Chapter 5
Summary and Conclusion

CHAPTER 5 SUMMARY AND CONCLUSION

Breast cancer is one of the main reasons of mortality in women. It has been associated with an overexpression of various molecular targets like estrogen receptor, progesterone receptor, HER2, ERK1/2 and CDK4/6. One of the critical forms of breast cancer is triple negative breast cancer which lacks overexpression of ER, PR and HER2, and thus considered as more lethal cancer. Inhibition of MAPK pathway provides one possibility for the management of both ER+ and TNBC sub-types of breast cancer. Although many chemotherapeutic agents are available nowadays but due to the problems associated with MEK and RAF inhibitors, focus has shifted onto the discovery and development of ERK1/2 inhibitors. ERK (extracellular signal regulated kinases), a tail member of MAPK pathway, is involved in survival, differentiation and progression of cells. Inhibition of ERK alone or in combination with MEK have been reported to bypass resistance mechanisms offered by other members of MAPK pathway. Therefore, in this study we have focused on the development of ERK inhibitors, which should overcome many of the observed resistance mechanisms involving MAPK pathway.

Firstly, a scaffold hoping-FBDD based hybrid virtual screening protocol was employed to screen putative ERK1/2 inhibitors. Ulixertinib, a ERK1/2 inhibitor was chosen as standard drug and subjected to scaffold hopping first which resulted in identification of novel scaffolds with superior ERK1/2 inhibitory activity. The top ten molecules with novel scaffold having optimum similarity score in comparison to Ulixertinib were selected. Ligand NSC39808 exhibited best shape similarity and feature mapping scores, 0.8528 and 0.442, respectively, in comparison to other hits. All the identified hits were validated by molecular docking within the catalytic domain of ERK2 protein. Total 14 X-ray solved protein structures were found to have resolution of less than 2Å. After the cross-docking experiment, PDB ID: 6GDQ, 1.86 Å (in complex with Ulixertinib) was found to be more suitable for the docking experiment with the lowest average root mean square deviation (RMSD) and thus, selected for *in-silico* study. Molecular docking experiment revealed Ligand NSC39808 as the top hit, which was then subjected for FBDD against FDA approved

fragment library. FBDD led to identification of ten top hits, which were again validated by molecular docking analysis. Docking studies revealed that all 10 molecules demonstrated good binding energy score (Autodock docking score), even better than standard Ulixertinib. Among all, only three molecules, i.e., Ligand **6**, **8** and **10** preserved the required essential H-bond interaction with Met108, which is established as an essential prerequisite for ERK2 inhibitory potential. Further, molecular dynamic simulation of Ligand **6**, **8** and **10** was performed for a time period of 100 ns, which showed that the complexes of all the three top hits with ERK2 were stable after minor adjustment. Subsequent MM-GBSA results showed the binding energy value of -21.92 kcal/mol, -27.89 kcal/mol and -32.22 kcal/mol, for Ligand **10, 6** and **8**, respectively, indicating Ligand **8** to possess highest binding affinity among all the hits. Lastly the top three hits were subjected to pharmacokinetic predictions *via* ADMET lab, a web server. Out of the top 3 hits, Ligand **8** having thiazolidinone-pyrimidine nucleus was chosen for further designing of compounds due to its simple chemistry. Due to the hydrophobic nature of the pocket, for the inhibitors mimicking Type-II binding mode, the binding affinity is dependent on both the overall hydrophobic packing of the ligand and conserved hydrogen bond interactions with key amino acid residues such as Lys54 and Met108 in the ATP-binding domain. Therefore, a library of around 100 derivatives of thiazolidinone-pyrimidine (Ligand **8**) derivatives was prepared by incorporating diverse hydrophobic substituents at different sites on the core structure and subjected to *in silico* evaluation to filter out derivatives with improved binding affinity for ERK2, in comparison to reference (Ligand **8**). Briefly, derivatives of Ligand **8** were firstly docked in the co-crystallized structure of ERK2. All the compounds showed good docking scores (glide scores) ranging from -6.57 to -4.54 Kcal/mol within the catalytic domain of ERK2. Top scoring Ligand **8** derivatives which were maintaining crucial interactions in the binding pocket of ERK2, such as H-bond interactions with Met108 were then forwarded to MD simulation analysis. The designed molecules which, both, retained this key interaction and maintained a stable complex with ERK2 after the production

run of 100 ns were then forwarded for the calculation of binding affinity in terms of MM-GBSA score. The obtained MM-GBSA scores clearly indicated that binding affinity correlated well with the levels of hydrophobic contacts of the designed compounds in the binding pocket, ranging from -44.49 to -34.01 kcal/mol. Fifteen molecules maintaining varied levels of contact with hydrophobic amino acid residues Met108, Lys114, Leu107, Lys54, Gly34, Asp106, Ser 153, Ala35, Gly37, Ile56 along with key H-bond interactions with Met108 and Lys54 were selected for synthesis (Formula I).

As second phase optimization, another series of analogues were also designed by increasing the ring size of the five-membered ring system (thiazolidin-4-one) present in Ligand **8,** to a six-membered ring system (1,3-thiazinan-4-one), to explore the binding pocket occupancy and its effect in the inhibition of ERK2. These analogues were also investigated through similar computational parameters and top 15 molecules with key interactions were synthesized (Formula II). The peculiar observation made during the *in-silico* analysis of increased ring size derivatives was that although increasing the ring size did tighten the fitting in the pocket, it completely altered the interaction network of the derivatives from the five membered predecessors. For molecules pertaining to both Formula I and II, a three-step synthetic protocol was followed. Synthesized compounds were structurally elucidated by ^{1}H-NMR, ^{13}C-NMR and Mass spectrometry. Briefly, IR spectrum for the thiazolidinone-pyrimidine derivatives exhibited a characteristic absorption band for C=O from ~1705 to ~1715 cm^{-1} and absorption bands for aromatic C=N from ~1690 to ~1550 cm^{-1} were also observed. While for 1,3-thiazinan-4-one-pyrimidine derivatives, IR spectrum exhibited a characteristic absorption band for C=O of amide from ~1680 to ~1695 cm^{-1} and absorption bands for aromatic C=N from ~1690 to ~1550 cm^{-1} were also observed. The ^{1}H-NMR spectrum for all the 1,3-thiazin-4-one-pyrimidine derivatives showed characteristic singlet around ~8.2 ppm for one proton presents in the pyrimidine nucleus, while the ^{1}H-NMR spectrum for all the thiazolidinone-pyrimidine derivatives showed characteristic singlet from ~7.7 to ~8.2 ppm for one

proton presents in the pyrimidine nucleus. Accordingly, mass spectra also showed the presence of the expected fragmentation pattern with quasi-ion peaks at the required m/z values. Characteristic M + 2 and M + 4 values were also recorded in each mass spectrum due to presence of halogen atoms throughout compounds of both Formula I and II.

Thereafter, *in-vitro* enzymatic assay was performed against ERK2 to evaluate their inhibitory potential, using ulixertinib as standard drug. In the enzymatic assay, compound **8j**, one of the top scorers in MM-GBSA analysis, with methoxy substitution at para position of aryl ring coupled with the thiazolidinone nucleus and methoxy-aryl ring substituted on the pyrimidine ring showed most potent ERK2 inhibition with an IC_{50} = 0.347μM. Compound **10h**, having the highest binding affinity score (MM-GBSA score) amongst all the 30 designed compound, was found to be the most potent compound with an IC_{50} value of 0.103 μM. Results indicated that although increasing the ring size did improve the binding pocket occupancy but it also altered the binding conformation and hampered the previous protein-ligand interactions. Finally, the most potent 10 compounds, as per enzymatic assay, were forwarded to anti-proliferative assay against MCF7, MDA-MB-231 and A549, *in-vitro*. Against MCF7 cell line, **8j** showed most potent cytotoxicity potential with 85.42%, 64.48%, 42.76% and 30.66% cell viability at 10μM, 20μM, 30μM and 40μM concentration, respectively. Similarly, **8k** also showed commendable anti-proliferative potential with 72.72%, 53.73%, 39.77% and 21.68% cell viability at 10μM, 20μM, 30μM and 40μM concentration, respectively. Against A549 cell line, compounds **8k** and **10i** showed commendable inhibitory potential. Out of the pool, **8k** showed significant anti-proliferative potential with 89.65%, 79.64%, 65.27%, and 43.36% cell viability at 10μM, 20μM, 30μM and 40μM concentration, respectively. Compound **8k** was followed by **10i**, which also showed commendable anti-proliferative potential with 86.31%, 74.95%, 57.18%, and 43.82% cell viability at 10μM, 20μM, 30μM and 40μM concentration, respectively. Against MDA-MB-231 cell line, compounds **10d**, **10f**, **10i**, **8j**, and **8c** showed significant inhibitory potential. Out of the pool, **10d**

showed significant anti-proliferative potential with 88.56%, 65.44%, 40.70%, and 20.29% cell viability at 10μM, 20μM, 30μM and 40μM concentration, respectively. Compound **10d** was followed by **10f**, which also showed commendable anti-proliferative potential with 95.63%, 81.67%, 65.41%, and 40.79% cell viability at 10μM, 20μM, 30μM and 40μM concentration, respectively. Against all the three cell lines, **10h** showed significant inhibition with IC_{50} values of, 29.41(±2.34), 49.30 (±2.12) and 29.94 (±2.07) μM, respectively. Some of the designed compounds were found comparable to the standard Ulixertinib in anti-proliferative assay. Finally normal cell toxicity of the compounds was also determined using HBL-100 normal breast cell line. All the compounds were found to be non-toxic at 40μM, while some were non-toxic even at 80μM.

On the basis of this study, it can be concluded that targeting MAPK pathway is a useful target for the management of triple negative and other sub-types of breast cancer. Inhibiting ERK can overcome the resistance due to cross-talk and mutation in other members of MAPK pathway. To address this problem, exhaustive *in-silico* protocol was successfully implemented to identify ERK2 inhibitors. The designed molecules were synthesized and characterized *via* spectral analysis. *In-silico* results were validated by evaluating designed molecules against ERK2 kinase employing enzymatic and cell-based assays. This *in-silico* study yielded two molecules (**8j** and **10h**) with significant therapeutic efficacy against ERK2. This study is further open for exploration of the therapeutic potential *via in-vivo* studies, pharmacokinetic and toxicity profiling of designed molecules and further lead optimization exercise to improve pharmacokinetics and pharmacodynamics of the designed molecules.