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DESIGNING NOVEL MEK1 INHIBITORS AS ANTICANCER AGENTS

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ABSTRACT

MEK1 is a key player of Ras-Raf-MEK-ERK pathway, a widely studied pathway in cancer biology. MEK1 inhibitors therefore are promising agents for cancer treatment. Recently, the combination of MEK1 inhibitors and PPARgamma agonists are emerging as effective anticancer therapy. Here, we studied the designing of novel allosteric-MEK1 inhibitors using common feature pharmacophore protocol of DS3.5. The best hypothesis, Hypo1 was selected based on rank and max-fit value and validated with Güner-Henry scoring method. The validated model was used as template to screen an in-house database in order to retrieve potential hits. Top ranking hits were subjected to docking to analyze their interactions with MEK1. Based on the interaction energy and binding mode, 115 compounds were selected for in vitro assay against MIA PACA-2 and PC-3 cells. Five compounds show percent inhibition of 45.8-52.2% against MIA PACA-2 cells. Compounds were further tested for inhibition of p-ERK expression, the immediate downstream kinase of MEK1. Compound **72** shows 35% inhibition of p-ERK expression.

KEYWORDS: ***MEK1, Pharmacophore, Docking, Cytotoxicity, Downstream kinase, PPARgamma***

INTRODUCTION

Mitogen-activated protein kinase kinase 1 (MAP2K1, MEK1) is a part of a signalling pathway called the Ras-Raf-MEK-ERK (RAS/MAPK) pathway. Dysregulation of this pathway leads to oncogenesis in humans. Constitutively active MEK1 proteins are present in a relatively high number of human tumors, particularly those from the colon, lung, pancreas, ovary and kidney[1]. MEK1 is known to be activated by several kinases, including Mos, A-Raf, B-Raf, Raf-1 and MEKK. At the same time, ERK1 is the only known substrates of MEK1. Therefore, inhibition of MEK1, rather than the Raf kinases, might offer a more effective approach to block signal transduction through the ERK pathway. Inhibitors of MEK1 operate either in an ATP-competitive fashion, targeting the ATP-binding site, or in a non-ATP-competitive fashion, binding to an alternative site of MEK1 that prevents either its activation (phosphorylation) or the activation of ERK1 through blocking the docking of its upstream effectors or downstream substrate, respectively[1]. Peroxisome proliferator-activated receptor gamma (PPARG), a nuclear receptor that promotes differentiation and apoptosis is one of the targets of MAPK/ERK cascade. It has been proved that thiazolidinediones, a series of PPARG agonist exerts the anti-proliferative affect against multiple type of cancers, though it is unclear whether this mechanism is PPARG dependent or independent[2]. Prominently, rosiglitazone, a thiazolidinedione is undergoing multiple phase 2 and 3 trials for cancers of diverse origin. The synergic activity of MEK1 inhibitors and PPARG agonists is already tested against various cancer cell lines[3].

Schematic representation of the RAS/MAPK pathway and its involvement in cancer

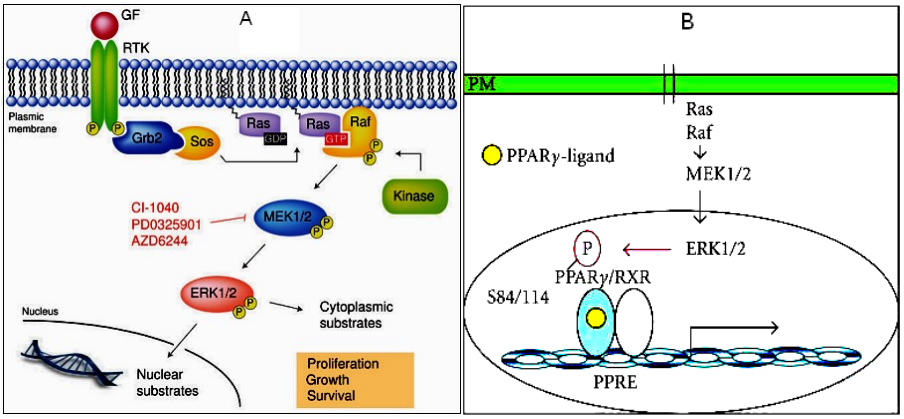


Figure I A
Ras-Raf-MEK-ERK (RAS/MAPK) pathway[4]. B: Upstream and Downstream kinases of MEK1 and PPARG-ERK crosstalk[5].

The present study is undertaken to design novel allosteric (non-competitive to ATP binding site) inhibitors of MEK1 using the structure activity relationship of already known inhibitors. Few highly active inhibitors from the literature were used as a template for generating a pharmacophore model to retrieve databases of drug like compounds. Upon retrieval, hits were subjected to docking against allosteric site of MEK1 followed by in vitro screening against 2 cancer cell lines i.e. MIA PACA-2 and PC-3. In order to prove the allosteric mechanism of inhibition computationally, newly designed compounds were subjected to docking against ATP binding site of MEK1. Further, to confirm MEK1 mediated anticancer activities, few promising compounds emerged from in vitro cell proliferation studies were tested for inhibition of MEK1 downstream kinase expression in MIA PACA-2 cells. In the second step of our study, we tested the combined antiproliferative effect of these newly designed MEK1 inhibitors with a known PPARG agonist in MIA PACA-2 cells, however results are not disclosed here.

MATERIALS AND METHODS

In silico studies

Common feature pharmacophore model

The common feature hypothesis (HipHop pharmacophore model) is an automated tool within Discovery studio version 3.5 (DS3.5) based on the alignment of common features present in a set of highly potent compounds termed as training set. HipHop generates a 'qualitative model' from a set of compounds without using activity data. This qualitative model represents the vital 3D arrangement of functional groups common for the set of compounds to form specific biological targets interactions. Molecules in the training set were evaluated based on the types of chemical features they contain, along with the ability to adopt a conformation that allows those features to be superimposed on a particular configurations. The partial features of molecules also mapped by HipHop in an aligned set, which is important to identify diverse and more significant models reducing the risk of missing a molecule which does not map to all the pharmacophore features. The generated pharmacophores were ranked from 1 to 10 (Hypo 1 to 10) as they built. The ranking indicates the mapping of the compounds onto the proposed pharmacophores[6]. Five allosteric inhibitors of MEK1 were carefully selected from literature by considering the activity, structural rigidity, and diversity to generate pharmacophore model. The structures and conformations of these 5 compounds were built within the HipHop module. The Poling algorithm implemented was used to generate conformations for all of the compounds. Possible diverse sets of conformations were generated for each molecule over an energy range 20 kcal/mol using the "BEST/Flexible" conformation generation option in HipHop.

Pharmacophore validation and database search

An external method of validation called Güner-Henry (GH) scoring method was used to validate the top ranked hypothesis i.e. Hypo1 using a set of active and inactive compounds (test set) to evaluate its ability to discriminate active compounds from inactive compounds. This method consists of computing the following: the percent yield (%Y), which is a measure of the selectivity of the model, the percent active (%A), which represents the

coverage of activity space by the model, the enrichment factor E, and the Güner-Henry (GH) score. These variables are determined using information derived from the total number of compounds in the drug database (D), the number of actives in the database (A), the number of actives retrieved by the model (Ha), and the total number of hits retrieved by the model (Ht). The formula to evaluate the GH score is

$$[((Ha/4HtA)(3A + Ht)) \times (1 - ((Ht - Ha)/(D - A)))], \text{ where } \%A = Ha/A \times 100;\ \%Y = Ha/Ht \times 100;\ E = (Ha/Ht)/(A/D)$$ [7].

The representative common pharmacophore hypothesis (Hypo1) was used as search query to retrieve potential hits with novel structural scaffolds and desired chemical features from the ~1M compounds of GVK BIO's in-house database with drug like properties. These libraries were loaded into DS3.5 and "Search 3D database" protocol was used to screen the hits with the exact maps with the features.

Cluster analysis and docking studies

Primarily screened compounds were further refined by cluster analysis, a methodology used for arranging the derivatives under same scaffold (central moiety) followed by a substructure search. Here, cluster representatives were explored for the probable features responsible for interactions with MEK1 with reference to the crystal ligand complexes from PDB. MEK1 crystal structure from PDB (ID: 3PP1) was used for the docking studies using CDOCKER program of DS3.5. CDOCKER, a molecular dynamics (MD) simulated-annealing-based algorithm is preferable where ligand set is large and ligand complexity is low. 3D structure from protein data bank was downloaded and "Prepare Protein" protocol was applied to minimize the energy. The ligand side chains are conformationally sampled and were subjected to core-constrained protein docking, using a modified CHARMm-based CDOCKER method to generate top poses along with CDOCKER energies[8]. Before subjected to docking, the ligand set was prepared using "Prepare Ligands" protocol preceded by the minimization using "Smart Minimizer", a CHARMm based force field method. Using "Prepare Ligands" protocol, charges of ligands were standardized, possible ionization states were explored at pH 6.5-8.5, and tautomers and isomers maintaining original stereochemistry were generated. Docking was also performed at ATP-binding site of the 3PP1 crystal structure using the CDOCKER program as discussed above. CDOCKER interaction energy of the newly designed compounds at allosteric binding site were compared to that of ATP-binding site in order to prove the allosteric nature of inhibition where energy at allosteric site are expected to be significantly lower.

In-vitro studies

Cytotoxicity assay

Cell line selection

MIA PaCa-2 (Human pancreatic carcinoma) and PC-3 (Human prostate cancer) cell lines were selected for in vitro assay to test the cytotoxic activity of newly designed MEK1 inhibitors. MIA PaCa-2 cells over-express MEK1 and being widely used in MEK1 inhibitor studies[1,9], while PC-3 cells are already been tested for synergic activity assays of MEK1 inhibitors in combination with PPARgamma agonists[2]. The selection of PC-3 cells was influenced by the long term objective to perform a synergic assay with newly designed MEK1 inhibitors from this study in combination with known PPARG agonist. As discussed previously, it is evident that when combined with a PPARG agonist, MEK1 inhibitors show higher anticancer activity and can be an effective treatment for specific drug-resistant cancers[3].

Cell culture

MIA PaCa-2 and PC-3 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained in the appropriate growth media [RPMI 1640 (Gibco) containing 10% heat-inactivated FBS (Thermo- Fisher scientific) at 37°C in a humidified atmosphere of 5% CO2. For subculture and experiments, cells were washed with 1 × Dulbecco's PBS (DPBS, Gibco), detached using trypsin-EDTA, 1X (Corning), collected in growth media and centrifuged. All experiments were performed in growth media using sub-confluent cells in the exponential growth phase.

MTT assay

Cytotoxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described[10]. Cells were seeded in 96-well plate at 2500-3000 cells/well. After overnight incubation, cells were treated with indicated compounds for 72 hrs. After

the treatment, MTT (Amresco, Solon, OH) solution was added to the well to a final concentration of 0.3 mg/ml, and incubated for 4 hrs at 37°C. At the end of incubation, the supernatant was removed and 100 ul of DMSO was added to each wells. Then the plates were placed on a shaker for 15 minutes at room temperature and absorbance was measured at 570 nm. Cytotoxicity was assessed by the cell viability rate as {1-(At-Ab)/(Ac- Ab)}×100 (At , Ac and Ab were the absorbance values from cells which were treated with compound, cells which were not treated with compound and blank, respectively). IC50 values were calculated by plotting concentrations (log) of the compounds versus corresponding % inhibition values.

Downstream effector assay

SDS-PAGE and Western blotting

4×10^5/well of MIA PaCa-2 cells were cultured in 6-well tissue culture plates. Cells were treated with compounds at 20 uM for 4 hrs. After treatment, cells were lysed with cell lysis buffer (1% Triton X-100, 25 mM Tris pH 7.4, 150 mM NaCl, supplemented with proteinase inhibitor and phosphotase inhibitor cocktail) at 4°C for 30 minutes and centrifuged (12000 rpm, 10 minutes, 4°C). Protein concentrations of supernatants were measured with BCA protein detection kit (Thermo Fisher Scientific). 40 ug protein per sample was subjected to SDS-PAGE analysis.10% polyacrylamide gels with Tris/glycine/SDS running buffer (Bio-Rad) were used for separation of proteins in the sample. Proteins were then electro transferred to methanol activated immobilon-FL PVDF membranes (EMD Millipore, Billerica, MA). Membranes were blocked with blocking buffer (Thermo Fisher Scientific) for 1 hr at room temperature and incubated with primary antibodies (Cell Signaling, Danvers, MA) in 5% BSA overnight at 4°C.Primary antibodies p-stat3 (9145L, cell signaling,1:1000), Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® (4370S, Cell signaling, 1:1000), GAPDH (2118L, Cell signaling, 1:2000), p-AKT [(Phospho-Akt (Ser473) (587F11)], (4051S, Cell signaling, 1:1000) were used for immunoblotting. Membranes were then washed with TBST (10 minutes × 3 times), incubated with Dylight 800-conjugated secondary antibodies (Thermo Fisher Scientific, Rockford, IL) 1:7500 dilutions in 5% milk for 1 hr at room temperature, and washed with TBST (5 minutes × 3 times) and TBS (5 minutes). Fluorescent signal was then scanned by Odyssey Imaging Systems (LI-COR Biosciences, Lincoln, NE). Band intensities were quantified using Image studio software for Odyssey (Li-Cor Bioscience) where GAPDH levels were used to normalize the band intensities. Percentage inhibition of p-ERK were calculated and plotted in Microsoft-Excel.

RESULTS

In silico studies

Designing common feature pharmacophore model

A common structural pattern was observed in MEK1 allosteric inhibitors throughout the structure activity relationship study from literature. PDB entries reveal the inhibitor-receptor interactions involving specific amino acids i.e. Lys97, Ser212 and Val211 in ligand binding domain of MEK1 allosteric binding site. Specially, inhibitor-Lys97 interaction(s) appeared to hold the key as PDB entries studied here retain the interaction in common. Presence of electronegative H-bond acceptor(s) in the inhibitor structure facilitates the H-bond interactions with the above mentioned residues. Moreover, a hydrophobic feature towards the pocket encircled with residues Met143, Val127, Ile141, and Phe209 seems to have the structural importance to the inhibitors. Figure II shows the receptor-ligand interactions from 2 PDB IDs i.e. 3OS3[11] and 3PP1[12], shaded shapes schematically represents the key inhibitor features and interactions (Triangles: H-bond acceptor, Oval: hydrophobic).

Crystal ligand interactions with amino acid residues in allosteric binding site of MEK1

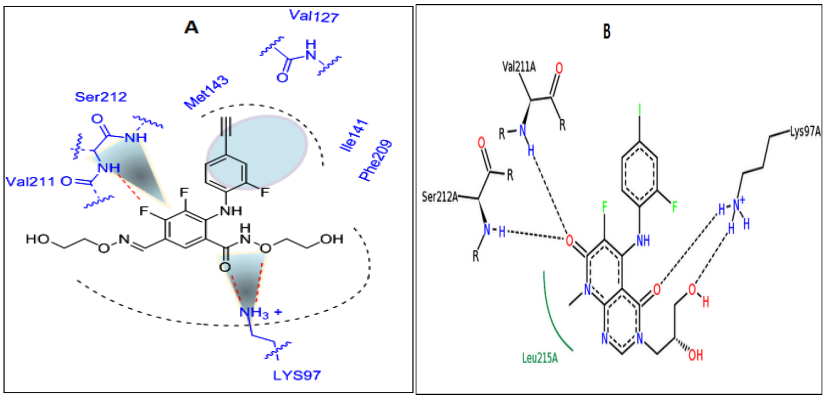
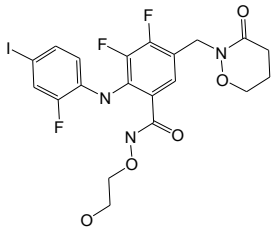


Figure II

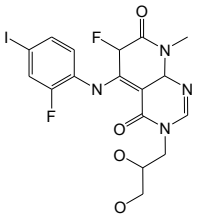
Receptor-Ligand interaction: PDB IDs 3OS3 (A) and 3PP1 (B) [http://www.rcsb.org].

The training set compounds (Fig III) selected for pharmacophore generation, were chemically diverse and expected to have similar binding mode in the allosteric binding site of MEK1. The pharmacophore features were selected based on (a) the structural and chemical features of the training set inhibitors, (b) the architecture of MEK1 allosteric binding site, and (c) the critical interactions observed between the crystal ligand and the key residues present in the binding site (PDB ID: 3PP1). The considered features for the generation of pharmacophore model were H-bond acceptor (HBA), H-bond donor (HBD), hydrophobic (HYA) and ring aromatic (RA) feature. Ten optimal pharmacophore hypotheses were generated by each of the HipHop run. These pharmacophore models were validated internally after mapping with each of the training set compounds and out of the best mapped models, Hypo1 (Fig IV) has been selected. Hypo1 carries all the important features as discussed above i.e. two electronegative (HBA) and two hydrophobic (HYA) features.

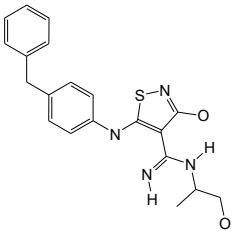
Training set



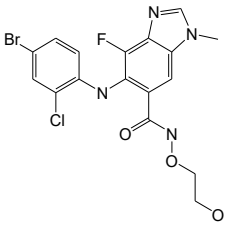
Compound **116**
IC50=5.2 nM[11]



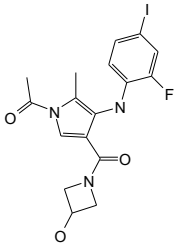
Compound **117**
IC50=3.2 nM[12]



Compound **118**
IC50=38 nM[13]



Compound **119**
IC50=12 nM[14]



Compound **120**
IC50=30 nM[15]

Figure III

High active training set molecules.

Validation of common feature pharmacophore model and database screening

A test set database of 51 compounds with wide range of activity profile against MEK1 was built to validate the Hypo1 for its ability to discriminate active compounds from inactive compounds and Güner-Henry (GH) score was calculated. Hypo1 model generated in this study is resulted with a GH score 0.65 (Table I), whereas GH score 0.6-1 indicates an optimal model. Once validated, Hypo1 was used as 3D query to screen ~1M compounds with drug like properties from in-house database to retrieve 5226 compounds, automatically sorted with the fit value it possesses corresponding to the pharmacophore mapping.

Table I
GH score for Hypo1

Total compounds in database (D)	51
Total number of actives in database (A)	37
Total hits (Ht)	39
Active hits (Ha)	35
% Yield of actives (%Y)	89.74
% Ratio of actives in the hit list (%A)	94.60
Enrichment factor or enhancement (E)	1.24
False negative	2
False positives	4
GH score (goodness of hit list)	0.65

3D representation of the features in the Hypo1 model and ligand mapping

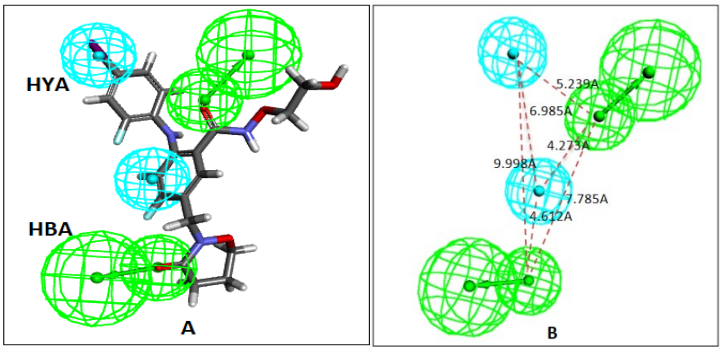


Figure IV A
Hypo1 is mapped onto compound 116. B: 3D representation of the features in the Hypo1 model i.e. H-bond acceptor (HBA) and hydrophobic (HYA) along with the intra-feature distances.

Cluster analysis and docking studies

All 5226 compounds i.e. primary set retrieved upon the Hypo1 screening were clustered based on central moieties. Promising cluster representatives were subjected to a substructure filter as discussed previously and a set of 564 compounds (~10% representatives from each cluster) was selected for docking studies. Docking was performed into the allosteric binding site of MEK1 (PDB ID: 3PP1). Coordinates of MEK1 from the MEK1/TAK-733 crystal structure were used after removing TAK-733 from the complex. Here, we used interaction energy of <-45 kcal/mol as threshold value for further refinement of novel ligand set. Based on this cut-off, 285 out of the primary set of 564 compounds were selected and further analyzed for the all important H-bond interactions as discussed previously. A distance criterion of 3.5 A^0 was used to monitor the H-bond interactions. Finally, a set of 115 compounds were selected for in-vitro screening. All of these compounds were showing H-bond interactions towards the MEK1 residues namely Lys97 and Ser212/Val211 with distance ranging from 2.5 A^0 to 3.5 A^0. Compounds were ranked manually from 1 to 115 based on 3 criteria i.e. a) distance of H-bond interaction it possesses with Lys97, b) CDOCKER interaction energy and c) presence of desired substructure (key feature) and its orientation in docking complex with reference to the crystal ligands in PDB complexes studied. Docked complexes were sorted by the CDOCKER interaction energy. In Table II, top 10

complexes are reported with CDOCKER interaction energy along with the distances of H-bond interactions towards Ser212 and Lys97 residues. Figure V A represents the interactions between compound **22** and binding site residues. Superimposed image of TAK-733, compounds **22** and **72** showing the overlapping of key features responsible for H-bond interactions is represented in Figure V D. Crystal ligand TAK-733 was also re-docked into the MEK1 allosteric binding site. CDOCKER interaction energy for TAK-733/MEK1 re-docking was found to be -57.126 kcal/mol where as H-bond distances of 2.252 A^0 and 2.501 A^0 were found towards Lys97 and Ser212 residues respectively.

Docking interactions of newly designed ligand and superimposition with crystal ligand

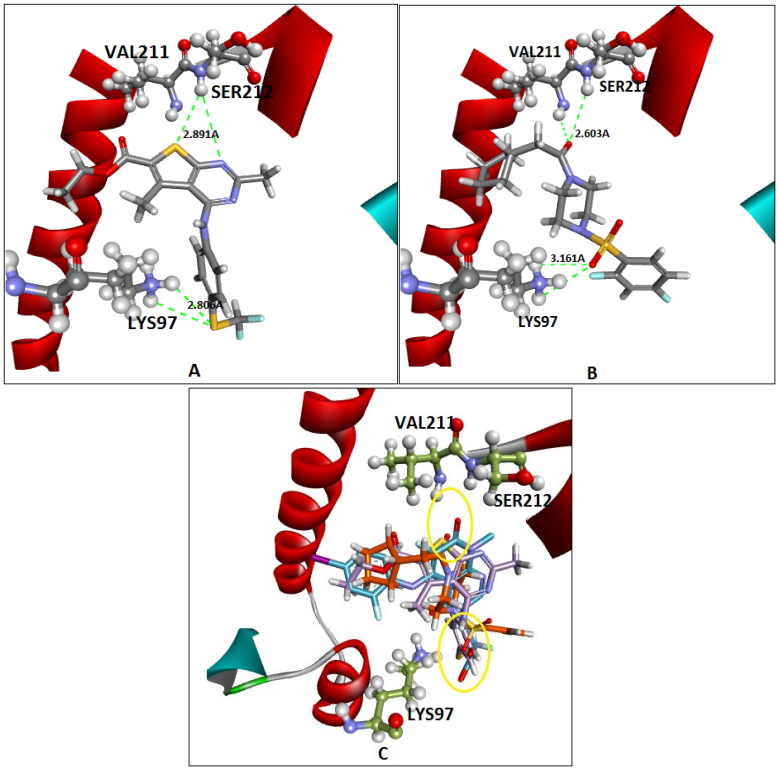


Figure V A

MEK1 interactions with compound 22. B: MEK1 interactions with compound 72. C: Superimposition of TAK-733 (blue), compound 22 (purple) and 72 (saffron) in MEK1 allosteric binding site.

In order to prove the allosteric nature of inhibition, an important aspect of this study, the newly designed compounds were docked into ATP-binding site of the MEK1 using the CDOCKER program as discussed above. We used already prepared MEK1 protein crystal structure (3PP1) for this purpose. Here, coordinates of MEK1 from the MEK1/ATP complex were used after removing ATP moiety from the complex. All of these compounds show no or minimal docking with CDOCKER interaction energy ranging from -4 to -12 kcal/mol. Hence, the allosteric pattern of inhibition was proved computationally for these compounds. Table II represents the interaction energy at an ATP binding site in comparison to the allosteric site for top 10 docking complexes.

Table II

CDOCKER interaction energy and H-bond distance of top ranked 10 compounds

Compound #	CDOCKER interaction energy at allosteric site/ATP binding site (-kcal/mol)	H-bond distance from Lys97 (A^0)	H-bond distance from Ser212 (A^0)
22	52.886/8.142	2.806	2.891
1	52.709/4.985	2.512	3.405
72	52.556/9.225	3.161	2.603
28	52.312/6.783	2.997	2.752
86	52.215/4.582	3.212	2.625
89	52.054/4.869	3.382	2.645
35	51.929/6.665	3.088	2.714
90	51.772/5.886	3.219	2.845
14	51.559/8.188	2.795	3.275
95	51.351/10.632	3.241	2.859
TAK-733	57.126/4.118	2.252	2.501

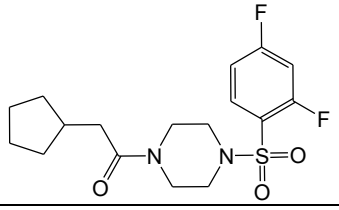
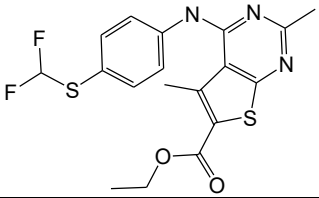
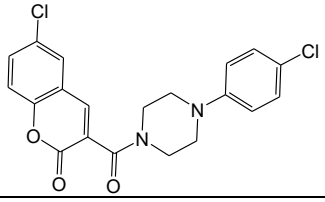
In vitro studies

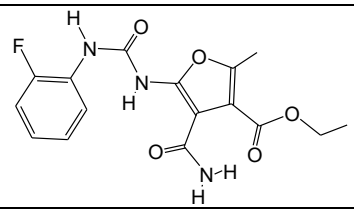
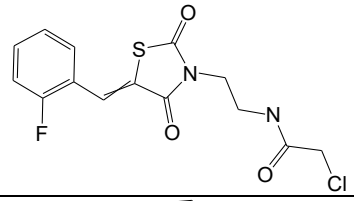
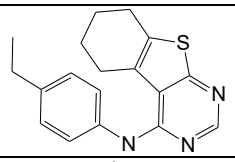
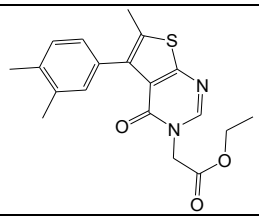
Cytotoxicity assay

115 compounds were selected for in vitro assays and tested against MIA PaCa-2 and PC-3 cell lines for their anti-proliferative activities. Compounds 86, 14, 72, 22, 90, 1 and 35 shows relatively higher inhibition rate against both the cell lines tested. MTT assay results i.e. % inhibition of cell proliferation and IC50 values are reported in Table III. Compounds 22, 86 and 72 shows IC50 of 12.8, 13.8 and 17.8 uM respectively against MIA PaCa-2 whereas compounds 1, 86 and 35 shows IC50 of 19.8, 22.4 and 37.0 uM respectively against PC-3 cells.

Table III

Anti-proliferative activities against MIA PaCa-2 and PC-3 cells

Mean % inhibition* @ 30 uM and IC50 values against MIA PaCa-2 and PC-3 cells					
Compound #	**Structure**	**% inhibition (MIA PaCa-2)**	**% inhibition (PC-3)**	**IC50 (MIA PaCa-2)****	**IC50 (PC-3)****
72		52.2	29.6	17.6	19.8
22		49.6	29.2	12.8	NA
90		45.8	46	NA	NA

35		48.8	48.3	46.9	37
86		47.7	66.6	13.8	22.4
14		23.7	NA	49.2	NA
1***		20.3	59.7	NA	NA

Values are mean of 3 different runs, **uM, ***concentration at 20 uM, NA – not available

Downstream effector assay

MEK1 mediated anticancer activities of the newly designed compounds were evaluated by expression of MEK1 downstream effectors i.e. p-ERK, p-STAT3, GAPDH and p-AKT in MIA PaCa-2 cells. Western blot images are reported in Figure VI.

Western blot of analysis of MEK1 downstream effectors

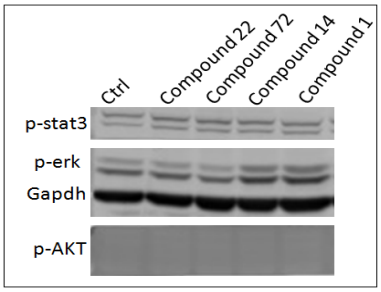
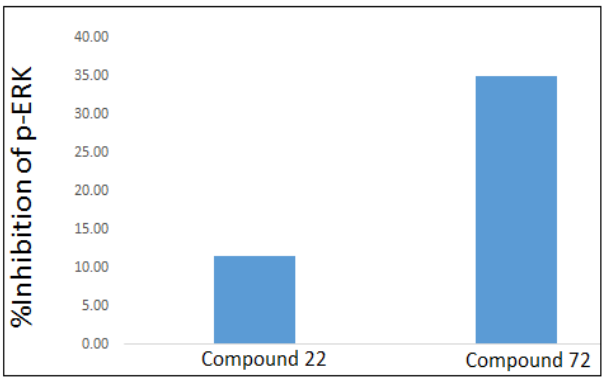


Figure VI
Expression of p-ERK, p-STAT3, GAPDH and p-AKT in MIA PaCa-2 cells.

Graph I represents the quantified values of western blot analysis. At a concentration of 20 uM, compounds 72 and 22 shows 35.06% and 11.53% inhibition respectively against p-ERK expression, the immediate downstream kinase of MEK1. As indicated in Figure V, compounds 14 and 1 show non-significant inhibition of p-ERK expression, hence values are not quantified. Compounds 35 and 90 were not tested for its ability to inhibit MEK1 downstream effectors owing to the low cytotoxic activities against MIA PaCa-2 cells (Table III), where as compound 86 was not available in sufficient quantity to perform this assay.



Graph I
Analysis of p-ERK expression

DISCUSSION

Inhibitory activities of the newly designed compounds against MIA PaCa-2 cells are correlated with the inhibition p-ERK expression. MIA PaCa-2 cells show higher rate of inhibition when treated with compounds 22 and 72. In downstream kinase assay, compounds 22 and 72 shows 11.53%and 35.06% inhibition of p-ERK expression respectively, where as compounds 14 and 1 showing no inhibition against p-ERK expression correlating the result of cell proliferation assay with minimal inhibition (Table III). This indicates the most valuable finding of the study that anticancer activity of compounds 22 and 72 is mediated by MEK1. This study also succeeded to achieve the eminent correlation between in silico and in vitro assay results as compounds with lower interaction energies appeared to have higher in vitro activities. Compounds 86, 14, 72, 22, 90 and 35 are among the best ranked 10 compounds as per the interaction energies are concerned (Table II). Hence, it is believed that computational optimization of these compounds can be resulted in the development of highly potent anticancer agents specially when combined with a PPARG agonist.

CONCLUSION

This study identified a fair number of compounds possessing growth inhibitory activities against pancreatic and prostate cancer cell lines using standard in silico drug designing methods followed by in vitro assays. Considering the nature of treatment resistance in these types of cancers, newly designed MEK1 inhibitors can be very useful leads. Compounds **22** and **72** are undergoing optimization and further development to get more drug-able leads.

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