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Original Article

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Three-Pronged Approach to Curb Cancer Metastasis

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ABSTRACT

BACKGROUND & OBJECTIVE: Extracellular signal-regulated kinases 1 and 2 [ERK1/2] have been reported to promote cancer spread through receptor tyrosine kinase (RTK)/Ras/Raf/MEK/ERK1/2 pathway hyperactivation. The extracellular signal-regulated kinase ERK5 has also been linked to cancer. However, inhibition of ERK1/2 has been reported to cause compensatory hyperactivation of the ERK5 pathway. Therefore, there is a need for simultaneous inhibition of this trio by a common inhibitor. This study aimed to find a novel common inhibitor for ERK1, ERK2 and ERK5, with a special focus on phytochemicals.

METHODOLOGY: All the available co-crystallized inhibitors of MEK1, ERK1/2 and ERK5 were used as references for 2D search across zillions of compounds. One hundred molecules with the best matching pharmacophores were extracted per virtual chemical space. A total of 20,000 new structurally diverse chemical entities with scaffold hopping ability were sifted out from these chemical spaces. Virtual screening of ERK1/2 and ERK5 was performed against these compounds. The successfully docked molecules with estimated affinities less than 500 nm were filtered. These filtered protein-molecule complexes of ERK1/2 and ERK5 were exported as Excel sheets, which were then compared to find any overlapping inhibitors. Four novel common/overlapping potential inhibitors were identified. Their pose views were generated, and binding interactions were analyzed. These novel compounds were compared for their absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) properties.

RESULTS: The molecules m240690bcc215667167368734, rxn109fEMOL37110279EMOL314046334 and LIND027BT1904LN00213276AK0086 showed good binding affinities to the conventional ATP binding pockets of the kinases ERK1/2 and ERK5.

CONCLUSION: These novel compounds may be proposed as potential common inhibitors of ERK1, 2 and 5. Further in silico analysis and in vitro testing of proteins are required to confirm their inhibitory potential.

KEYWORDS: ERK1/2, (RTK)/Ras/Raf/MEK/ERK1/2, ERK5, Metastasis, MAPK kinase kinase (MAPKKK) Raf.

INTRODUCTION

Cancer metastasis poses a challenge for the scientific community. Cancer cells undergo nonlinear dissemination from focal lesions for seeding and colonization of distant sites with the help of a series of overlapping cascades. Protein kinases have been found to be crucial for cancer metastasis. This study aimed to find a common inhibitor for three protein kinases [ERK1, ERK2 and ERK5] implicated in cancer spread. The distinct cascades of extracellular signal-related kinases (ERK1/2) and ERK5 merge into the collective mitogen-activated protein kinase (MAPK) signaling pathway [1]. ERK1/2 and ERK5 are isoforms of classical MAPK. Both of these proteins possess the Thr-Glu-Tyr (TEY) activation motif and a similar N-terminal domain. However, ERK5 is double the size, with a unique extended carboxy terminal domain that contains the transcription activation domain (TAD). ERK5 can autophosphorylate its TAD, thereby controlling gene expression directly [2].

ERK1/2 are present inside the cytoplasm and mediate the processes of cell proliferation and apoptosis through receptor tyrosine kinases (RTKs) on the cell membrane [3]. RTKs are activated by growth factors, cytokines, mitogens, hormones, and oxidative or heat stress [4]. The activation of RTKs triggers signal transduction by engaging the SOS (son of seven less). SOS activates the conversion of GDP to GTP and subsequent stimulation of the adapter protein growth-factor-receptor-bound-2 (Grb2). Gb2 in turn stimulates Ras.

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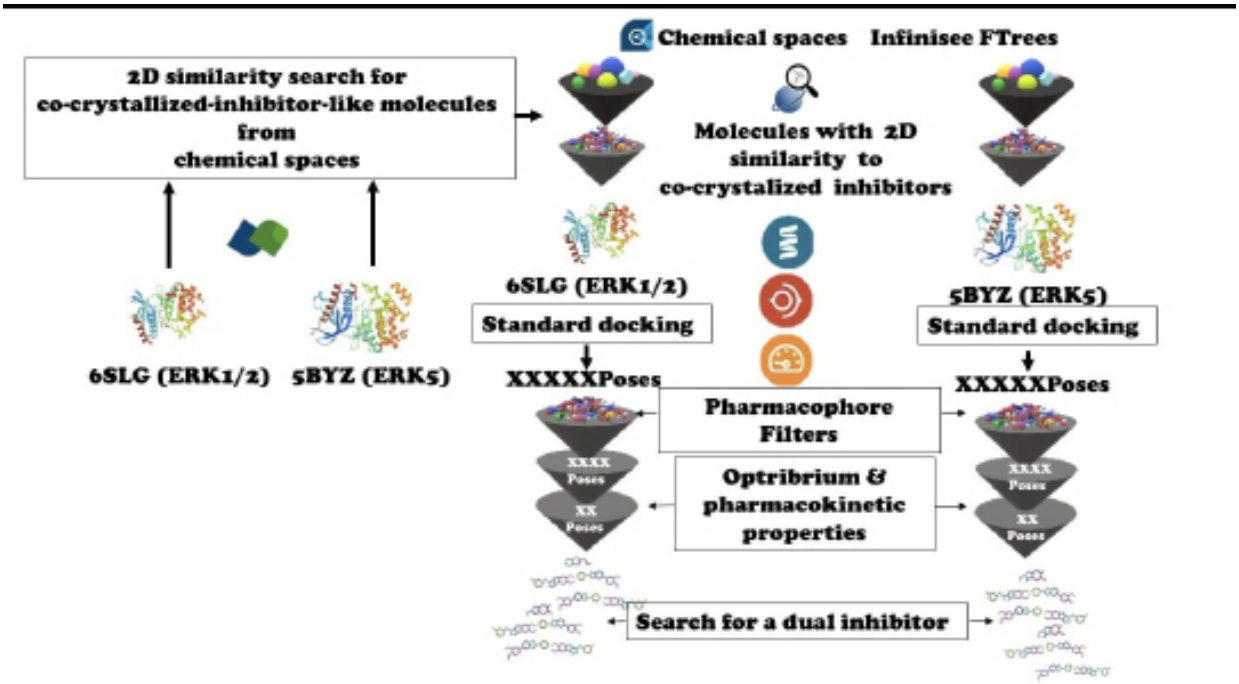


Figure-I: Graphical Abstract.

Figure-I depicts the execution of work starting from finalization of protein structures to mining of molecules with 2D similarity to known inhibitors followed by high throughput screening using standard docking protocol.

Activated Ras, in addition to interacting with a wide range of downstream effector proteins, induces a conformational change in the MAPK kinase kinase (MAPKKK) Raf. Raf moves to the plasma membrane [5]. Activated Raf phosphorylates MAPKK (MEK1/2), leading to its activation, which then phosphorylates and activates MAPK (ERK1/2). These activated ERK1/2 proteins translocate to the nucleus and phosphorylate and activate multiple transcription factors, which in turn regulate gene expression, mitosis, embryogenesis, cell differentiation, movement, metabolism and apoptosis. The MAPK (RAS/RAF/MEK/ERK) pathway has a defining role in cell survival [6].

ERK5 mediates cell proliferation by inducing molecules linked to the cell cycle [7]. Phosphorylation of the TEY motif of ERK5 by MEK5 activates the ERK5 kinase domain. This activated kinase domain mediates phosphorylation of the C-terminal half at multiple residues, resulting in a conformational change. As a result of the conformational change, the nuclear localization signal (NLS) becomes exposed, leading to dissociation from Hsp90 and its translocation into the nucleus [8]. Once inside the nucleus, ERK5 can directly or indirectly (through the TAD in its C-terminal tail) phosphorylate a variety of transcription factors.

Under normal physiological conditions, ERK1/2 inhibits ERK5 activation [9]. ERK1/2, MEK1/2 and ERK5 have clear roles in cancer spread.

METHODOLOGY

The search terms "ERK1/2 ERK5 and cancer metastasis" were searched in https://pubmed.ncbi.nlm.nih.gov/ and Google Scholar https://scholar.google.com/ for literature search. It was an in silico study where the database https://www.rcsb.org/ was used for acquisition of crystal structures of MAP kinases of interest. The known inhibitors of these kinases were used as templates to mine compounds with diverse structures but the same warheads/pharmacophores using the infinisee tool/chemical space navigator of http://www.biosolveit.com/. A library of 20000 compounds was retrieved from infinisee. This library was subjected to qualitative structure-activity relationship analysis through high-throughput screening and lead optimization using the drug design dashboard (SeeSAR) of http://www.biosolveit.com/. Recently, Zaib S et al. used the same software for the identification of selective and potent monoamine oxidase-B (MAO-B) inhibitors [10].

Ethical approval for the current study was taken from University of Karachi under reference number IBC kU-2932022

Figure-II represents the step-by-step approach and the milestones achieved. Milestone-I was the creation of a library of 20,000 new structurally diverse chemical entities possessing warheads for the binding sites of MAP kinases of interest. The second milestone was high-throughput screening, and milestone three was the discovery of overlapping inhibitors.

Crystal structures of ERK1/2 (6SLG) ERK5 (5BYZ) were used. Before docking, 5BYZ was closely analyzed to appreciate its hinge region, p-loop and activation loop with help from the published work of Chen H et al [11], according to which the salt bridge between Lys84 (β3 strand) and Glu102 (αC helix) defines active ERK5. Furthermore, the sites

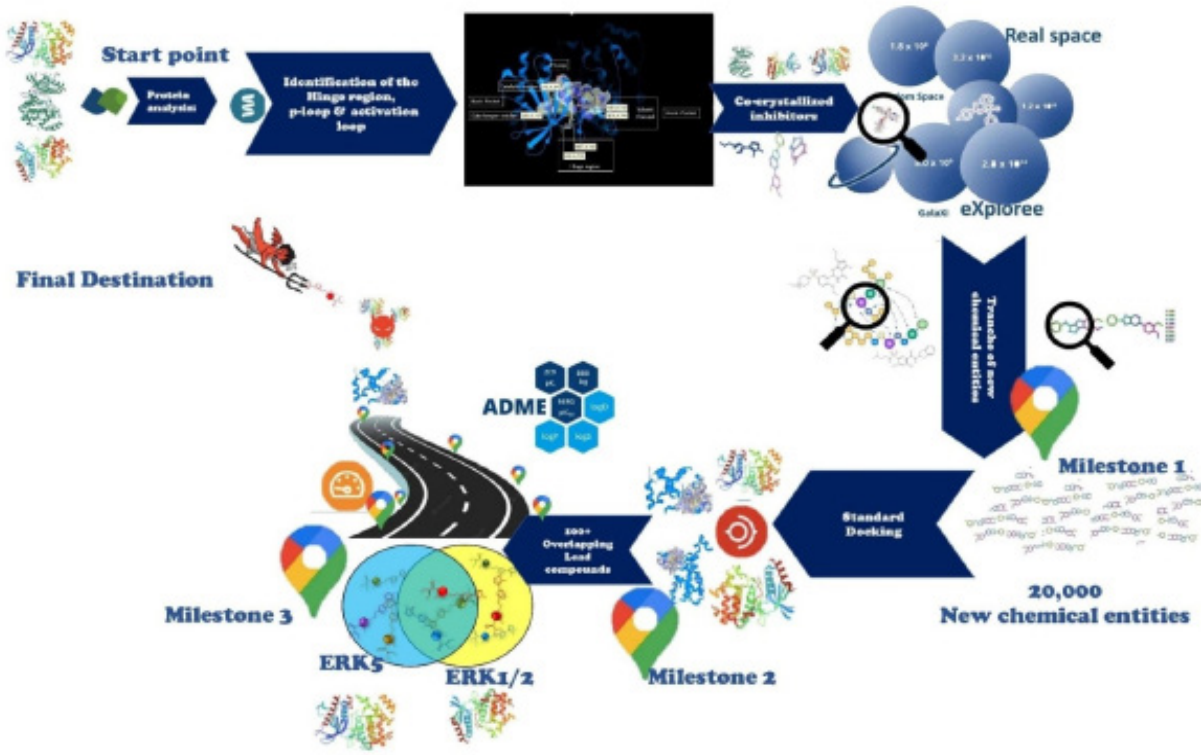


Figure-II: Graphical representation of methodology.

occupied by reported inhibitors were thoroughly analyzed. The conventional ATP binding site was marked with help of its reported active amino acid residues, including the gate keeper residue Leu137, solvent channel (Asp143 & Gln146), catalytic Lys84 and hinge region Met140 and Leu139 in the ATP binding site. Furthermore, the allosteric binding site located in a recess between the p-loop and αC helix, with Lys84, Val68, Glu102, Arg98 and Tyr66, was identified with help of the literature [11].

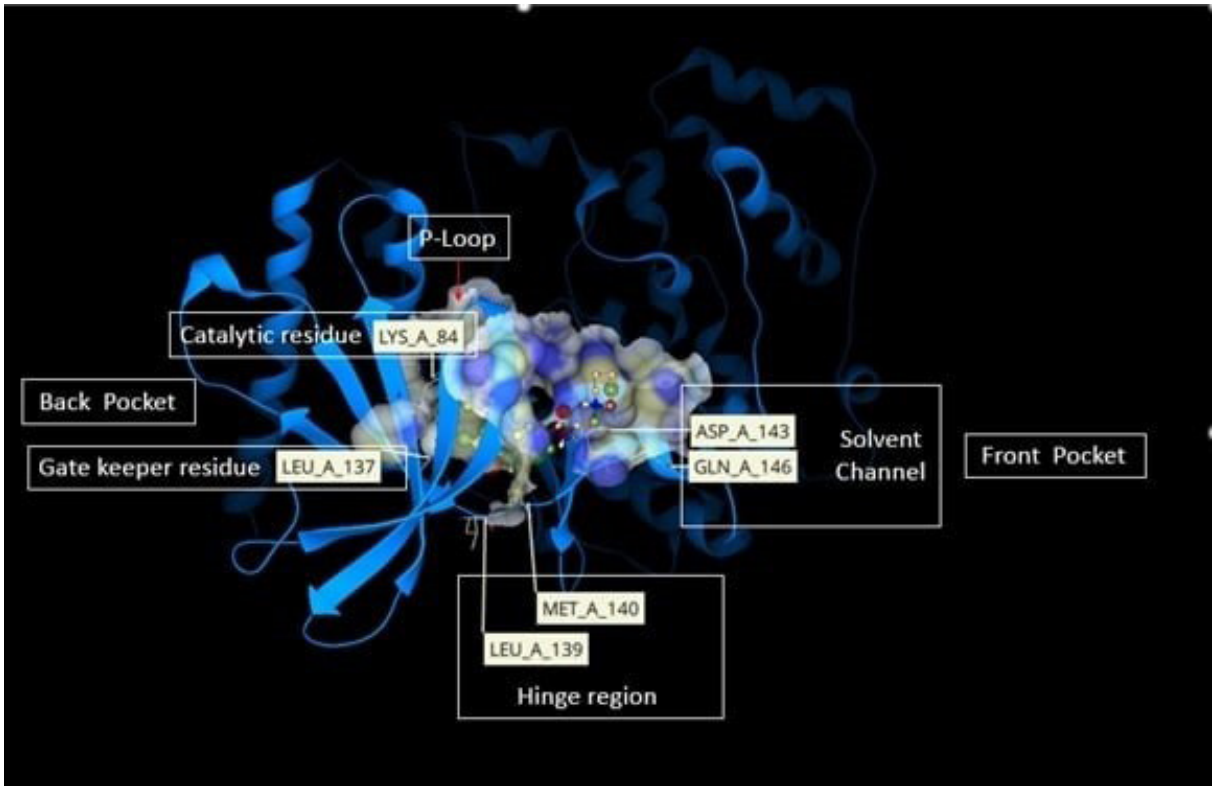


Figure-III: ERK5 (5BYZ) ATP binding site.

Figure-III shows active amino acid residues occupying the ATP binding site of ERK5.

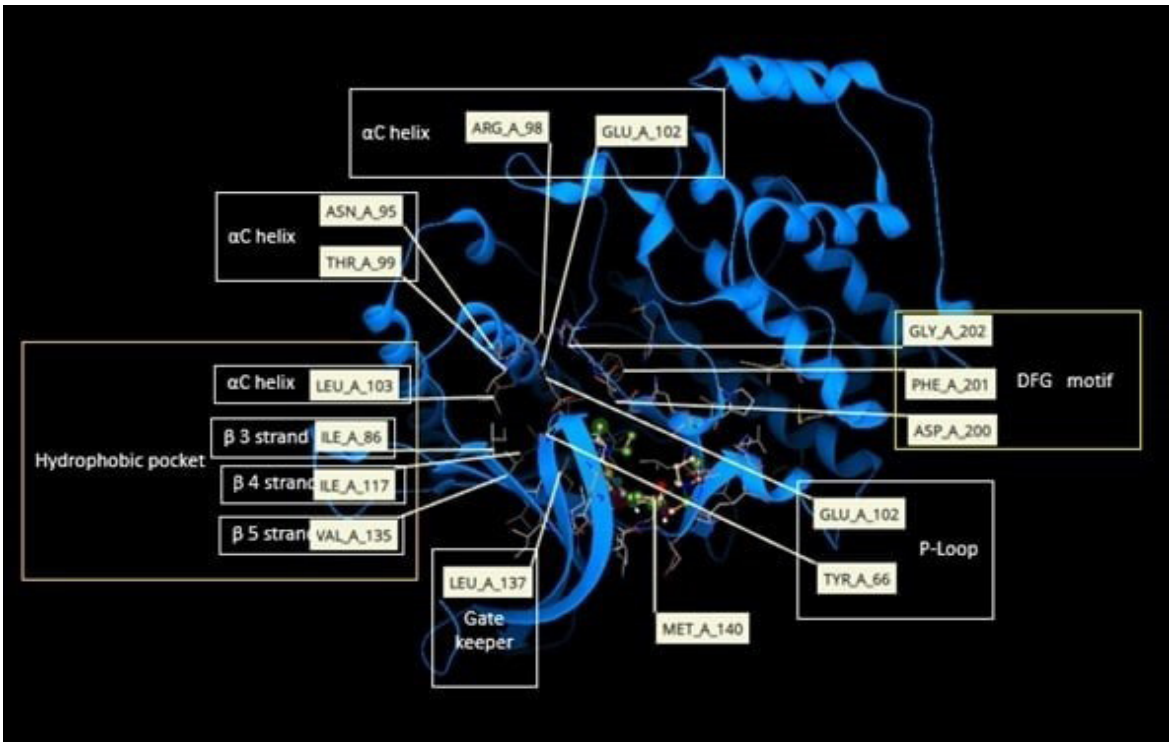


Figure-IV: The allosteric binding site depicted by the p-loop and αC helix.

Figure-IV shows the active amino acid residues in the allosteric binding site of ERK5.

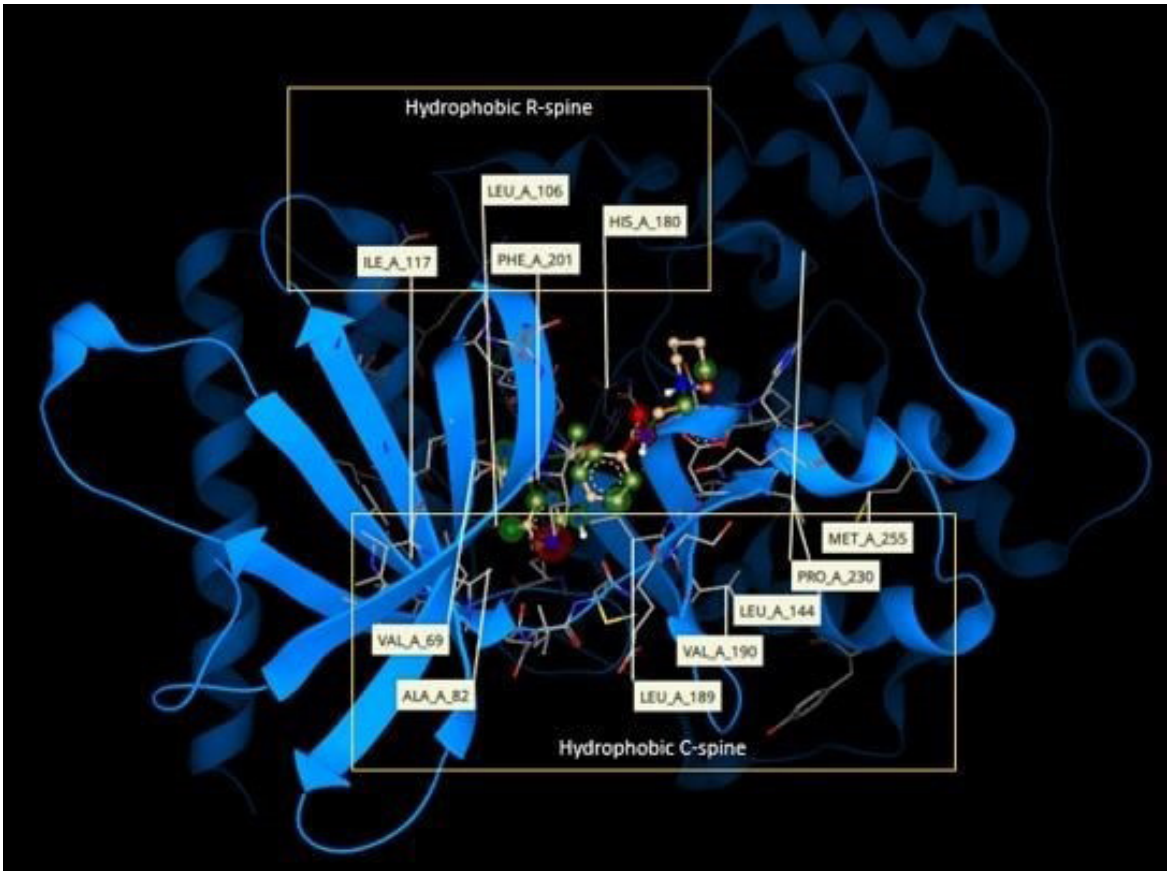


Figure-V: ERK5 hydrophobic spine in the binding pocket.

Figure-V represents the hydrophobic amino acid residues. Hydrophobic R- and C-spines were identified because some conventional and allosteric inhibitors have been shown to bind ERK5 in which the hydrophobic R- and C-spines are intact [11]. Next, pose views for ERK1/2 and ERK5 and their co-crystallized ligands were generated by importing their PDB IDs 6SLG and 5BYZ into the www.proteins.plus.com Next, a library of structurally diverse potential inhibitors was sifted from chemical spaces. The following protocol was used: The known inhibitors of these kinases were retrieved from the protein data bank www.rcsb.org [11,12] and PubChem https://pubchem.ncbi.nlm.nih.gov/ [13].

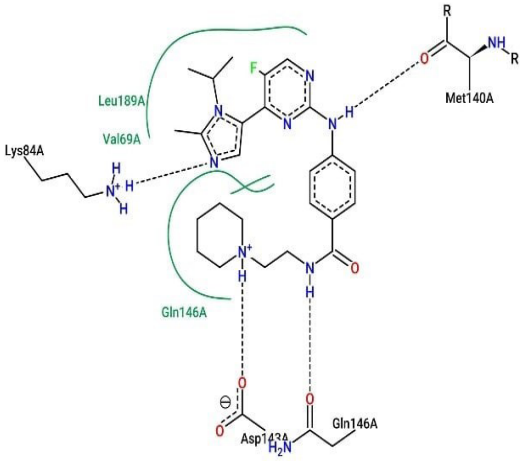


Figure-VI: Pose view 5ERK

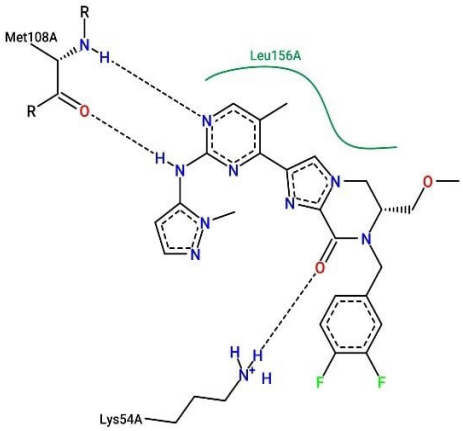


Figure-VII: Pose view ERK1/2

Figure-VI represents the pose view for [5BYZ] and the co-crystallized ligand. Fig-VII shows the pose view for [6SLG] and the co-crystallized ligand.

The following -crystallized inhibitors were taken from protein data PDB IDs: ERK5 [5BYZ, 5BYY, 4ZSG, 4ZSJ,4ZSL,4B99], ERK1/2[6SLG], MEK2[1S9I], MEK1[7JUS,1S9J] and related kinases interleukin-2-indicible T-cell kinase ITK[4M0Y] and cyclin-dependent kinase-2 CDK2 [3PY1,3PY0]. Furthermore, the reported inhibitors for the following kinases were obtained from PubChem. ERK1/2 [(SCH772984), (SCH900353), (ulixertinib/BVD-523), (GDC-0994/ravoxertinib), (LY-3214996), and (CC-90003), (AZD0364)], as well as drugs that target the V600E mutation of BRAF oncoproteins (vemurafenib) and (dabrafenib)], BRAF and MEK inhibitors [(trametinib), (suleminitib)], and MEK1 inhibitor (cobimetinib). The structures of these inhibitors were drawn with help of the eesketch tool of infinisee and subjected to a 2D similarity search, with their pharmacophores acting as catch points to search and navigate the chemical spaces in infinisee software http://www.biosolveit.com/ [14]. The search parameters were tweaked to 100 results per space with a similarity scale of 0.7 to 0.9.

The following chemical spaces were searched [afronp subset of ZINC database, consisting of phytochemicals from African medicinal plants], REAL space ($3.2x10^{10}$ molecules), Galaxi space (8x109 molecules), FreedomSpace ($1.5x10^{8}$ molecules), CHEMriya ($1.2x10^{10}$ molecules), knowledge space ($2.9x10^{14}$ molecules), and DrugBank consisting of 11000 molecules. In summary, all the known inhibitors of ERK1/2, ERK5 and MEK1 were used as templates for sifting. Pharmacophores were used as reference points for the search. A cache of 20,000 compounds was retrieved from zillions of compounds in virtual chemical spaces. These compounds were then docked separately in batches of 5000 against the PDB ID for ERK1/2 [6SLG] and ERK5[5BYZ].

Keeping in mind the structural similarities of these ERK1/2 &5 kinases. The primary purpose of this search was to sift structurally diverse molecules with scaffold hopping abilities due to the possession of pharmacophore warheads.

Next, the crystal structure of 5ERK [5BYZ] was imported into the SeeSAR docking interface, and the binding site was defined by its co-crystallized ligand 4 WE. Standard docking was performed by loading the molecules from new chemical entities in batches of 5000. The same library of molecules was then docked against 6SLG with standard docking. The docked molecules were shortlisted by applying a filter for an estimated affinity of less than 500 nm. These shortlisted docking results for ERK1/2 and ERK5 were exported as two separate Excel sheets. These were then compared for any common overlapping inhibitor.

Next, property plots were constructed for these potential inhibitors for molecular weight [MW], LogP, topological polar surface area [TPSA], estimated affinity, LE, LLE, torsion quality, intramolecular clash, intermolecular clash, odd torsions, H-bonds, acceptors, donors, heavy atoms, aromatic atoms, N & O, rings, aromatic rings, maximum ring size, halogens, stereocenters, stereo bonds, rotatable bonds, total charge, 2C9pKi[cytochrome p450Ki prediction], BBB log[an indicator of CNS penetration], LogD[logarithm of n-octanol-water partition coefficient at physiological 7.4, which describes the relationship between lipophilicity and hydrophilicity of an ionized compound], LogS[logarithm of intrinsic aqueous solubility in µM for neutral compounds], logSpH7.4[logarithm of intrinsic aqueous solubility in µM for ionizable compounds].

2D structures of proposed inhibitors				
Proposed Inhibitors	LIND027BT0263LN00170235AN4197	m240690bcc215667167368734	rxn109fEMOL37110279EMOL314046334	LIND027BT1904LN00213276_AK0086
Pose view				
Binding Pockets 5BYZ [ERK5]				
LogP MW & TPSA triangle				
Property Plot				

Table-I: The Proposed inhibitors for 5BYZ [ERK5].

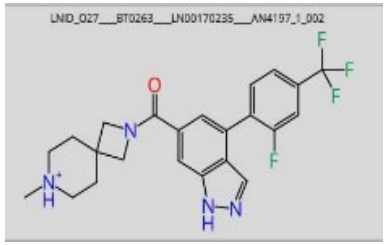
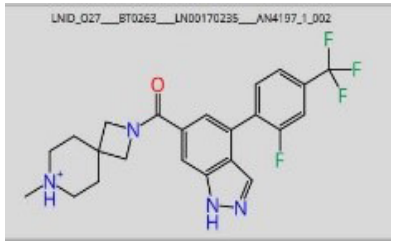
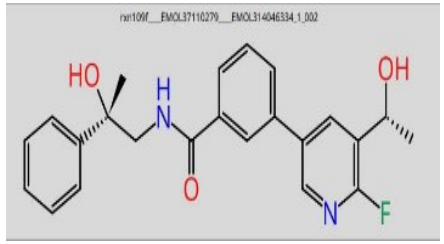
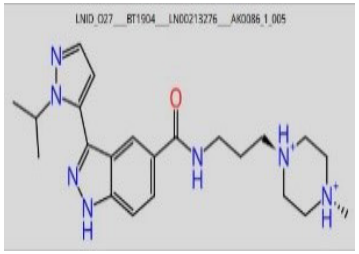
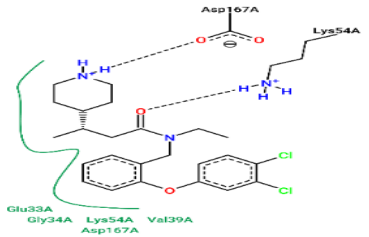
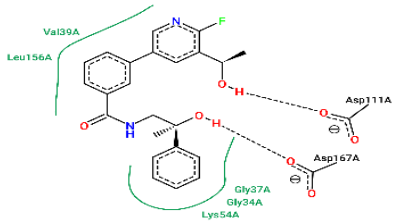
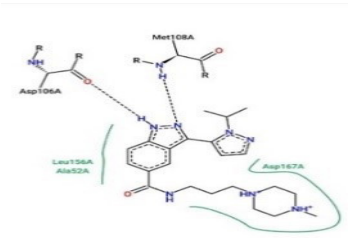
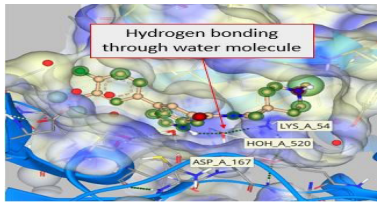
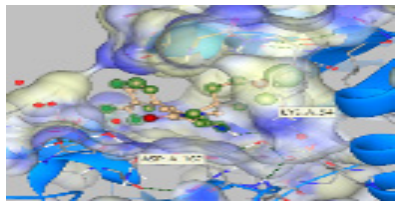
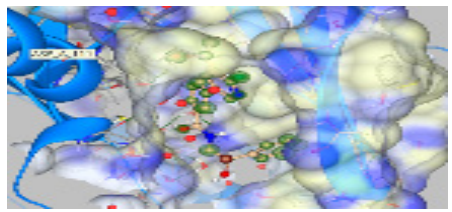
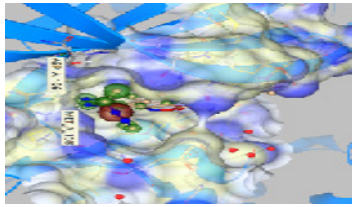
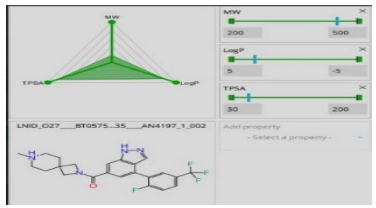
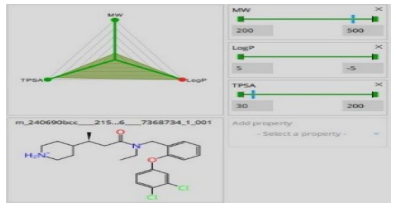
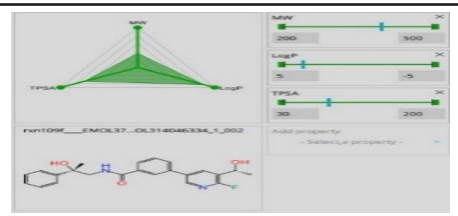
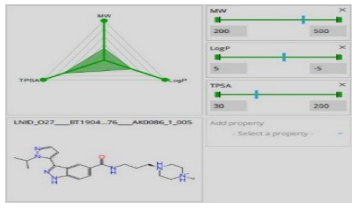
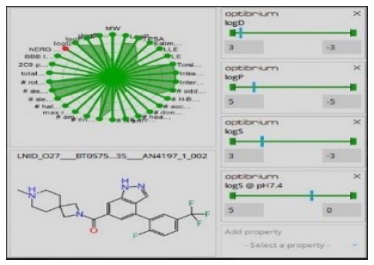
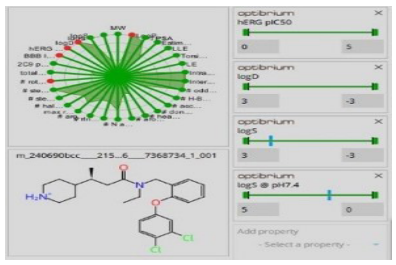
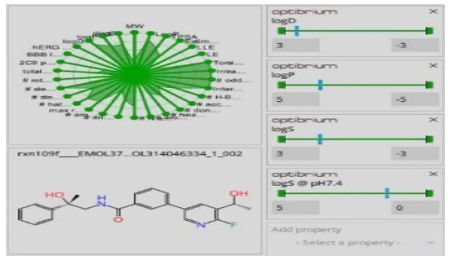
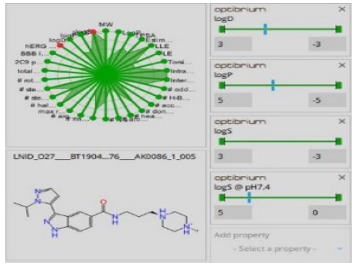
2D structures of proposed inhibitors				
Proposed Inhibitors	LIND027BT0263LN00170235AN4197	m240690bcc215667167368734	rxn109fEMOL37110279EMOL314046334	LIND027BT1904LN00213276_ AK0086
Pose view	Pose view could not be generated			
Binding site 6SLG [ERK1/2]				
LogP MW & TPSA triangle				
Property plot				

Table-II: The Proposed inhibitors for 6SLG [ERK1/2].

For 5BYZ & m240690bcc215667167368734, except for LogP, LogD, number of rotatable bonds, BBB log, and hERG, all properties appeared in the favorable range. For 5BYZ & rxn109fEMOL37110279EMOL314046334, except for the number of H bonds, all properties appeared fine. For 5BYZ and LIND027BT1904LN00213276AK0086, except for odd torsions, inter and intramolecular clashes, and hERG, all properties appeared in the favorable range.
For 6SLG and LIND027BT1904LN00213276AK0086, except for hERG and LogS, all properties appeared in the favorable range.

RESULTS

After lead optimization using the inbuilt tools for absorption, distribution, metabolism, excretion and toxicity [ADME-Tox] properties, four novel molecules emerged as dual inhibitors for the ATP binding sites of ERK1/2 and ERK5. Their greatest advantage was the occupancy of the ATP binding site because although ERK5 appears to have an allosteric binding site, inhibition of the allosteric site of ERK5 signaling with small molecules is challenging because of the paradoxical activation of the transcription activation domain [15]. ATP may be used as a positive control for molecular dynamic simulations in future studies.

DISCUSSION

MEK1/2 inhibitors can inhibit both the ERK1/2 and ERK5 pathways+nd MEK2 have the ability to phosphorylate the T-E-Y motif in the kinase activation loop of ERK1/2, resulting in their activation. MEK1 dysfunction is strongly linked to cancer [18].

The RAF-MEK1/2-ERK1/2 signaling pathway regulated by RAS is commonly dysregulated in human cancers. In addition to MEK1/2 inhibitors, ERK1/2 inhibitors have also been developed, but recent evidence suggests that parallel activation of ERK5 may be responsible for resistance to inhibitors of MEK1/2 and ERK1/2. The transcription factor MYC has been implicated in pancreatic cancer growth. ERK1/2 helps mutant oncoproteins upregulate MYC. This makes ERK1/2 inhibition a lucrative therapeutic target. However, ERK1/2 inhibition has been shown to prevent suppression of MYC because of a compensatory increase in EFGR-SRC-ERK5 signaling. Vaseva et al, [19] recently demonstrated that combined inhibition of ERK1/2 and ERK5 leads to synergistic loss of MYC and suppression of pancreatic cancer growth. Furthermore, Jiang W et al [20] demonstrated that increased ERK5 expression in lung cancer is linked to metastatic and invasive potential. There are reports about the overexpression and upregulation of ERK5 in several advanced stage tumors with metastasis and poor prognosis. It has been recently demonstrated that inhibition of MEK5/ERK5 reduces the proliferation of lung cancer cells [21]. Downregulation of DUSP6 by miRNA-211 has been reported to increase ERK5 phosphorylation with resistance to inhibitors of ERK1/2 [22]. In addition, the pharmacological inhibition of ERK1/2 in melanoma cell lines has been reported to cause significant upregulation of ERK5 [23].

Further abrogation of ERK1/2 in colorectal cancer has been shown to stimulate compensatory activation of ERK5 [24]. Lee B and Shahoo A demonstrated that downregulation of DUSP6 in non-small cell lung carcinoma (NSCLC) cell lines resulted in increased ERK5 activation and enhanced epithelial-mesenchymal cell transition (EMT)[22]. ERK5 plays a role in cancer migration and metastasis. ERK5 is an important regulator of cytosolic Akt, p90RSK, SGK and focal adhesion kinase (FAK) involved in cytoskeleton remodeling. The link between the MEK5/ERK5 axis and the invasive capability of triple-negative breast cancer (TNBC) has been recently established [25]. The inhibition of ERK5 has been reported to upregulate p27 and p15, leading to a reduction in the proliferation rate and an increased number of cells in G0/G1 phase in hepatocellular carcinoma [26]. It has been demonstrated that pharmacological inhibition of both ERK1/2 and ERK5 decreases the proliferation rate and tumor growth [24]. Recently, ERK5 has emerged as a promising therapeutic target because of its role in tumorigenesis and tumor malignancy [27].

Both the ERK1/2 and ERK5 pathways are linked to cancer spread. Therefore, inhibitors have been developed for MEK1/2, ERK1/2 and the related oncoprotein BRAF. MEK1/2 inhibitors are sensitive to MEK1/2 inhibitors, but ERK5 is less sensitive [12]. Recent evidence also suggests that parallel activation of ERK5 may be responsible for resistance to inhibitors of BRAF/MEK and ERK1/2. Binding of the allosteric inhibitor has been shown to push away the P-loop from the αC helix, leading to blockade of the ATP-binding site. Chen et al. verified competitive inhibition of ATP binding by allosteric inhibitors. Some conventional ERK5 inhibitors form hydrogen bond interactions with the hinge region Met140 and the solvent channels Asp143 and Gln146.

This study aimed to find some potent common inhibitors for ERK1/2 and ERK5 through exploitation of their structural similarities, with a special focus on phytochemicals. However, no overlapping phytochemical inhibitors were found. However, four synthetic chemicals showed overlapping inhibition.

Out of these compounds, LIND027BT0263LN00170235 AN4197 bound to the conventional ATP binding pocket of ERK5 through hydrogen bonds with Asn187A, Asp138A, Met140A and Asp138A, which is adjacent to the gatekeeper residue Leu139A.

Its hydrogen binding with Asp138A and Met140A implied that it binds at the hinge region of the ATP binding site, and the hydrophobic interactions with Tyr66A [p-loop and hydrophobic spine], Val69A, Ile61A and Leu189A, Ala82A [hydrophobic interactions with 82A reflected that it is in close proximity to the catalytic residue Lys84A]. In addition, it apparently bound to the ATP binding pocket for ERK1/2

with an affinity of less than 40 nm. However, a pose view could not be generated for it, as it showed hydrogen bonding to Lys54 and Asp167 through a water molecule, so it had to be dropped out of the race of potential inhibitors.

The compound m240690bcc215667167368734 showed an estimated affinity of less than 300 nm for 5BYZ. It appeared to bind in the ATP binding pocket and showed hydrogen bonding with Asp143A [solvent channel] and Ser186A and hydrophobic interactions with Val69A, Leu137A [gate keeper residue], Leu189A [hydrophobic C-spine], Lys84A [catalytic residue], and Tyr66A[p-loop]. 6SLG showed hydrogen bonding with Lys54A and Asp167A and hydrophobic interactions with Glu33A, Gly34A, Lys54A, Val39A, and Asp167A. For 6SLG, the co-crystallized ligand also shows hydrogen bonding with Lys54A. This supports the prediction that it may inhibit the activities of both 5BYZ and 6SLG.

The third molecule, rxn109fEMOL37110279EMOL314046334, and the 5BYZX complex showed hydrogen bonding with Lys84A [the catalytic residue], Asp143A, Tyr66A and hydrophobic interactions with Tyr66A[p-loop], Leu189A [hydrophobic C-spine] and His145A. On account of its hydrogen bonding to Lys84A, it can be predicted that it not only binds in the ATP binding site but also disrupts the salt bridge between Glu102 and Lys84A, which defines the active enzyme. It also showed binding to Asp143, which is part of the solvent channel. For 6SLG, rxn109fEMOL37110279EMOL314046334 showed hydrogen bonding with Asp111 and Asp167 and hydrophobic interactions with Val39A, Leu156A, Lys54A, Gly34A, and Gly37A. The co-crystalized ligand for 6SLG also shows hydrophobic interactions with Leu156A. Furthermore, its proximity to Lys54A supports the prediction that it is likely binding at the active site.

The fourth moleculeLIND027BT1904LN00213276AK0086 and 5BYZ showed hydrogen bonding with Met140A [hinge region] and Ile61A and hydrophobic interactions with Leu189A [hydrophobic C-spine], Ile61A, Gln146A [solvent channel front pocket], and Val69A [hydrophobic C-spine]. π-π interaction with Tyr66[p-Loop]. This implied that it could act as a potent inhibitor of the ATP binding site. In complex with 6SLG, it showed hydrogen bonding with Met108A and Asp106A. Hydrophobic interactions with Leu 156A, Ala52A and Asp167A. The co-crystallized inhibitor for 6SLG also shows hydrogen bonding with Met108A and hydrophobic interactions with Leu156A. Therefore, it can be predicted that it also occupies the conventional ATP binding site.

CONCLUSION

The structurally diverse novel compounds m240690bcc215667167368734, rxn109fEMOL37110279EMOL314046334 and LIND027BT1904LN00213276AK0086 may be proposed as potential common inhibitors for ERK1, 2 and 5. However, this cannot be coined with surety until this study is validated by some other docking software and as well as by molecular dynamic simulations with at least one positive and two negative controls. Therefore, further in silico analysis studies and in vitro testing on proteins and cancer cell lines are required to confirm their inhibitory potential.

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Author's Contribution:

Jariya Kalsoom: Designed the study, did computational analysis.

Sadaf Naeem: Supervised the study.

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