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Molecular docking studies shows tivozanib and lapatinib as potential inhibitors of EML4-ALK translocation mediated fusion protein in non small cell lung cancer

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Abstract:
Identification of activating mutations in non-small cell lung cancers (NSCLC) has been a focus in recent years. This led to successful evidence of using tyrosine kinase inhibitors (TKIs) over the standard platinum doublet based chemotherapy as the first line treatment in the metastatic setting.The rearrangements of fusion protein EML4-ALK in NSCLC lead to the use of crizotinib for this class of tumors. Preclinical and Phase 1 clinical studies show that ceritinib is more effective against both crizotinib sensitive and resistant tumors. Although robust responses to crizotinib are observed in NSCLC harboring ALK mutations, majority of tumors eventually become resistant, posing a major challenge in treatment course. Thus, there is a need for the identification and development of second-generation of ALK inhibitors. Computer aided molecular docking data show Tivozanib and Lapatinib bind EML4-ALK with high score. Tivozanib is in clinical trials for renal cell cancer and Lapatinib is a known dual tyrosine kinase inhibitor effective in breast cancer patients with HER2 over-expression. Additional data on these compounds for use in EML4-ALK positive NSCLC will provide evidence for use in patients treated with crizotinib. Data shows the importance of computer aided molecular docking in developing candidates with improved activity for further consideration in vitro and in vivo validation.

Background:
The therapeutic aspects of non-small cell lung cancer (NSCLC) have undergone a dramatic change over the last one decade due to the advent of targeted therapy. The armamentarium of active agents is expanding rapidly and several landmark trials and results of the targeted agents are being supported for their clinical use in NSCLC. Tyrosine kinases have a definite role in tumour development driving many different solid tumours including lung cancer. Research carried over the past decade has shed light on the expression of the proteins that are driven by the tyrosine kinases. There has been increased success in tyrosine kinase based therapeutic agents in several types of cancers including lung cancers.

Echinoderm microtubule associated protein like 4 (EML4) - anaplastic lymphoma kinase (ALK) is a fusion type protein tyrosine kinase found in 4-5% of NSCLC **[1-3].** The EML4-ALK fusion gene was identified as tumorigenic in NSCLC in 2007 **[1, 4].** The EML4-ALK fusion oncogene arises from a recurrent inversion on the short arm of chromosome 2 (Inv (2) (p21p23) that joins exons 1-13 of EML4 to exons 20-29 of ALK. The resulting chimeric protein EML4-ALK contains an N- terminus derived from EML4 and a C-terminus containing the entire intracellular tyrosine kinase domain of ALK. ALK rearranged NSCLC is a molecularly defined subgroup of NSCLC but is heterogenous due to the different isoforms of EML4-ALK fusion proteins and other fusion partners for ALK protein. One of such proteins was NPM-ALK protein identified as a

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neoplastic agent in anaplastic large cell lymphoma. (ALCL) **[1, 5]** ALK has been later linked to several fusion partners including EML4. The EML4-ALK fusion oncogene provides a potential target for therapeutic intervention. Cells harboring the EML4-ALK translocation are effectively inhibited by small molecule inhibitors that target the ALK tyrosine kinase **[2].**

Crizotinib is a multi-targeted small molecule tyrosine kinase inhibitor which had been originally developed as an inhibitor of the mesenchymal epithelial transition growth factor (c-MET) and is also a potent inhibitor of ALK phosphorylation and signal transduction. It was identified as a clinic ready inhibitor of EML4-ALK at a rapid pace and shown promise **[6].** Crizotinib has been identified as an orally active small molecule inhibitor of ALK and c-Met receptor tyrosine kinase. Crizotinib has been shown to be highly selective for ALK and c-Met kinases with 50% inhibitory concentrations 5-20 nM compared with values atleast 20 fold higher for other kinases **[7, 8].** Mode of action of the crizotinib is by binding to the adenosine triphosphate binding site (ATP binding site) of the ALK enzyme and preventing binding of ATP thus inhibiting its auto phosphorylation. Crizotinib has been shown highly effective as a specific inhibitor of receptor tyrosine kinase c-MET (hepatocyte growth factor receptor) and was shown as c-MET inhibitor **[9].**

However, it is currently the only approved drug available for NSCLC patients who harbor EML4-ALK translocation. In contrast to patients with EGFR mutations, patients with ALK fusion oncogene do not appear to respond to EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib or gefitinib. Therefore there is an urgent need to explore if any of the tyrosine kinase inhibitors currently in use would be effective inhibitor for EML4-ALK fusion protein as well. Therefore, in the similar lines of c-MET inhibitor identified as EML4-ALK inhibitor crizotinib we have attempted to identify tyrosine kinase inhibitors that are already in clinical use and that can find use as EML4-ALK kinase inhibitor as well by molecular docking studies.

EML4-ALK has up to 11 different variants, but the all the different variants retain the cytoplasmic portion of the ALK with truncation of EML4 at several points **[10-13].** We have therefore chosen the ALK portion of the fusion protein for the docking studies and hypothesize that the findings of the study may be suitable for all the different EML4-ALK variants.

Methodology:

Details: EML4-ALK isoform 3a and 3b were selected for the study because of the frequent expressions in different lung cancer types and also the size of the protein was compatible for in-silico studies. The structures were modeled using Ab-initio modeling approach because of the lack of suitable template structure.

Modeling of Eml4-alk isoform 3a and 3b

The protein sequence of EML4-ALK isoform 3a and 3b with accession no. BAG55003.1 of length 785 AA and BAG55004.1 of length 796AA respectively were retrieved from NCBI. The structure of isoform 3a and isoform 3b was determined using **Robetta server**. Robetta provides both *ab initio* and comparative models of protein domains. Domains without a detectable PDB homolog are modeled with the Rosetta de novo protocol **[14, 15].** Comparative models are built from template PDBs detected and aligned using locally installed versions of HHSEARCH/HHpred, RaptorX, and Sparks-X. Alignments are clustered and comparative models are generated using the RosettaCM protocol. The procedure is fully automated.

Energy Minimization and assessment of stereochemical properties

The models of Eml4-alk isoform 3a and 3b obtained from Robetta were energy minimized using Chiron an online server. Chiron performs rapid energy minimization of protein molecules using discrete molecular dynamics with an all-atom representation for each residue in the protein. The energy minimized structures were analyzed for stereochemical properties using Rampage server.

Docking studies

The docking studies were performed using Ligand Fit function of Accelry's Discovery Studio 2.4. Ligand conformations generated using Monte-Carlo techniques are initially docked into an active site based on shape, followed by further CHARMm minimization. The models of Isoform 3a and 3b were prepared for docking by adding hydrogens and atomic valencies were corrected using Ligand Fit's valence check function. The binding sites were detected using Ligand Fit's flood filling algorithm. The binding site present in the catalytic domain of the ALK region was selected for the docking purpose. Grid generated near the potential binding sites was extended to accommodate larger ligands. Crizotinib an approved drug for the treatment of NSCLC with EML4-ALK mutation was taken as the standard control for all the docking sites in isoform 3a and 3b. Flexible dockings were performed with the default parameters of Ligand Fit using Dreiding ForceField. The docked poses were evaluated with the set of Ligand Fit's scoring functions. The docking interactions were checked in the graphics window 5 (Accelrys Software Inc.,) and the 2-D interactions were plotted using Ligplot+v.1.4.

Ligand Selection

The tyrosine kinase inhibitors already in use as a drug for various types of cancer were selected for the study as shown in Table 1.The structure of the drugs were retrieved from NCBI pubchem database and the structural geometry was cleaned using Discovery studio clean geometry function.

Results & Discussion:

Modeling of EML4-ALK isoform 3a and 3b

Figure 1 shows the EML4 ALK fusion protein due to the inversion in chromosome 2. The isoforms 3a and 3b are also shown with exon variant 6a and 6b respectively. The presence of additional 11aminoacids from 224-234 in isoform 3b distinguishes it from isoform 3a. The sequences of EML4-ALK isoform 3a and 3b (accession number BAG55003.1, BAG55004.1) were retrieved from NCBI for analysis. The structures of the fusion protein isoform 3a and 3b were modeled using Robetta server. The modeled protein isoforms **(Figure 2a & 2b)** were checked for geometry and stereochemical properties using Ramachandran plot. This plot had 94.4% and 93.5% of isoforms 3a and 3b in the most favoured region respectively. The

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structure of the ALK region for both the models was well resolved making the docking studies more consistent.

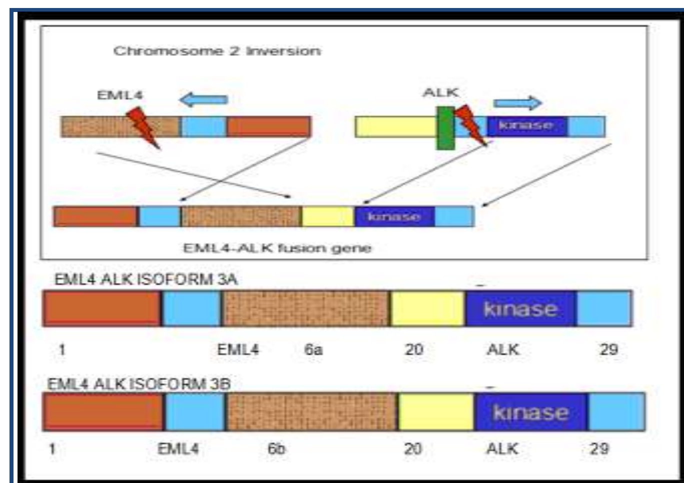

Figure 1: Schema of EML4-ALK fusion.

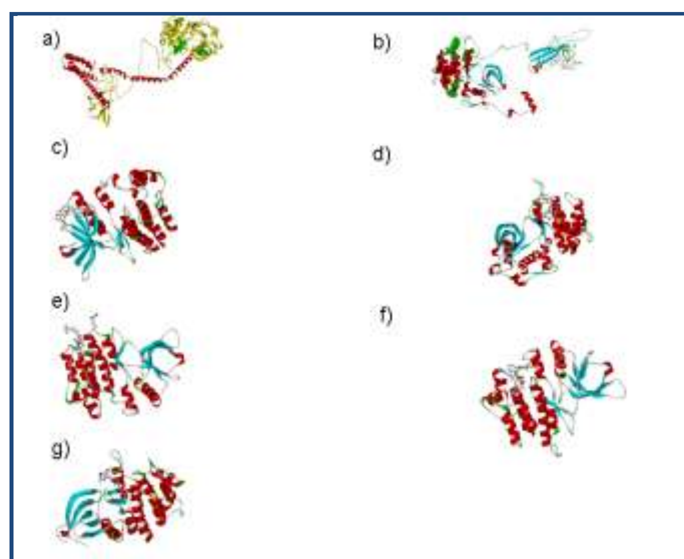

Figure 2: a) Structure of EML4-ALK isoform 3a with ALK region highlighted in yellow and binding sites in green; **b)** Structure of EML4-ALK Isoform 3b with the binding site highlighted in green; **c)** Binding of Isoform3a with Tivozanib at Site 7 with the drug represented in ball and stick model; **d)**Binding of Isoform3a with Lapatinib at Site 20 with the drug represented in ball and stick model; **e)** Binding of Isoform 3b with Lapatinib at site 9 with the drug represented in ball and stick model; **f)** Binding of Isoform 3b with Lapatinib at site 10 with the drug represented in ball and stick model; **g)** Binding of Isoform 3b with Tivozanib at Site 17 with the drug represented in ball and stick model.

Docking studies using crizotinib as a reference to identify catalytic sites

Catalytic domain of ALK region (aa 274-550) of isoform 3a and (aa 285-561) of isoform b were selected for the docking purpose. The binding site search showed 26 sites with 17/26 sites present in ALK region for isoform 3a and 28 sites with 12/28 sites present in ALK region for isoform 3b shown in **Table 1 (see supplementary material)** shows the list of tyrosine kinase inhibitors used for docking study. Initially the docking was performed on all sites present in ALK region for both isoforms using Crizotinib as a reference standard. Of all the sites, for isoform 3a - site 7, 20 and for isoform 3 b- sites 9, 10, 17 were found to be binding to crizotinib with good affinity. These sites were further selected for docking with the other TKIs from the database. The amino acid residues involved in interaction at site 7, site 20 for isoform 3a and site 9, 10, 17 for isoform 3b is shown in **Table 2 (see supplementary material)**. The Dock score of Isoform 3a and 3b for all the tyrosine kinse inhibitors are listed in the **Table 3a** and **3b (Available with author)**.

Our study identified Tivozanib and Lapatinib as potential inhibitors **(Figure 2c,d,e,f,g)** of EML4-ALK based on the consensus docking score for both isoform 3a and 3b compared to all the TKIs taken for the study **(Figure 3a,b)**. The drugs with the top 3 scores for both the isoforms were compared. For Isoform 3a site7, the binding order was Lestaurtinib < Tivozanib < Radotinib. For site 20, the binding score order was Tivozanib < Cabozanitib < Regorafenib. Tivozanib showed good consensus binding score in both the sites. For isoform 3b site9, the binding was of order Erlotinib < Lapatinib < Nilotinib. For site 10, the binding score order seen was Lapatinib < Nilotinib < cediranib. For site 17, the binding score order was found to be Tivozanib < Dasatinib < Neratinib. Lapatinib and Nilotinib were within the top 3 category for 2 sites whereas the binding of the other drugs were not consensus in all the sites. Of the 2 drugs, Lapatinib and Nilotinib, Lapatinib demonstrated a better binding score than Nilotinib. Further analysis revealed, Lapatinib showed good consensus score on all the 3 sites of isoform 3b. The interactions between the selected drugs and the EML4-ALK isoforms were demonstrated using Ligplot+v.1.4.5 **(Figure 4a,b,c,d,e)**.

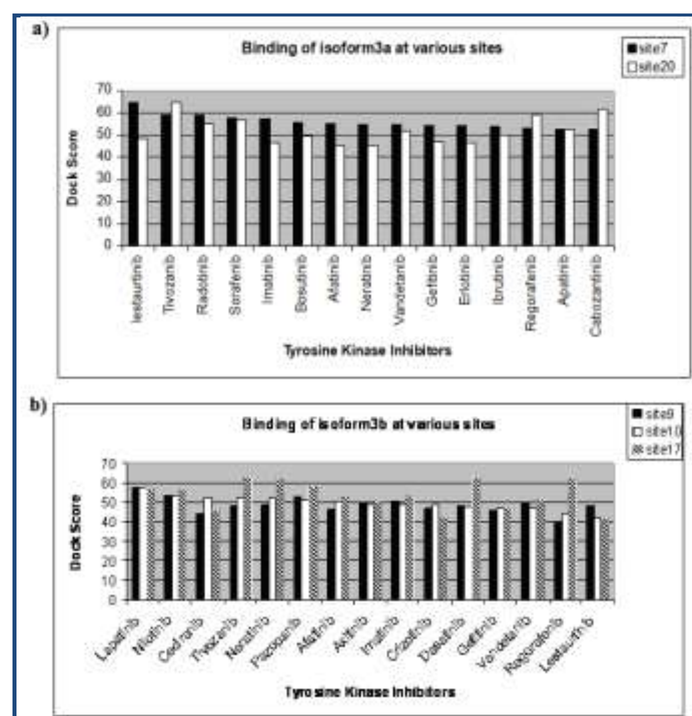

Figure 3: a) Binding of Isoform 3a with top 15 tyrosine kinase inhibitors; **b)** Binding of Isoform 3b with top 15 tyrosine kinase inhibitors.

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Tivozanib, oral VEGF receptor TKI suppresses angiogenesis by being selectively inhibiting all the three VEGF receptors **[16, 17]**. Tivozanib has been evaluated in several clinical trials including a Phase I and Phase II trial demonstrating safety and efficacy for patients with advanced clear cell renal cell carcinoma (RCC) **[18].** Treatment using Tivozanib in the phase I clinical study of patients with advanced solid tumors that were resistant to other therapies showed promising trends of decreased intratumoral vascularity with 35% of patients showing shrinkage in tumour size. Another phase III clinical trial in RCC done for Tivozanib versus Sorafenib as initial targeted therapy showed a longer progression free survival for Tivozanib **[19].** Tivozanib has been recently shown to inhibit multidrug resistance mediated by ABCB1 (p-glycoprotein) and ABCG2 (BCRP) **[20].** The other promising tyrosine kinase inhibitor binding to EML4-ALK fusion protein in this study was Lapatinib, originally identified as the first dual epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) tyrosine kinases approved by the FDA in 2007 **[21].** This drug is indicated for use in combination with capecitabine for the treatment of patients with advanced breast cancer or metastatic breast cancer with tumours that overexpress HER2 and whose previous treatment had included an anthracycline, a taxane and Trastuzumab **[22].** Lapatinib can bind to EGFR inactive form unlike gefitinib and erlotinib and therefore has a slower dissociation rate compared to other tyrosine kinase inhibitors. This is important for greater duration of the effect at the target site compared to gefitinib and erlotinib. A recent report **[23]** shows that Lapatinib in combination with trastuzumab (anti-HER2 antibody) and bevacizumab (Anti-VEGF antibody) helped achieve an impressive and durable anti-tumour activity in a heavily pre-treated NSCLC patient. Therefore use of Lapatinib in NSCLC strengthens our hypothesis that probably it should be explored further for ALK rearranged NSCLC as well. Our study has identified this dual inhibitor to be binding with a good docking score with EML4-ALK fusion protein.

Studies shows that this is the most promising oral tyrosine inhibitor that is effective in HER2 over expressing breast cancers and can probably tried for NSCLC patients harboring the EML4-ALK translocation as well in future after studying its role further. EML4-ALK fusion protein has several variants but the common aspect of all variants is the ALK portion of fusion protein that is constant comprising the ATP binding site. Therefore we docked the chosen agents in the ALK region so that the tyrosine kinase inhibitors chosen could be useful for targeting the different EML4-ALK variants with the common ALK active site. The future studies should involve biochemical assays for Tivozanib and Lapatinib to determine if this can indeed bind to the ALK region of EML4-ALK and if so the IC_{50} should be evaluated in NSCLC cell lines harboring EML4-ALK. Clinical trials are currently ongoing to evaluate the safety and antitumour activity of Tivozanib in breast, lung and colorectal cancer.

Crizotinib was originally synthesized as the MET inhibitor, at the ATP binding site of the MET kinase domain obtained from the co-crystal structure. Evaluation of Crizotinib against a panel of more than 120 kinases in biochemical assays and twelve cell based phosphorylation assays and was determined to be nearly 20 fold more selective for ALK and MET compared to other kinases evaluated. The phenomenon of acquired resistance is known for crizotinib and most frequent mutations involve the gatekeeper residue – aminoacid substitutions that can hinder the drug binding. Since the docking sites have been derived based on crizotinib as reference, it needs to be checked whether the different gatekeeper mutations identified for crizotinib mediated resistance can hinder the binding of tivozanib and lapatinib as well.

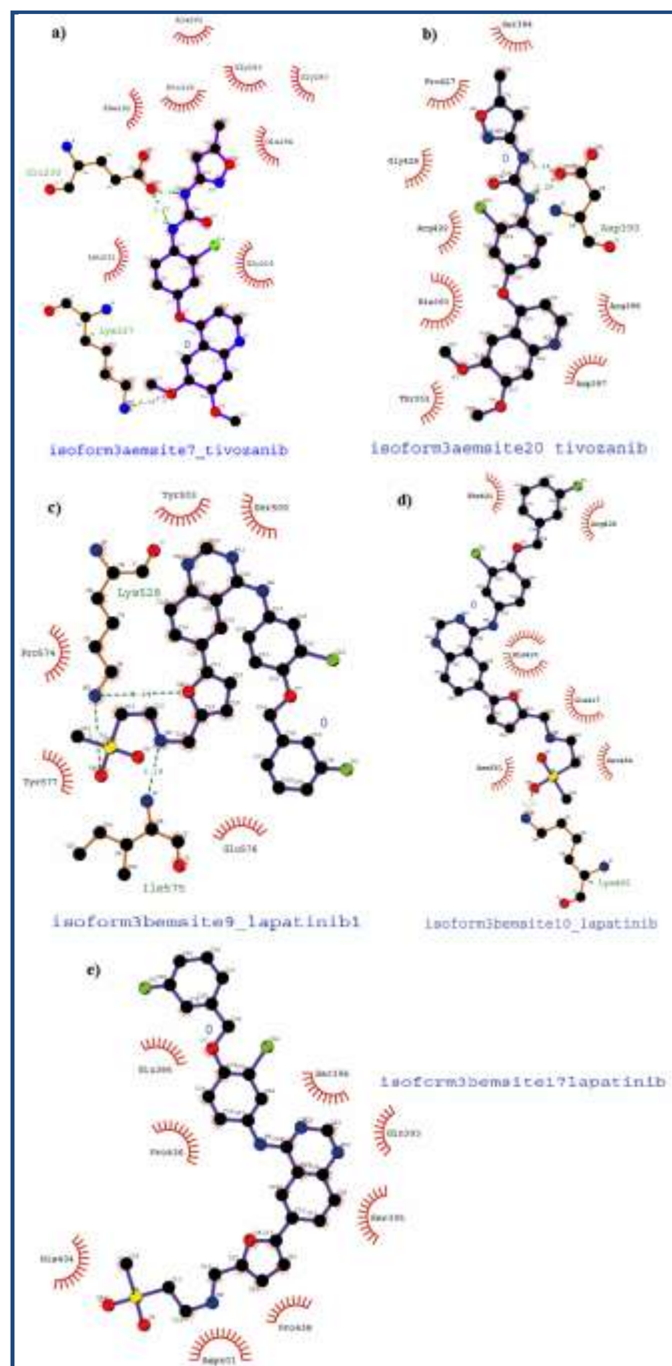

Figure 4: a) Ligplus showing interaction of Isoform3a with Tivozanib at site 7; **b)** Ligplus showing interaction of Isoform3a with Tivozanib at site 20; **c)** Ligplus showing interaction of Isoform3b with Lapatinib at site 9; **d)** Ligplus showing interaction of Isoform3b with Lapatinib at site 10; **e)** Ligplus shows interaction of Isoform3b with Lapatinib at site 17.

Conclusion:

Developing resistance to EGFR and EML4-ALK based TKIs augments the need to develop therapeutic strategies to overcome the resistance mechanisms by blocking of multiple members of ErbB family. Identification of Tivozanib and Lapatinib as potential inhibitors of EML4-ALK needs to clearly pursued further. Our study thus suggests the probable direction that could be further explored in inhibiting EML4-ALK fusion protein harboring NSCLCs.

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Supplementary material:

Table 1: Tyrosine Kinase Inhibitors used for the docking study

Drug	NCBI Accession number
Crizotinib	11626560
Imatinib	5291
Gefitinib	123631
Erlotinib	176870
Sunitinib	5329102
Sorafenib	216239
Pazopanib	10113978
Lapatinib	208908
Axitinib	6450551
Nilotinib	644241
Dasatinib	3062316
Afatinib	10184653
Diranib	9933475
Lestaurtinib	126565
Neratinib	9915743
Regorafenib	11167602
Tivozanib	9911830
Vandetanib	3081361
Bosutinib	5328940
Ponatinib	24826799
Ibrutinib	24821094
Radotinib	16063245
Apatinib	11315474
Cabozanitib	25102847
Quizartinib	24889392

Table 2: Amino acid involved in interaction of Tivozanib and Lapatinib at various sites of Isoform 3a and Isoform 3b

EML4-ALK Isoform 3 a	Site pocket residues involved in interaction
SITE 7	Y224,K227,E230,L231,F292,G293,Y294,Y296,T316,P318,E319,V320, P356.
SITE 20	S384,S385,L389,D390,H393,R396,D397,V430,P427,R429,G428
EML4-ALK Isoform 3 b	
SITE 9	W496,S500,Y503,P526,P527,K528,P574,I575,E576,Y577
SITE 10	E417,H420,F421,I422,R424,F482,T483,S484,K485,N551
SITE 17	E386,T387,Q393,S395,S396,D401,H404,P436,P438,R440

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