PHOSPHORYLATION OF THE P65 SUBUNIT OF NUCLEAR FACTOR KAPPA B (NF-κB) BY USING A CHEMICAL SELECTED FROM INDIAN PLANT ANTICANCER COMPOUNDS DATABASE.

Sher Muhammad Zaman[1], AqeelJaveed[1], Aamir Ghafoor[2], Muhammad Ashraf[1], Muzammad Hussain[1], Muhammad Hassan Mushtaq[3], Muhammad Anees[4],MuhammadYaqoob[5]

[1]Department of Pharmacology and Toxicology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[1]University Diagnostic Lab, University of Veterinary and Animal Sciences, Lahore, Pakistan

1. Department of Epidemiology and Public Health, University of veterinary and Animal Sciences, Lahore, Pakistan
2. Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan
3. Department of Clinical Sciences, PMAS Arid Agriculture University, Rawalpindi, Pakistan.

Contact:

ABSTRACT:*Nuclear Factor Kappa B (NF-κB) is a transcription factor that is activated in many types of cancer, and has been recognized as an important target for anticancer therapeutics. In particular, the phosphorylation of p65 subunit at serine 276 phosphorylation regulates the expression of different types of genes including vascular cell adhesion molecule-1(VCAM-1) and Interleukin-8(IL-8), which plays a crucial role in tumor-associated angiogenesis and metastasis. Thus inhibition of serine 276 phosphorylation may prevent metastasis and angiogenesis, thereby, inhibiting certain types of cancerous growth. In this study, a virtual screening was performed against a structural pocket near serine 276 with 125 compounds from Indian Plant Anticancer compounds Database (InPACDb) using Autdock 4.02 software. The docked complexes were ranked according to their docking score and binding pose using methodology that was shown to achieve maximum accuracy. Finally, one potent compound was obtained with best Autodock score (Artemisinin: -4.12 kcal/mol). Artemisinin was further tested for anti-angiogenic activity by using chorio-allantoic membrane assay (CAM assay) that showed significant anti-angiogenicresults. Therefore, Artemisinin could be a promising inhibitor of serine 276 phosphorylation.*

1. INTRODUCTION

Angiogenesis is the fundamental physiological mechanism required for the growth, reproduction, wound healing and response to ischemia. Angiogenesis is mediated by different factors including vascular endothelial growth factors(VEGF)[1].Pathological angiogenesis is important for metastasis of solid tumors[2].Today's research efforts in anticancer drug discovery are mainly focused on developing targeted drug therapy.Nuclear Factor kappa B (NF-κB) is one of the transcription factor that initiate immuno-inflammatory responses inside the body. NF-κBis a family of transcribing subunits likep50, p52, c-Rel, Rel A (p65), and Rel B in its homo and heterodimeric forms. NF-κBis one of the responsible factor in tumor developmental processes like apoptosis, malignancy, cellular proliferation and hence angiogenesis[3]. Regarding mechanism of action of NF-κB, its phosphorylation is necessary at Serine 276 site of p65subunit[4].Furthermore, this phosphorylation will be ended up in the activation of IL-6, IL-8 and Vascular cell adhesion molecules (VCAM-1)[5].VCAM-1 is important for metastatic activity and IL-8 for angiogenic and metastatic both. If NF-κB is specifically inhibited at Serine 276 site, then it could be useful in preventing metastasis and cancer. Almost more than 50 inhibitors of NF-κBhas been isolated uptilnow[6], but no one is proved to be successful in selective inhibition of NF-κB regulated genes and hence showing undesirable effects. Our objective is to build up a molecule that would selectively inhibit NF-κB at its Serine 276 site and hence treatingcancer more selectively by suppressing angiogenesis and metastatic activity in a safe and effective way.

2. MATERIAL AND METHOD

2.1 *In-silico* molecular modeling

Virtual screening or virtual high throughput screening (vHTS) is an alternative approach to experimental high through put screening- that is the technique used for the identification of new lead compounds in drug discovery by physical screening of large libraries of chemicals against a biological target. Indian Plant Anti-Cancer Compounds Data base (InPACDb), comprising of 125 compounds of phytochemical origin, was filtered by excluding those compounds for which NF-κB was described as a target for the anticancer activity.Ligand-Protein docking was performed by using the software AutoDock 4.02 [7] to predict the binding orientation and binding energies of the compounds obtained after the filtration step from InPACDb. AutoDock4.02 is parameterized to use a model of the protein and ligand that includes polar hydrogen atoms (bonded to electronegative atoms), but not non-polar hydrogen atoms (bonded to carbon atoms). The program AutoDockTools 1.5.4 [7] was used for creating the needed pdbqt files from traditional pdb files.

The compounds with at least one cluster showing the best pose and docked energy in the structural pocket of Serine 276 phosphorylation was then tested for anti-angiogenic activity through a wet-lab study involving a Chorio-allantoic membrane (CAM) assay for angiogenesis.

2.2 Chorio-allantoic Membrane (CAM) Assay

Fresh fertilized eggs were taken from local hatchery. Eggs were sprayed with 70% ethanol and air-dried. Eggs were divided into five groups containing four eggs per group. Four groups were subjected to Artemisin solutions and one group served as control. Eggs were incubated at 37C°and at

60-70% humidity. At day 4 of incubation, 4-5 ml of albumin was removed with the help of syringe. A window was made by removing shell and shell membrane and was sealed with sterile parafilm. At day 5 of incubation windows were opened and 200µl of each sample solutions was applied on developing CAM. Windows were sealed again with adhesive tape and eggs were kept in incubator for 24 hours. Then CAM was separated and processed for further macroscopic and microscopic evaluation.Different concentrations of Artemisinin solutions such as 0.1mM, 0.5mM, 0.8mM were prepared using Miglyol Oil as a Solvent. These concentrations were prepared immediately before use in CAM assay. These concentrations were filtered through 0.22 micro meter pore filters. The pH of Artemisinin solution was adjusted to 7.0. Subsequently, 200 micro liter of this solution was applied to CAMs on day 4 of incubation. Thus the effects of Artemisinin on angiogenesis were screened.

3. RESULTS

Virtual High throughput screening (vHTS) and docking studies results

Onecompound out of 125 compounds was obtained after the result of the 10 rundocking procedure and is shown in the table 1 along with the binding energy.

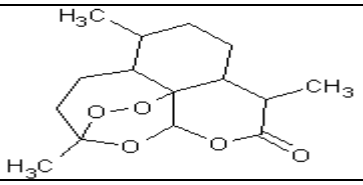
CAM Assay Results

In this study we have examined the anti- angiogenic effect of Artemisinin using chicken CAM assay. A significant reduction in blood vessels formation was observed by the application of various concentrations of Artemisinin with reference to control group. The following results were observed;

Macroscopic vascular transformations in CAM:

Applying different concentrations of Artemisinin caused marked changes in micro vasculature of the CAMs. Anti-angioenic activities were observed after applying different concentrations of Artemisinin, with optimal effect was observed on the dose adjusted by Autodock software, which resulted in thinning of primary and secondary blood vessels and fading of tertiary blood vessels of CAMs. It resulted in marked reduction in the complete vascular network of CAM. Among all treated groups like 0.1mM, 0.5mM and 0.8mM concentration of Artemisinin, the most significant and crucial anti- angiogenic response was seen in 0.8mM concentration (Figure 2, 3) which was the same concentration as specified by the Autodock software.

Table 1. The compound selected after 10 run docking studies.

Accession Number	Compound Name	Binding Energy (Kcal/mol)	Chemical Structure
ACD000 4	Artemisinine	-4.21	

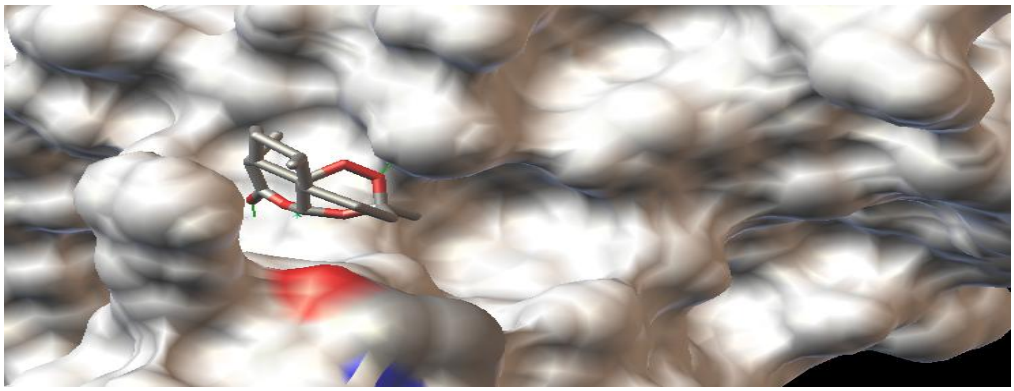


Fig 1 Docked representation of Artemisinin to the structural pocket of Serine 276 phosphorylation in NF-Kb

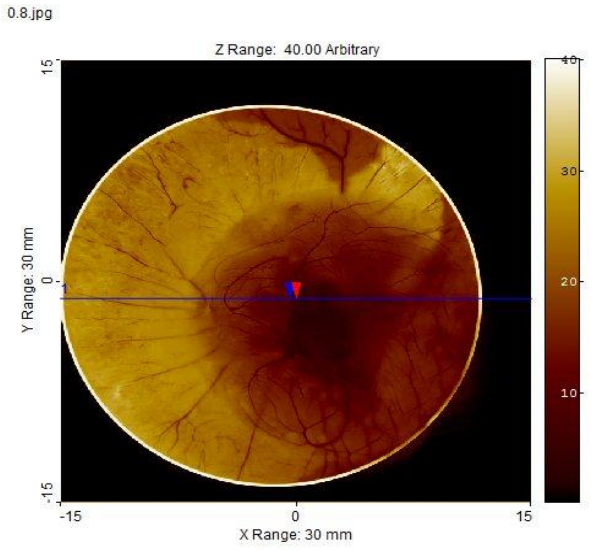


Fig 2.CAM treated with diluent only

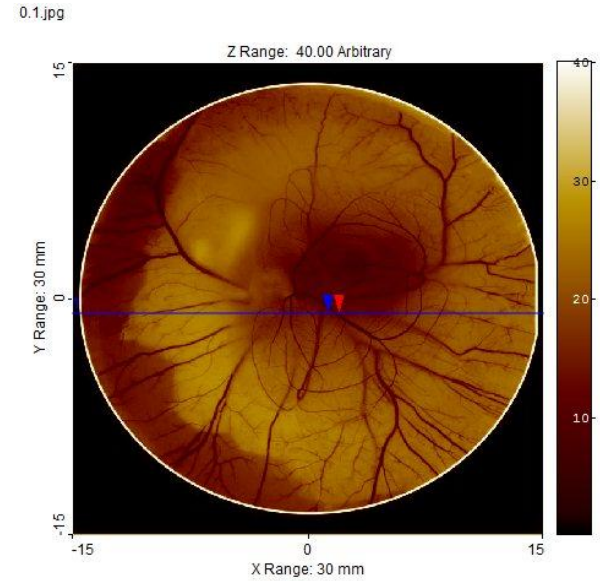


Fig 3.CAM treated with 0.8mMArtemisinin

Out of 125 compounds, the Compound Artemisinin (Accession Number ACD0004), showed lowest docked energy i.e. -4.21Kcal/mol and best binding pose to its targeted Serine 276 phosphorylation structural pocket of NF-κB. The software predicted inhibitory constant Ki value of 800µg.

Quantitative analysis of structural changes in the microvasculature of CAM:

A novel image probing system (IPS) was utilized for the assessment and quantification of structural changes in CAMs caused by applying different concentrations of Artemisinin.

Reduction in diameter of blood vessels:

A significant reduction in diameters of primary, secondary and tertiary blood vessels was evident and recorded among all Artemisinin treated groups as compared to control groups.

4. DISCUSSION

The drug development process involves the design and synthesis of small molecule entities, either acidic or basic in nature, showing optimal interactions and binding ability to their macromolecular targets, specifically to target proteins. The ultimate goal of this whole process is to correct the disease / pathological conditions of the body. The NF-κB family of transcription factors plays a vital role in expressing a set of genes that play a key role in inflammation, cell proliferation and cell survival. NF-κB transcription factors also play a critical role in the pathogenesis of many diseases, especially the cancer [8, 9, 10]. Therefore, it is more likely that developing NF-κB inhibitors could have beneficial effects in the treatment of diseases involving NF-κB as the major pathogenic factor. To this regard, many NF-κB inhibitors have been identified till now including inhibitors of IKK (the kinases that phosphorylate IkB) and the inhibitors that block nuclear translocation of NF-κB[6]. These inhibitors, however, have unwanted side-effects because of their influence on the global activity of NF-κB, resulting in altered transcription of most or all of the NF-κB-regulated genes. Recently, a group of researchers [11] took a unique approach, *in-silico* molecular docking, to identify/develop more selective NF-κB inhibitors. *In-silico* molecular docking is a primary method for the discovery of ligands, and has been used successfully in the discovery of many novel ligands, including carbonic anhydrase II inhibitors, aldose reductase inhibitors and the retinoid receptor inhibitors [12]. Law et al. (2010) used in-silico molecular docking to screen a catalog of more than 200,000 molecules against a structural pocket adjacent to serine 276 of p65 subunit of NF-κB. Since the serine 276 phosphorylation is very critical to the regulation of a defined group of genes [13, 14, 15], the results of that study showed promising effects against the activity of p65 subunit of NF-κB.

NF-κB is one of the tumor promoting transcription factor families, and is known to have its role in carcinogenesis. Thus, if we could inhibit the NF-κB transcription factor specifically it will beeasy to control carcinogenicity mechanisms involving NF-κB as the key player [16]. In this study, same *in-silico* molecular docking technique has been used to screen a small database of natural anticancer compounds (the Indian Plants Anticancer Compounds Database) comprising 125 molecules against the serine 276 phosphorylation site. The interaction mode between each candidate ligand and Serine 276 site of NF-κB was explored by the docking program AutoDock 4.2 [17]. The compounds were distinguished on the basis of least binding energy or interaction energy and were listed. Artemisinin was the compound with best interaction and least binding energy as smaller binding energy gives more stable drug-target interaction. The software also predicted the concentration that may give optimum level of effect.The selected compound was then further tested in Angiogenesis Lab using CAM assay. Different concentrations of the compound were prepared around the optimal concentration specified by AutoDock software during docking process. The compound was only soluble freely in Miglyol oil. After CAM Assay, the results showed significant anti angiogeniceffect of

Artemisinin around that optimal concentration (0.817mM) that was specified by AutoDock software during docking.

5. CONCLUSION

In the present study a docking based virtual screening was developed in order to verify whether artemisinin as an inhibitor of transcription factor NF-κB, which could be profitably used for finding ligands against cancer by a computational procedure. The study revealed that Artemisinin can be used successfully to inhibit angiogenesis and hence lead to tumor suppression. Artemisinin concentration is also important to adjust around optimal concentration while using for anti-angiogenic purposes, as it has high risk of toxicity to the normal growing cells.

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