ABSTRACT

Background: Breast cancer is the second most common cause of cancer-related deaths in women. It is categorized into various sub-types including ER+/PR+, which shows overexpression/upregulation in either estrogen receptor (ER) or progesterone receptor (PR), HER2+, which shows overexpression of human epidermal growth factor receptor 2 (HER2). There is another subtype that corresponds to overexpression of both ER+/PR+ and HER2 but importantly all these sub-types have their characteristic molecular targets which could be inhibited to mitigate the complications. However, there is one critical form of breast cancer that lacks overexpression of all these molecular targets (ER, PR, and HER2), known as triple-negative breast cancer (TNBC) and is considered a more lethal subtype of breast cancer. Although TNBC lacks overexpression of usual molecular targets, studies have revealed that several key signaling pathways remain overexpressed and their inhibition has proved fruitful. One such signaling pathway is the MAPK (mitogen-activated protein kinase) pathway, which provides one possible option for the management of TNBC. Additionally, due to its involvement in the downstream signaling *via* cell surface receptors such as HER2, inhibition of MAPK signaling pathways is also useful in the management of other subtypes of breast cancer (ER+/PR+ and HER+). Literature reveals that several chemotherapeutic agents focussed on different members of the MAPK pathway are available. But the molecules focussed on commonly targeted members of the MAPK pathway such as RAF (a serine/threonine-protein kinase named after rapidly accelerated fibrosarcoma), RAS (a small GTPase named after Rat sarcoma virus), and MEK (mitogen-activated protein kinase kinase) suffer from several issues including acquired mutation dependent resistance and cross-talk signaling. Therefore, hitting the tail member of the MAPK pathway, *i.e*. ERK (extracellular signal-regulated kinases), has gained an increasing interest. ERK is involved in cell survival, differentiation, and progression. Many other signaling pathways, activated by different cell surface receptors, also execute their function *via* activating ERK, which then moves into the nucleus to alter the transcription factors and translation process of different proteins. Inhibition of ERK alone or in combination with MEK has been reported to overcome the resistance offered by the upstream kinases in the MAPK pathway. Though many preclinical ERK1/2 inhibitors are reported, there is still a need to identify novel hits to increase the number of molecules in clinical trials.

In-silico approaches are one of the rational and successful ways of designing drug-like molecules. Some of the advanced *in-silico* methodologies with more focussed aims include scaffold hopping, fragment-based drug designing (FBDD), etc., which provide a valuable platform for the identification of new hits. These strategies help in diversifying the chemical space for a single pharmacological profile and also lead to enhanced clinical efficacy. Therefore, in this study, we utilized *in-silico* assisted rational drug designing approaches to design and develop ERK2 inhibitors, targeting MAPK pathway to mitigate cancer. Followed by synthesis of the designed compounds, their characterization, and biological evaluation to establish their ERK2 inhibitory potential and anti-proliferative potential against different cancer cell lines.

Methods: A systematic virtual screening protocol was designed to identify putative hits with ERK2 inhibitory potential for the management of cancer. The drug, Ulixertinib, a well-known ERK2 inhibitor, currently in phase 2 of clinical trials, was selected for exhaustive computational investigations. First, Ulixertinib was subjected to scaffold hopping exercise, which helps in replacing scaffolds with similar ones based on molecular 3D (three-dimensional) similarity calculation. In the current study, ChemMapper, a freely accessible web server, was used to perform scaffold hopping. It works *via* the identification of hits using SHAFTS, a method in which the feature of molecular shape superposition and chemical feature matching are combined. This was followed by validation of the top-scoring hits with high similarity scores *via* molecular docking analysis. The structure of ERK2 protein was retrieved from the RCSB protein data bank. Based on resolution value cut-off (2.00 Å) and cross-docking, PDB ID: 6GDQ, 1.86 Å (in complex with Ulixertinib) was selected for further analysis. The top hit was then subjected to FBDD to identify favorable substructures which could enhance the binding affinity of the top scaffold in the catalytic domain of ERK2. FBDD is a lead discovery methodology involving interlinking different bioactive fragments with each other. FBDD was carried out using ACFIS (Auto Core Fragment In silico Screening) server. Again, docking analysis was performed to validate and determine their binding affinity. Further, the top hits identified after docking analysis were subjected to molecular dynamic simulations, MM-GBSA (Molecular Mechanics Generalized Born Surface Area) calculation, DFT (Density Functional Theory), and ADMET studies. Upon completion of virtual screening protocol, out of the top three hits, one of the top hits was selected as a lead compound to optimize and improve its binding affinity for

ERK2. A library of derivatives (Formula I) of selected hit was prepared and subjected to *in-silico* evaluation to filter out derivatives with improved binding affinity for ERK2, in comparison to reference (selected hit). Analogs maintaining interactions with key amino acid residues in the ATP binding domain of ERK2 were selected and duly synthesized. In parallel, to explore the binding pocket occupancy and its effect on the inhibition of ERK2, another set of molecules (Formula II) was designed by increasing the ring size of the selected hit. Similar virtual screening parameters were considered for the second set of molecules to identify the improved hits, which were then forwarded for synthesis. A three-step synthetic protocol was utilized to synthesize the desired compounds. All the synthesized compounds were characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry. These compounds were then forwarded for the evaluation of their *in-vitro* potential as ERK2 inhibitors using a FRET-based enzymatic assay. Considering the predicted activity of the designed compounds against ERK2, three concentration points (0.1 µM, 1 µM, and 10 µM) were chosen for the enzymatic assay. The top molecules were then evaluated for their anti-proliferative potential by performing the MTT assay using MCF-7, MDA-MB-231, and A549 cell lines. The toxicity of the compounds was determined against normal breast cells using the HBL-100 cell line.

Results: A hybrid scaffold hopping-FBDD based approach was employed to identify novel putative ERK1/2 inhibitors. The catalytic domain of the ERK2 was explored by binding different fragments obtained from databases onto a new scaffold identified *via* scaffold hopping of Ulixertinib. Top hits showed good binding affinity, similar to Ulixertinib, and also maintained the key interaction with residue Met 108. MD simulations validated both the binding mode and stability of the formed complex between ERK2 and identified hits, thereby supporting the claim. *In-silico* study resulted in the identification of three hits Ligand **6**, Ligand **8**, and Ligand **10**. All the three hits, Ligand **6**, **8**, and **10**, preserved the required essential H-bond interaction with Met108, parallel to that of Ulixertinib, which is established as an essential prerequisite for ERK2 inhibitory potential. Out of three hits, Ligand **8** containing thiazolidinone-pyrimidine scaffold was selected as a lead compound for further designing of derivatives. A library of derivatives was generated and subjected to structure-based drug designing experiments (molecular docking, dynamics, and binding affinity calculations). Out of all derivatives present in the library, a total of 15 molecules (**8a**-**8o**) were found to possess equal or more than equal docking scores

(glide docking scores) in comparison to the lead (Ligand **8**), ranging from -6.57 kcal/mol to -4.54 kcal/mol, within the catalytic domain of ERK2. Similarly, from the second library of compounds bearing thiazinanone-pyrimidine scaffold, a total of 15 molecules (**10a**-**10o**) were found to exhibit a good docking score, ranging from -7.18 kcal/mol to -4.33 kcal/mol, within the catalytic domain of ERK2. The RMSD plot of the designed compounds in the complex with the protein revealed complexes to be fairly stable. MM-GBSA calculations scores indicated that binding affinity correlated positively with the degree of hydrophobic interactions between the designed compounds and catalytic domain of ERK2. ADME results revealed that the compounds possess a favorable drug-likeness profile with a maximum of two violations per molecule for Lipinski's rule of five. A total of 30 compounds were synthesized and characterized by spectral techniques. The ^{1}H-NMR spectrum for all the thiazolidinone-pyrimidine derivatives showed a characteristic singlet from ~7.7 to ~8.2 ppm for one proton present 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 the presence of -Cl and -Br atoms throughout the series. While the ^{1}H-NMR spectrum for all the thiazinanone-pyrimidine derivatives showed characteristic singlet around 8.2 ppm for one proton present in the pyrimidine nucleus. Mass spectra also showed a similar pattern of M+2 and M+4 in each mass spectrum due to the presence of -Cl and -Br atoms throughout the series. *In-vitro* ERK2 enzymatic study results revealed that compound **8j** exhibits the highest ERK2 inhibitory activity with IC_{50} = 0.347μM among all synthesized thiazolidinone-pyrimidine derivatives (Formula I). The results correlated well with the *in -silico* analysis of the molecules. Except for one or two outliers, in general the compounds predicted to bind well as per the MM-GBSA analysis showed good potency in the biochemical assay. From Formula II (thiazinanone-pyrimidine derivatives), compound **10h** was found to be the most potent compound with an IC_{50} value of 0.103μM. In general, the packing of compounds improved upon increasing ring size but bulky substituents led to steric clashes and therefore unsubstituted or compounds with smaller substituents showed better potency. The anti-cancer evaluation revealed compound **8j**, **8k**, **8l**, **10f**, and **10h** were found to possess potent anti-proliferative potential with IC_{50} values of 18.51 (±2.84), 20.09 (±2.64), 19.29 (±2.54), 27.10 (±3.02) and 29.41 (±2.34) μM, respectively against MCF7 cell line. Against A549 cell

line, **8k**, **10i**, and **10h** were found to possess significant inhibitory potential with IC_{50} values of 32.63 (±2.44), 34.43 (±1.89), and 49.30 (±2.12) µM, respectively. Against MDA-MB-231 cell line, **10d**, **10i**, and **10h** were found to possess significant inhibitory potential with IC_{50} values of 21.78 (±1.25), 24.80 (±1.51), and 29.94 (±2.07) µM, respectively.

Conclusion: This research work encompassed *in-silico* assisted designing of novel ERK2 inhibitors. One of the top hits identified after rigorous computational exercises was further optimized to increase potency and binding affinity, resulting in two (**8j** and **10h**) derivatives with sub-micromolar ERK2 inhibitory potential, along with optimal anti-proliferative activity. Overall, the work led to the identification of diverse pyrimidine based small molecule heterocycles as potent ERK2 inhibitors using *in-silico* approaches. This work may guide researchers in utilizing *in-silico* protocols to identify small molecule heterocycles for other targets. Also, researchers can carry forward exploration of these hits to develop more potent ERK2 inhibitors with anti-cancer potential.