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Short communication

Molecular dynamics guided development of indole based dual inhibitors of EGFR (T790M) and c-MET

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ABSTRACT

Secondary acquired mutation in EGFR, *i.e.* EGFR T790M and amplification of c-MET form the two key components of resistant NSCLC. Thus, previously published pharmacophore models of EGFR T790M and c-MET were utilized to screen an *in-house* database. On the basis of fitness score, indole-pyrimidine scaffold was selected for further evaluation. Derivatives of indole-pyrimidine scaffold with variedly substituted aryl substitutions were sketched and then docked in both the targets. These docked complexes were then subjected to molecular dynamic simulations, to study the stability of the complexes and evaluate orientations of the designed molecules in the catalytic domain of the selected kinases. Afterwards, the complexes were subjected to MM-GBSA calculation, to study the effect of substitutions on binding affinity of double mutant EGFR towards these small molecules. Finally, the designed molecules were synthesized and evaluated for their inhibitory potential against both the kinases using *in vitro* experiments. Additionally, the compounds were also evaluated against EGFR (L858R) to determine their selectivity towards double mutant, resistant kinase [EGFR (T790M)]. Compound **7a** and **7c** were found to be possess nanomolar range inhibitory (IC_{50}) potential against EGFR (T790M), **7 h** showed good inhibitory potential against c-MET with IC_{50} value of 0.101 μM. Overall, this work is one of the earliest report of compounds having significant dual inhibitory potential against secondary acquired EGFR and cMET, with IC_{50} values in nanomolar range.

1. Introduction

Kinases form a major class of molecular targets in lung adenocarcinomas harbouring activating mutations. These kinases result in over-activation of downstream signalling pathways that regulate the process of cell growth, proliferation, and survival. The identification of these driver kinases has previously led to the clinical use of small molecule kinase inhibitors such as erlotinib and gefinitib [1,2]. These molecules act as competitive inhibitors of ATP and have been proven efficacious over conventional chemotherapies in patients harbouring epithelial growth factor receptor (EGFR) overexpressed adenocarcinomas [3]. However, clinical responsive success of these kinase targeted inhibitors has been observed to be short lived due to development of acquired resistance to these drugs. Multiple mechanisms have been identified as the cause for the failure of these molecules which include: (1) alteration in the driver oncogene such as acquired secondary mutation T790M in EGFR (2) activation of signalling pathway(s) *via* parallel signalling, as in case of amplification of wildtype c-MET (hepatocyte growth factor receptor) in the L858R mutant EGFR overexpressed lung cancer and (3) reactivation of signalling pathways downstream of a driver oncogene, Nuclear factor-κB (NFκB)-containing complex activation is one such example. One more mechanism of resistance involves transformation of cell lineage such as epithelial (Non-small cell lung cancer) to another *i.e.*, mesenchymal (Small cell lung cancer) [4].

Among all, secondary acquired gate keeper residue mutation, T790M in EGFR, is observed in ~50% of EGFR-mutant patients who develop resistance to EGFR inhibition and is a pivotal mechanism of resistance. Initially, researchers suggested that gatekeeper mutation results in steric hindrance towards small molecule inhibitors thereby leading to development of resistance [5]. Later, studies disclosed that secondary acquired kinase do not possess resistance due to steric hindrance but rather the affinity of the kinase is altered back in favour of ATP and hence, ATP competitively inhibits the inhibitors [6]. To counter this secondary acquired mutation, researchers focussed on developing second generation kinase inhibitors, covalent inhibitors. Several studies focused on developing covalent inhibitors for EGFR T790M, via analysis of catalytic mechanism of binding with Cys797 [7] and utilization of different cysteine-trapping fragments [8] such as

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isothiocyanates [9], have been conducted. However, they suffer with lack of selectivity and therefore have poor safety profile. This further led to the development of third generation kinase inhibitors, which are covalent but are selective towards double mutant EGFR. Selectivity in these agents is claimed due to the hydrophobic interactions between the inhibitors and mutated residue M790 in the catalytic domain of the double mutant kinase. Osimertinib, recently reported EGFR (T790M) inhibitor, was also based on the same concept that molecules with hydrophobic interaction with the mutant methionine-790 gatekeeper residue could result in potent and selective inhibitors. It is also potent inhibitor of EGFR (L858R) due to its increased affinity towards small molecule inhibitors in place of ATP [10]. However, as covalent inhibitors are site specific in nature, any alteration in target residues can limit their efficacy. In recent years another acquired mutation, C797S in EGFR, has been disclosed and reported to make third generation inhibitors ineffective. Additionally, another mutation in the P-loop residue (L718Q) has been reported to result in resistance against third generation inhibitors such as osimertinib. One of the mechanism suggests that mutant Gln718 affects the conformational space of the EGFR–osimertinib complex, preventing its interaction with Cys797 [11].

Another key mechanism of resistance against kinase inhibitors in NSCLC (Non-small cell lung cancer) is amplification of c-MET, upon administration of first generation inhibitors. It has been one of the earliest mechanism of resistance which rendered EGFR inhibitors ineffective [12]. Amplification of c-MET leads to the reactivation of PI3K/Akt signalling pathway by forming a MET-ErbB-3 heterodimer, previously formed by EGFR. Basically, c-MET behaves as a substitute for EGFR in the signalling pathway. Thus, despite continuous suppression of EGFR by the inhibitor, a critical downstream signalling pathway continues, and resistance to the inhibitor emerges [13]. In patients with resistance to first-generation EGFR tyrosine kinase inhibitors generated by c-MET amplification, it is unlikely that a third generation covalent inhibitor would be effective, but the combination of a double mutant EGFR inhibitor and a c-MET/mTOR/PI3K/Akt pathway inhibitor may prove to be an effective strategy [14]. Clinical trials are underway to test the effect of dual inhibition of c-MET and EGFR to overcome this mode of resistance [15].

Thus, in our study we focussed on targeting c-MET and secondary acquired mutant EGFR (T790M) via non-covalent inhibitors. Focus was laid on the fact that sensitivity towards inhibitors is not lost in T790M EGFR rather the affinity towards ATP increases. Special attention was also given to the fact that molecules having hydrophobic interaction with mutant residue M790 provide selectivity to the molecules and thus, may also enhance the binding affinity towards EGFR T790M. Thus, we attempted to design molecules with higher affinity towards T790M EGFR along with c-MET using *in silico* techniques and further synthesis and biological evaluation.

2. Results and discussion

2.1. In-silico analysis

Previously reported ligand-based pharmacophore models for EGFR (T790M) and cMET, generated using Discovery studio were employed to screen and cross screen an *in-house* small molecule database containing around 200 molecules with diverse scaffolds such as benzimidazole, oxindole, indole, flavones and thiazolidinones. Pharmacophore mapping tool was employed for this purpose and molecules possessing significant fit score in mapping via both pharmacophores were selected for further analysis. This screening yielded a total of 18 molecules with indole scaffold, similar compounds have been reported previously to possess antimicrobial and cytotoxic potential [16–18], which were then subjected to docking analysis using co-crystallized 3-D structure of both the target kinases followed by molecular dynamic simulations and calculation of MM-GBSA score (binding energies). For molecular docking, the PDB were selected utilizing resolution as cut-off followed by cross docking protocol (Supplementary Tables s1–s4). Molecules selected after docking analysis, in EGFR T790M, showed that an amino group present at the second position of the pyrimidine containing molecules acts as donor group and interacts *via* hydrogen bond with Gln791, Met793 and Lys745 in many of the designed compounds while the ring of indole formed the hydrophobic interactions with various other hinge region residues. Similarly, in cMET, free NH_2 in many of the designed compounds formed hydrogen bond with Asp1231 and the hydrophobic region was occupied by benzyl group substituted at N^1 of the indole. All the compounds showed good docking scores (Glide XP G-score) in both the kinases; ranging from −8.22 to −6.98 kcal/mol within the EGFR (T790M) protein and −7.42 to −4.59 kcal/mol in cMET. Docking was followed by molecular dynamic simulations of the designed molecules in complex with EGFR (T790M) as well as cMET, for a period of 30 ns. Simulations studies disclosed that almost all the molecules were maintaining key H-bond interactions with hinge region amino acids. A key disclosure was the fact that nine molecules were maintaining varied levels of hydrophobic interactions with mutated gate keeper residue M790 which is essential for the selectivity and potency of molecules against double mutant EGFR (T790M), which were then forwarded for synthesis and *in vitro* evaluation. Additionally, presence of bromine in the side ring was found to be vital as it occupied small hydrophobic cavity in the catalytic domain of EGFR (T790M) enhancing the overall hydrophobic and van der waal interactions, while in some derivatives it also formed halogen bond with neighbouring residues. The RMSD values of the protein and ligands in the complex with top compounds (**7c** and **7h**) reflected the overall stability of the complex for the given period of 30 ns and the graphs for the same are represented in Figs. 1 and 2.

The 3D interaction diagram of the designed molecules **7c** in EGFR T790M and **7h** in cMET are shown in Figs. 3 and 4, respectively. Finally binding energy scores, calculated using MM-GBSA protocol, reflected significant affinity for the designed molecules in both the targets. The score of all the compounds lied in the range of −75.706 to −49.003 in EGFR (T790M) and −84.334 to −66.319 in cMET (Table 1).

2.2. Chemistry

The designed compounds were synthesized according to Scheme 1. In the first step, alkylation of indole-3-carbaldehyde was performed by reacting it with variedly substituted benzyl chlorides in the presence potassium carbonate to yield different *N*-substituted indole-3-carbaldehydes. Followed by claisen-schmidt condensation of the obtained *N*-substituted indole-3-carbaldehydes with *p*-bromoacetophenone using piperidine as catalyst in methanol to afford 1,3-diaryl/heteroaryl propenones. The obtained 1,3-diaryl propenones were purified via recrystallization. Then, 1,3-diaryl/heteroaryl propanones were treated with guanidine hydrochloride, using sodium hydroxide as catalyst in methanol to afford desired products. Finally column chromatography was performed to obtain pure derivatives. All the compounds were characterized by IR, Mass, ^{1}H NMR and ^{13}C NMR. In IR spectrum, the indole-pyrimidine derivatives showed the presence of strong absorption bands of multiple C=N from ∼1680 to ∼1580 cm^{-1}. The synthesis of final compounds were confirmed in ^{1}H NMR. Almost each spectrum showed singlet of only proton present in pyrimidine nucleus, ranging from ∼7.3 to ∼7.8 ppm according to