



## IN SILICO STUDIES OF INDAZOLE PYRIDINE ANALOGS AS POTENT INHIBITORS OF AKT PROTEIN IN CANCER

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### ABSTRACT

AKT/Protein kinase B (PKB) signaling cascades are important in both normal cellular physiology and various diseased states, especially its expression is observed as hikes in few cancers. A series of invention of AKT inhibitors lead to the discovery of Indazole pyridine analogues. These analogues are more potent in binding with AKT protein and are ATP competitive. Aim of this study is to identify few indazole pyridine compounds that can effectively inhibit AKT protein activity. Our methods include minimization of analogues with commercial software's like HyperChem 8.0 followed by docking against the target (AKT) using GOLD. From docking analysis, we found that substitutions like Methyl linkage, Thiophene, Thiozole, Imidazole and Amino groups at the third position of indazole pyridine are more efficient in inhibiting the AKT activity. Among all the substituents, Thiophene was found to be most potent lead and has the highest fitness score of 64.5. Absorption, Distribution, Metabolism, Excretion (ADME) and Toxicity profiles are found favorable for majority of these analogues.

**Keywords:** AKT/PKB, Indazole pyridine, PI3K, ADME, toxicity, Gold, Hyper Chem, Discovery Studio Client.

### INTRODUCTION

The success of DNA damaging drugs used in anticancer therapy depends on ability to induce growth arrest in cells and apoptosis<sup>1</sup>. Apoptosis which is induced by DNA damage associated with the activation of the proteases and caspases, which results the sequence of mitochondrial events. Release of cytochrome 'c' will activate the caspases, several proteins like apoptosis inducing factor and endonuclease G are also released from mitochondria which eventually leads to the cell death. Cell cycle regulation is also one of the important determinants for cell death in response to DNA damage<sup>2</sup>. DNA damage check points are in G1 and G2 phases of cell cycles. In fact here are some mechanisms of cells which will result resisting the DNA damage<sup>3</sup> For instance resistant lung cancer cells have increased DNA dependent protein kinase (DNA-PK) activity and DNA repair with decreased incidence<sup>4</sup> This suggests that there is an involvement of DNA-PK in tumor survival<sup>5-6</sup>. From decades, much of the research in cancer has been focused on the first identified oncogene Ras and mitogen activated protein kinase (MAPK'S) which plays major role in neoplastic cell proliferation in human. By recent studies it has been found that there is second pathway which is downstream of the Receptor Tyrosine Kinase (RTK) that involves phosphatidylinositol 3-kinase (PI3K) and AKT which is found to have some importance in regulation of mammalian cell proliferation and survival. Dysregulation of the several components in the pathway of the PI3K and AKT are observed in wide spectrum of human cancers.

Through molecular cloning methods it is clear that PI3K's are large complex families that have three classes with

multiple subunits and isoforms. Class1 PI3K's will phosphoralise the inositol-containing lipids, known as phosphatidylinositols (PtdIns). In vivo primary substrate is PtdIns (4,5) P2(PIP2) Which are converted to PtdIns (3,4,5)P3 (PIP3). Class 1 PI3K's has two sub groups 1A and 1B. Class 1A PI3K's is clearly involved in oncogenesis.PI3K's has separate regulatory and catalytic subunits, It has phosphoprotein substrate of many cytoplasmic and receptor kinase. P58 regulatory subunit of PI3K is associated directly with many tyrosine kinases through SH2 DOMAIN with its phosphoprotein residue. In some cases the interaction between P58-RKT is indirect by the intermediate phosphoproteins such as insulin receptor substrates IRS1 and IRS2<sup>7</sup> P58 has two SH2 domains that bind constitutively to the 110 catalytic subunit residues.

### Activation of PI3K

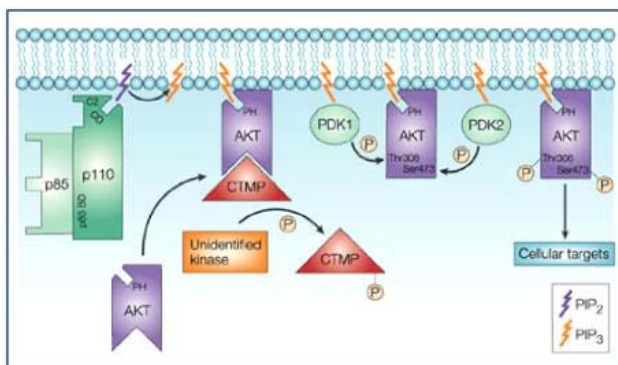
After the activation of receptor by the specific cytokines, PI3K can be activated by two mechanisms. Firstly it is the phosphorylation of the tyrosine residue on the receptor which serves as the docking for the P58 followed by the addition of the p110 to this complex.<sup>8</sup> Secondly it is by the activation of the receptor by cytokine, further shc binds to the receptor which will enable Grb2 and sos to form complex which inturn activates Ras.<sup>9-12</sup> Ras will induce the membrane localization<sup>13</sup> followed by the activation of the P110 subunit of PI3K, Which binds to the sequence on PI3K-gamma referred as Ras binding domain(RBD)<sup>14</sup> Recently it was shown that PI3K has two regulatory domains: GTPase responsive domain (GRB) that mediates PI3K activation through binding small GTPase (Ras, Rac1) and an inhibitory that blocks these binding events. This



inhibition is by certain tyrosine kinases such as Src, Lck and Abl which phosphorylate the S668 residue contained within the inhibitory domain<sup>15</sup> Activation of PI3K will lead to the conversion of phosphatidylinositol (4,5)-phosphate [PI(4,5)P<sub>2</sub>] in to phosphatidylinositol (3,4,5)-phosphate [PI (3,4,5) P<sub>3</sub>], This results in membrane localization of Phosphatidylinositol-dependent kinase-1 (PDK-1) by its pleckstrin homology (PH) domain.

### Activation of AKT

AKT (protein kinase B, PKB)<sup>16-18</sup> is also recruited to lipid rich plasma membrane by its PH domain and its activation is phosphorylated by PDK1 at Thr308 and Ser473 (Figure 1). AKT is membrane of AGC family of kinases. The three isoforms of AKT are AKT1, AKT2, and AKT3. All three AKT's will show 85% homology in protein sequence and 100% homology<sup>19-21</sup> in ATP binding sites except for one amino acid in AKT3. AKT1 is the threonine/serine protein kinase whose activity is found to be elevated in many human malignancies<sup>22-23</sup>. It has been discovered that the AKT1 found in the human is homologue to the transforming gene in the AKT-8 oncogene virus which was isolated from spontaneous thymoma in AKR mouse<sup>24-25</sup>. All three AKT isoforms are over expressed in variety of human tumors like lung, prostate, breast, gastric, and pancreatic carcinomas<sup>26-28</sup> Hence, Akt is the good target for the cancer therapy.



**Figure 1:** After the cell stimulation and PtdIns (3, 4, 5)P<sub>3</sub> (PIP<sub>3</sub>) production AKT activity is initiated by membrane translocation. Accomplishment of AKT at plasma membrane is by an interaction between its pleckstrin-homology domain (PH) and PIP<sub>3</sub>. AKT association with caxyterminal modulator protein (CTMP) prevents it come becoming phosphorylated and fully active. CTMP phosphorylation by an unidentified kinase releases CTMP from AKT with allows AKT to be phosphorylated by PDK1 and PDK2 at Ser473 and Thr308, respectively. Phosphorylations at these particular sites lead to full activation of AKT.

### Inhibition of AKT activity

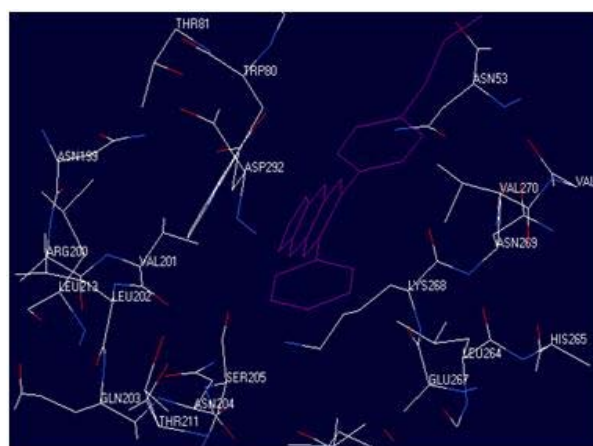
Inhibition of the activity of Akt or with the combination of the standard chemotherapeutics helps to preferentially kill the cancer cells. It have been reported that there are several ATP-competitive small molecule inhibitors with ATP selectivity's. It is found that phosphatidylinositol (PI) analogs will inhibit the activity of Akt, but these type of analogs have specificity problems with respect to other. Proteins containing pleckstrin homology (PH) domain and they may have poor bioavailability. In addition new inhibitors have been reported which are PH domain dependent and have selectivity for individual Akt

isoenzymes and inhibit the activity of Akt. Akt inhibitors with these classes have sufficient potency and specificity have efficacy in tumor xenograft models. After the series of preparation of heteroaryl-pyridine containing inhibitors of Akt1 leading to the discovery of Indazole pyridine, which are the ATP competitive and reversible inhibitors of Akt activity. It is 40 folds selective for Akt than for the other members of AGC family of kinases. No selectivity is observed among the AKT isoforms for this analog. The hinge binding interaction is observed as a common motif for most AKT competitive<sup>29</sup> kinase inhibitors. From the recent synthesis and SAR studies of different AKT inhibitors it has been identified that there are over all 65 compounds which reduce the activity of AKT protein. Here we carried out studies in docking, ADME, toxicity and molecular properties for these series of Akt kinase inhibitors to trace the best ligands that are highly effective in inhibiting the Akt activity.

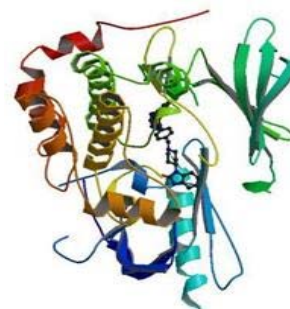
## MATERIALS AND METHODS

### Selection of target protein

Protein Akt was retrieved from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb/>), with PDB Id – 3O96 (Figure. 2). Protein reports of Akt were obtained from the RCSB Protein Data Bank. Identification of active site is primarily done using online tools like RCSB Ligand Explorer. Further active sites prediction is done by using SPDBV viewer and we found active site amino acids as ASP292, TYR272, VAL270, LYS268, LEU210, SER205 and TRP80, which are shown in the (Figure. 3) in our studies we found that LYS268 as best active site.



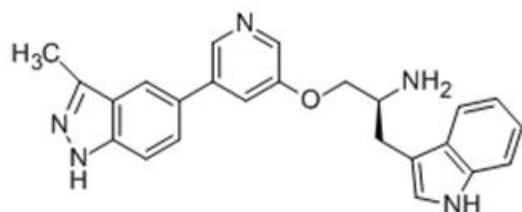
**Figure 2:** Identification of active binding site of AKT protein Using SPDBV viewer (PBD ID 1O6L).



**Figure 3:** PBD structure of AKT

## Selection of Ligands

A series of 65 AKT inhibitors are retrieved from the available article on "synthesis and SAR studies of Indazole-Pyridine (Figure. 4) based protein kinase B/Akt Inhibitors". It has been concluded from their work that Indazole Pyridine is potent inhibitor for AKT protein. In our study all the 65 AKT inhibitors are subjected to docking and post docking studies.



**Figure 4:** Structure of Indazole Pyridine.

## Geometrical optimization and minimization of protein and ligands

Minimization of AKT protein is done in the Accelry's Discovery studio 2.5 by removing all the hetero atoms with parameters like force field Charm27, RMS gradient 0.1, maximum cycles 2000 and smart minimizer algorithm.

Drawing the structures of 65 AKT inhibitors and their Geometrical optimization is done using Hyperchem release 8.0 with parameters like force field Amber 2, RMS 0.01, maximum cycles 2000, invacuo and polak-ribere algorithm where we will get energy for optimized ligands.

## Conversion of ligand from .mol to .mol2

Ligands are converted to .mol file using Hyperchem release 8.0 with the parameters like bio Charm force field, RMS 0.01, Maximum cycle 2000, invacuo and polak-ribere algorithm which is followed by the single point.

Mol file ligands are converted to .mol2 using Discovery studio 2.5 with the following parameters like force field Charm 27, RMS gradient 0.1, maximum cycles 2000 and smart minimizer algorithm.

## Docking studies

The interaction between protein and ligands is observed using GOLD. This will predict the interactions between ligands and protein in biological system. Active site atom number of protein is identified before docking. In GOLD docking protocol, initially the protein is loaded in GOLD docking software, next the ligands which are in .mol2 format are loaded followed by giving atom number for active site amino acid as LYS268 (3648) and output options are selected. Finally the fitness scores of ligand-protein interaction are obtained. Results comprises of four parts: two inter atomic van der waals force, hydrogen bond interactions and two intra-atomic van der waals force, hydrogen bond interactions. It allows us to find the best complementary analogue and binding pose with target active site. Van der waals force and hydrogen bond forces will determine the fitness of the analogs.

$$\text{Gold fitness} = \text{Shb\_ext} + 1.3750 + \text{Svdw\_ext} + \text{Shb\_int} + \text{Svdw\_int}$$

## ADME studies

ADME (Absorption, Distribution, Metabolism and Excretion) studies are carried out in the Discovery studio client for assessing the disposition and potential toxicity of ligand with in an organism. It has published models that we computed to analyze blood brain barrier level, Absorption level, solubility level, hepatotoxicity level, CYP2D6 level and plasma protein binding level of all the ligands with ADME plot.

## Molecular properties

Molecular property protocol used to calculate many properties used to create QSAR model which is done in Discovery studio client. We have calculated the molecular properties which include Molecular weight, Molecular surface area, number of hydrogen bond donors, and number of hydrogen bond acceptors and AlogP of different ligands.

## Toxicity studies

Toxicity prediction is carried out in Discovery studio client using "toxicity prediction extensible" protocol which helps to predict the toxicity of drugs by using Bayesian model. We predicted the toxicity profiles like aerobic biodegradability, AMES mutagenicity, developmental toxicity potential, ocular irritancy, skin irritancy and prediction of carcinogenicity using various model organisms in Insilco.

## Hydrogen bond analysis

Hydrogen bond analysis is carried out in the Gold 4.1.2, where we uploaded docking results in the Hermes 1.3.1 to predict the number of hydrogen bonds and their bond distances.

## RESULTS

Protein AKT with PDB ID 3O96 is geometrically optimized and screened for its active binding sites. Structures of 65 AKT inhibitors are designed in Hyperchem release 8.0 followed by minimization in Discovery studio client. Docking results with fitness scores are tabulated in the table 1 and five of the best ligands are highlighted. Ligands with the highest score which are the substitutions at third position of Indazole Pyridine are shown in the table 2. In hydrogen bond analysis majority of ligands formed hydrogen bond with the following amino acids like TRP80, LYS268, SER205, SER266, and GLU267. Hydrogen bonding of Best five ligands based on their fitness is shown in the table 3, ligand 54 has 4 hydrogen bonds with the receptor residues like GLU265, GLU 267 and SER266 which can be visualized in the figure 5.

## Molecular properties

Molecular properties like molecular weight, surface area, and volume, number of hydrogen bond acceptors & donors and AlogP were found for all optimized ligands, only five with best dock score are tabulated in the table 4.

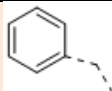







**Table 1:** Docking fitness and interaction energies of the inhibitors with the protein AKT.

Ligand	Fitness	Hb-ext	Hb-int	Vdw-ext	S(int)
1	38.72	0.00	0.00	34.96	-9.35
2	48.90	1.71	0.00	40.18	-8.06
3	53.58	0.00	0.00	44.55	-7.68
3b	51.93	0.03	0.00	42.61	-6.69
4	52.32	0.01	0.00	42.33	-5.91
4s	58.10	0.50	0.00	45.91	-5.52
5	31.64	6.52	0.00	19.48	-1.67
6	43.54	4.41	0.00	32.52	-5.58
7	47.18	0.14	0.00	40.63	-8.82
8	43.99	0.31	0.00	47.72	-21.93
10	28.22	1.23	0.00	23.94	-5.92
11	28.96	0.00	0.00	27.16	-8.38
12	31.18	0.72	0.00	27.19	-6.93
13	37.70	1.89	0.00	26.04	-0.00
14	38.71	1.89	0.00	26.78	-0.00
15	34.31	2.00	0.00	29.56	-8.34
16	36.82	2.23	0.00	25.16	-0.00
17	37.19	0.95	0.00	26.84	-0.68
18	38.92	1.21	0.00	27.43	-0.00
19	37.42	1.15	0.00	26.38	-0.00
20	44.53	0.00	0.00	36.11	-5.13
21	48.53	2.67	0.00	39.96	-9.09
22	31.86	0.00	0.00	23.24	-0.10
23	34.19	1.52	0.00	23.76	-0.00
24	36.97	2.00	0.00	28.39	-4.06
25	33.96	0.00	0.00	25.96	-1.74
26	35.74	1.75	0.00	24.89	-0.24
27	37.10	1.74	0.00	26.51	-1.09
28	32.74	2.00	0.00	23.04	-1.21
29	31.36	2.00	0.00	21.67	-0.43
30	44.32	2.00	0.00	33.00	-3.06
31	46.15	2.00	0.00	32.37	-0.35
32	30.49	0.00	0.00	22.90	-1.13
33	57.68	5.92	0.00	43.69	-8.31
34	21.97	0.00	0.00	15.98	-0.00
35	32.70	1.97	0.00	22.36	-0.00
36	47.46	2.08	0.00	34.01	-1.39
37	54.70	0.28	0.00	42.95	-4.64
38	50.85	1.83	0.00	41.52	-8.07
39	49.14	0.03	0.00	42.47	-9.28
40	50.59	2.02	0.00	41.45	-8.01
41	55.09	2.06	0.00	43.32	-6.53
42	54.62	0.00	0.0	47.43	-10.60
43	53.33	7.94	0.00	40.86	-10.79
44	51.63	3.76	0.00	40.45	-7.74
45	48.78	5.45	0.00	37.33	-8.00
46	49.89	5.75	0.00	38.99	-9.47
47	50.88	8.39	0.00	36.55	-7.77
48	52.85	1.03	0.00	40.69	-4.13
49	54.74	0.17	0.00	43.84	-5.71
50	59.57	0.13	0.00	47.05	-5.25
51	55.04	0.17	0.00	45.92	-8.26
52	59.38	0.09	0.00	46.05	-4.02
53	55.21	0.01	0.00	46.19	-8.31
54	60.06	7.14	0.00	44.17	-7.82
55	64.5	1.69	0.00	50.19	-6.55
56	63.94	0.10	0.00	51.26	-6.64
57	59.14	2.82	0.00	47.42	-8.89
58	62.42	2.75	0.00	46.29	-3.99
59	54.59	2.20	0.00	46.04	-5.42
60	39.36	0.03	0.00	43.21	-96.80
61	46.08	0.00	0.00	46.81	-18.28
62	55.61	0.93	0.00	46.81	-9.68
63	49.70	1.42	0.00	40.64	-7.60
64	61.49	3.34	0.00	48.29	-8.19
65	51.94	2.20	0.00	40.30	-5.67

**Table 2:** Ligands with best fitness scores

Ligand	Substitution at third position of indazole
54 (Methylene linkage)	
55 (Thiophene)	
56 (Thiozole)	
58 (Imidazole)	
64 (Amino)	NH

**Table 3:** Hydrogen bond analysis of best five inhibitors

Ligand	Pose	NO of H atoms	Amino acid name	HB distance
54	3	4	LYS268	3.082
			GLU267	2.945
			GLU267	2.174
			SER266	2.766
55	1	2	TRP80	2.927
			SER205	2.751
56	4	3	TRP80	2.554
			SER205	3.010
			GLU267	2.597
58	1	3	SER205	2.687
			TRP80	3.095
			LYS265	2.419
64	5	1	LYS268	2.980

### ADME and toxicity studies

ADME (Absorption, distribution, metabolism and Excretion) profile of five ligands according to their best fitness in docking is tabulated in table 5. Majority of inhibitors have solubility as 3 without blood brain barrier penetration and are Cytochrome P450 enzyme non-inhibitors. All inhibitors have shown low intestinal absorption and more plasma protein binding levels. Dose dependent hepatotoxicity is found in all of them. ADMET plot of ligand 55 is shown in figure 6.

**Figure 5:** Figure depicts the hydrogen bonding of ligand 54 (Thiophene) against AKT protein

**Table 4:** Molecular properties of best five AKT inhibitors substituted at third position of indazole

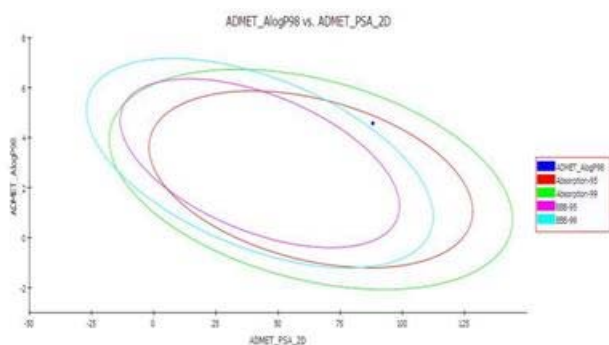
Ligand no	Mol MW	Mol surface area	Volume	No of HB donors	No of HB acceptors	AlogP
54	473.568	449.75	312.81	3	4	5.204
55	451.543	414.99	291.89	3	4	4.802
56	466.557	432.53	305.95	3	5	4.032
58	449.507	424.32	286.06	4	5	3.468
64	398.46	382.34	457.24	4	5	3.015

**Table 5:** ADME profile of the best five inhibitors of AKT.

Ligand No	BBB levels	Absorption level	Solubility Level	Hepatotoxicity Level	CYP2D6 level	PPB level
54	4	1	1	1	1	2
55	4	0	1	1	0	1
56	4	0	1	1	0	1
58	4	1	1	1	0	1
64	3	0	3	1	0	2

**Table 6:** Toxicity profile of five AKT inhibitors

Ligand NO	Aerobic biodegradability	Ames mutagenicity	Developmental Toxicity potential	Ocular irritancy	Skin irritancy	Carcinogenicity			
						Female Mouse	Male Mouse	Female Rat	Male rat
54.	NO	NO	YES	NO	NO	NO	NO	NO	NO
55.	NO	YES	NO	NO	NO	NO	NO	YES	YES
56.	NO	NO	NO	YES	NO	NO	NO	NO	NO
58.	NO	NO	YES	YES	NO	NO	NO	NO	NO
64.	NO	NO	NO	YES	NO	NO	NO	YES	NO

**Figure 6:** Figure shows the ADMET plot of ligand 55

In Toxicity studies all five inhibitors in (Table 6) shows as biodegradable. AMES mutagenicity and developmental toxicity is not observed in many of the inhibitors, further it is found that none of the inhibitors have shown skin irritancy and carcinogenicity, in fact ocular irritancy is seen in many.

## DISCUSSION AND CONCLUSION

It can be concluded that the substitutions at third position of indazole by 54, 55, 56, 58 and 64 compounds have better binding affinities with protein AKT1 when compared to other inhibitors because free N-H of the indazole is essential for this interaction. It is also confirmed from the binding energies of the protein-ligand interactions that these five inhibitors will potentially fit in to the active pockets of the receptor. Further ADME, toxicity and other post docking studies have shown that these respective inhibitors have better lead profile with respect to others. These can be better drug candidates that inhibit the AKT pathway in cancer disease.

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