactams and a fused γ-lactam moiety increases their activity as an inhibitor. Further enhancement of activity is bringing about by presence of dioxal moiety **[10]**. In present study we found that compounds are anchored in to the hydrophobic cage of IBS by a hydrogen bond between nitrogen atom of γ-lactam moiety and oxygen atom of LEU 83 whereas phenetherene ring is involved in to making week interaction inside the hydrophobic cage. In addition of this a higher binding energy (9.7okcal/mol) of compound B with CDK2 IBS is observed. These all evidences suggest that our present finding is in the agreement earlier in vitro findings.
CDK2 is involved in to initiation of DNA synthesis during G1/S transition phase **[11, 12]** and in S-phase CDK2/cyclin-A complex prevents unscheduled activation of transcription factor E2F and facilitates cell cycle to passes this phase without interruption **[13]**. The role of CDK2 during the S phase of cell cycle is not well defined. However, due to its rate-limiting role the cell cycle, it has been regarded as a potential target for cancer therapy. A study recent study claims that in comparision of compound B, compound A is more selective for CDK2 compared to other related kinases but CDC2 kinase has a close homology with CDK2 and compound A shows similar inhibitory activity. However A showed more selectivity against other kinases like CDK4, aurora-2 kinase and MAP-kinase **[10]**. In our study, we found that compound A shows 100% frequency in its best possible position inside IBS of CDK2, whereas it is only 73% for compound B. Since a frequency of a species in the population corresponds to the probability of occurrence. In other words later is a parameter of selectivity and specificity of compounds to IBS of CDK2. Hence, our finding is in agreement with the finding of **[10]**. The seemingly greater selectivity of compound A with CDK2 as earlier studies reported could make it a better lead for designing anticancer drugs.

Conclusion:
We have shown the possible interactions of compound A and B isolated from a Chinese herb Aristolochia manshuriensis with the IBS CDK2. This could provide a structural and molecular basis for the existing evidences for anticancer properties of compound A, B and related compounds. Such observations can also help to consider compound A and B as an effective scaffold for rational design of novel and potential drugs against cancer.

Abbreviations:
CDK2: cyclin-dependent kinase 2; OLO : Olomoucine; NW1: 6-Cyclohexylmethyloxy-5-Nitroso-Pyrimidine-2,4-Diamine; CMG: 6-O-Cyclohexylmethyl Guanine

Competing interests:
Authors declare that there is no competing interest among them.

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Supplementary material:

Table 1: List of software and servers used and their purpose of use

Softwares/Servers	Used for
Symyx Draw 4.0	Draw the compound molecules
Dundee PRODRG Server	File format conversion (.mol to PDB)
Docking Server	Molecular docking
PyMol	Visualization of results

Table 2: Comparison of different energies of compound A and B calculated during docking

Compounds	A	B	OLO	NW1	CMG
Est. Free Energy of Binding (kcal/ mol)	-7.38	-9.70	-7.58	-7.96	-7.37
Est. Inhibition Constant, Ki	3.88 μM	78.15 nM	2.87 μM	1.47 μM	3.97 μM
vdW + H bond + dissolve Energy (kcal/ mol)	-8.34	-10.48	-8.41	-8.45	-8.03
Electrostatic Energy (kcal/ mol)	-0.38	-0.50	-0.12	-0.27	-0.14
Total Intermol. Energy (kcal/ mol)	-8.72	-10.98	-8.54	-8.72	-8.16
Frequency (%)	100	73	20	6	1
Interact. Surface	689.748	913.126	745.763	631.495	604.061