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Quinazoline and tetrahydropyridothieno[2,3-*d*]-pyrimidine derivatives as irreversible EGFR tyrosine kinase inhibitors: influence of the position 4 substituent†

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Herein, we describe new quinazoline and tetrahydropyridothieno[2,3-*d*]pyrimidine derivatives with an acrylamido group at positions 6 and 7 respectively, and with variable anilino, sulfonamido and cycloalkylamino substituents at position 4. The lipophilic and steric properties of the position 4 substituent seem crucial for activity. Several compounds were more active than gefitinib in inhibiting the wild type EGFR enzyme, the autophosphorylation of the mutant EGFR expressing cell line (H1975), and the growth of cell lines with wild type and mutant EGFR tyrosine kinase. Moreover, a novel synthesis of the quinazoline nucleus from a formimidate derivative is described.

Introduction

Members of the epidermal growth factor receptor (EGFR) family were found to play a vital role in lung tumorigenesis being overexpressed in 40–80% of non-small cell lung carcinoma (NSCLC) tumors.[1–4] A series of downstream signaling events results from EGFR activation and can mediate cancer cell growth, proliferation, motility, adhesion, invasion, apoptosis inhibition and metastasis as well as resistance to chemotherapy. Accordingly, EGFR inhibitors would be valuable in cancer treatment.[1,2]

Gefitinib, erlotinib, and lapatinib (Fig. 1) are examples of small molecules, acting as kinase inhibitors, that have been approved in cancer treatment.[5] They are used clinically in the treatment of EGFR/HER2-dependent tumors which occur in non-small cell lung cancer (NSCLC) or breast cancer.[6] They belong to a class of compounds known as 4-anilinoquinazolines which are designed mainly to target the ATP binding pocket of the kinase domain.[6]

The quinazoline core is reported to be among the best scaffolds for the development of EGFR inhibitors.[7] This was justified by a hypothesis explaining the importance of quinazoline N3 in the formation of a water-mediated hydrogen bond to the side chain of the gatekeeper Thr790 of the EGFR.[8,9] This aided successfully in designing reversible and irreversible EGFR and HER2 kinase inhibitors.[10–13]

The tetrahydropyridothieno[2,3-*d*]pyrimidine nucleus is also among the scaffolds showing EGFR inhibitory activity.[4] The 4-(phenylamino) quinazoline core has also been used to develop several irreversible EGFR inhibitors by introducing a Michael acceptor functional group such as the acrylamide group attached at the C-6 or C-7 positions, *e.g.* **I** & **II** (Fig. 1). These groups form a covalent linkage with the sulfhydryl group of the Cys797 of EGFR and these compounds proved to be potent inhibitors of tumor growth relying on the overexpression of the EGFR.[14,15]

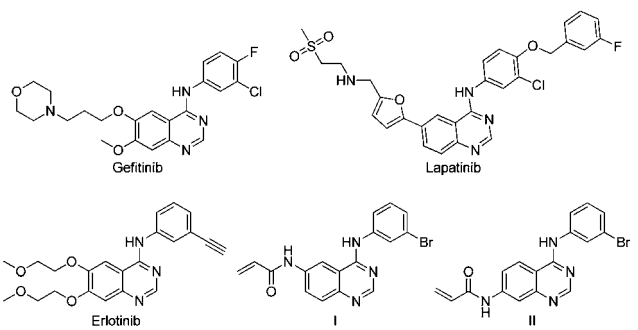

Fig. 1 Reversible and irreversible EGFR tyrosine kinase inhibitors.

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Drug resistance was found to develop in approximately half of the NSCLC cases that showed an initial response to reversible EGFR tyrosine kinase inhibitors. This was associated with the emergence of a secondary mutation leading to the substitution of a single amino acid threonine 790 by methionine (T790M) in the ATP binding pocket of the EGFR.[16–18] Several other mechanisms of resistance to reversible EGFR inhibitors have also been reported.[19,20]

The Thr790 residue in the EGFR is present at the entrance of the deep hydrophobic pocket of the ATP binding site. Therefore, its substitution with the bulkier methionine residue caused resistance towards the reversible tyrosine kinase inhibitors such as gefitinib and erlotinib and this was attributed to an increased enzyme affinity for ATP.[21] Several studies reported that the irreversible inhibitors[22–24] are able to overcome this mutation-associated drug resistance.[18,25–28]

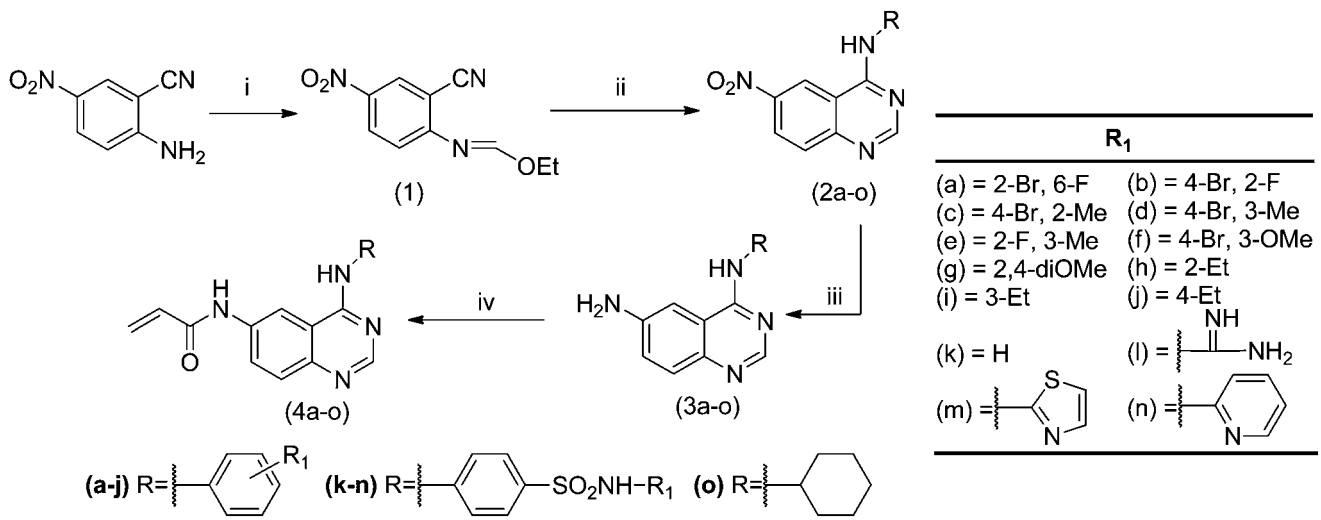
Although the T790M mutation takes place in Thr790 which is present in the deep pocket that is occupied mainly by the position 4 substituents of quinazoline derivatives, the introduction of a Michael acceptor group at position 6 of the quinazoline has proven to overcome this mutation-associated drug resistance. While the role of Michael acceptor groups in overcoming this resistance is justified and clear, the significant role of the position 4-substituents in the inhibition of the mutant EGFR in the presence of Michael acceptor groups is still not clear.

Therefore, we strived to investigate the effect of position 4 substituents on the potency of our potential irreversible inhibitors. In this study we aimed to provide a better understanding of the significant role of the nature and size of the position 4 substituents – that can be attached to a quinazoline scaffold in the presence of a potential covalent interaction – on the inhibition of the mutant as well as the wild type EGFR kinase. In addition, the importance of the quinazoline core was also tested by replacing it with a tetrahydropyridothieno[2,3-*d*]pyrimidine nucleus. Accordingly, for application in our study we synthesized quinazoline derivatives having an acrylamido substituent at position 6 and with diverse substituents at position 4. The acrylamido substituent is intended to potentially alkylate cysteine (C797) in the ATP binding site of the EGFR, to help in overcoming the mutation-associated drug resistance. Varied substituents at position 4 were added, namely haloanilines, alicyclic amines, alkylanilines, alkoxyanilines, and sulfonamide containing aniline derivatives **4a–4o**. Furthermore, a new cost-effective modification for the synthesis of the quinazoline nucleus is described. In addition, another series of compounds **10a–10f** was synthesized by replacing the quinazoline nucleus with a tetrahydropyridothieno[2,3-*d*]pyrimidine scaffold with also the same acrylamido substituent at position 7 while keeping the position 4 substituents showing potent inhibitory activity towards the quinazoline nucleus. All acrylamido derivatives **4a–4o** and **10a–10f** have been tested for their inhibitory activity on the recombinant wild type EGFR kinase as well as cell growth inhibition *versus* cancer cell lines, with mutant EGFR (H1975) and with wild type (SKBR3). In addition, cell based autophosphorylation inhibition was done for selected compounds.

Chemistry

Synthesis of the quinazoline nucleus was started by refluxing 2-amino-5-nitrobenzonitrile with triethyl orthoformate in the presence of drops of acetic anhydride to yield the formimidate derivative **1** (Scheme 1). Compound **1** was confirmed from its IR spectrum showing a band at 2228.6 cm^{-1} indicating the existence of the (C≡N) group. The ^{1}H-NMR spectrum of **1** in DMSO-d_6 revealed signals at 8.22 ppm (N=CH–) as a singlet, quartet at 4.36 ppm (CH_2) and triplet at 1.35 ppm (CH_3).

The second step in Scheme 1 shows a novel modification for the synthesis of the quinazoline nucleus, whereby the formimidate derivative **1** was refluxed in acetic acid with different amines to yield the nitroquinazoline derivatives **2a–2o** and the



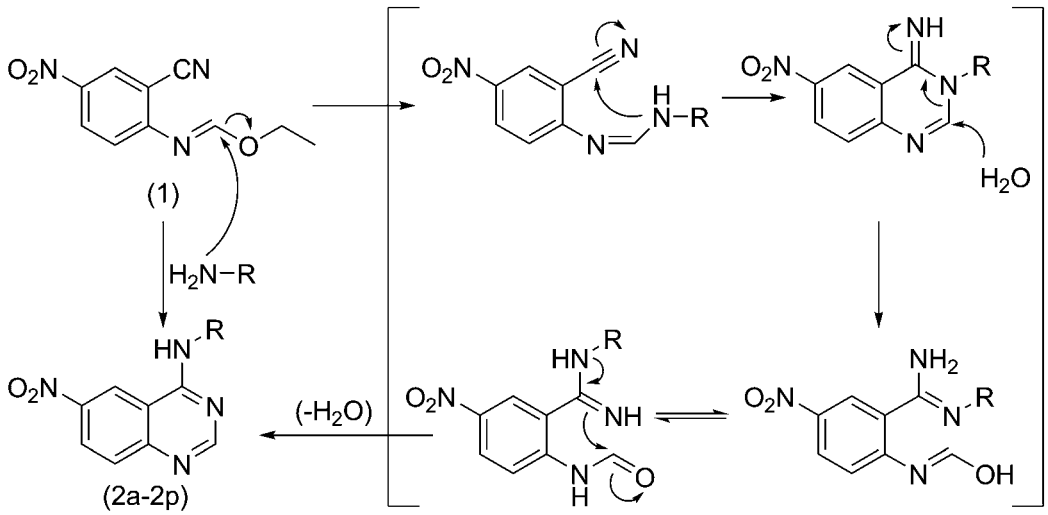
Scheme 1 *Reagents and conditions*: (i) TEOF, $(Ac)_2O$, reflux, 24 h; (ii) R–NH_2, CH_3COOH, reflux, 1 h; (iii) $SnCl_2$, MeOH, reflux, 1 h; (iv) CH_2=CHCOCl, $NaHCO_3$, acetone or DMF, 0 °C, 30 min.

cyclization was confirmed from the IR spectrum by the disappearance of the band for the cyano group. This novel modification is cost-effective since the quinazoline nucleus is synthesized from the formimidate derivative which is prepared from the much cheaper triethyl orthoformate instead of the usual *N*,*N*-dimethylformimidamide derivative prepared from the more expensive DMF–dimethyl acetal.[29]

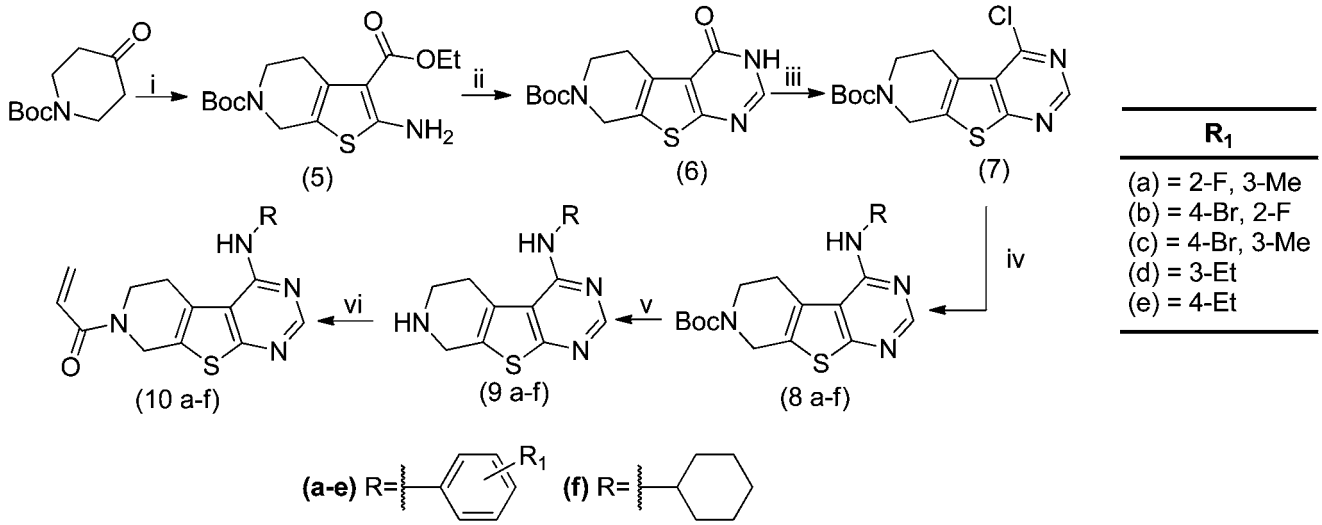
The suggested mechanism for the formation of the quinazoline nucleus from the formimidate derivative **1** is described in Scheme 2 as reported in the literature for a similar derivative.[30] It is assumed that the aromatic amines or the cyclohexylamine firstly attacks the carbon of the ethoxy resulting in ejection of the ethoxy group. An amidine intermediate is then formed which cyclizes into the quinazoline skeleton *via* Dimroth rearrangement where the endocyclic and exocyclic nitrogen atoms switched place to afford the 4-substituted aminoquinazoline.

Reduction of the nitroquinazoline derivatives was done by refluxing with $SnCl_2$ in methanol to yield the aminoquinazoline derivatives **3a–3o**, which then reacted with acryloyl chloride in acetone or DMF at 0 °C in the presence of $NaHCO_3$ to yield the acrylamide derivatives **4a–4o** (Scheme 1).

Synthesis of the tetrahydropyridothieno[2,3-*d*]pyrimidine derivatives is outlined in Scheme 3 according to the reported procedure.[4] It started by condensing the 4-oxo-piperidine-1-carboxylic acid *tert*-butyl ester with ethyl cyanoacetate under basic conditions followed by cyclization through a Gewald reaction[31] to construct the thiophene core. The construction of the thieno[2,3-*d*]pyrimidine ring system **6** was done using a modified Niementowski quinazoline synthesis by condensation of **5** with formamidine acetate. This was followed by chlorination of pyrimidone **6** with phosphorus oxychloride which gave the intermediate **7**. Nucleophilic reaction of **7** with appropriate



Scheme 2 Suggested mechanism for the formation of the quinazoline nucleus.



Scheme 3 *Reagents and conditions*: (i) $NCCH_2COOEt$, S_8, Et_3N, rt, 16 h; (ii) formamidine acetate, DMF, 100 °C, 16 h; (iii) $POCl_3$, Et_3N, 60 °C, 3 h; (iv) R–NH_2, EtOH, reflux, 8 h; (v) TFA, CH_2Cl_2, 0 °C → rt, 2 h; (vi) CH_2=CHCOCl, $NaHCO_3$, acetone, 0 °C, 30 min.

amines gave **8a–f**, which were then subjected to Boc deprotection using TFA resulting in the intermediates **9a–f**. The desired compounds **10a–f** were obtained by reacting the intermediates **9a–f** with acryloyl chloride in acetone at 0 °C in the presence of sodium bicarbonate to yield the acrylamide derivatives **10a–f**.

Biological results and discussion

All synthesized acrylamide derivatives **4a–4o** and **10a–10f** were tested for their ability to inhibit isolated recombinant wild type EGFR kinase. This was followed by testing the cell growth inhibitory activity on cancer cell lines with wild type EGFR (breast cancer cell line SKBR3) and the gefitinib-resistant (H1975) NSCLC cell line harboring the L858R and T790M mutations. In addition, to correlate the cell growth inhibition with the mutant EGFR kinase inhibition, selected compounds were tested for their ability to inhibit EGFR autophosphorylation in the mutant EGFR expressing cell line (H1975) (Table 1).

From the results, it can be seen that several compounds show significant inhibitory activity on the wild type as well as the mutant EGFR kinase which is correlated with the cell growth inhibition. Compounds like **4a**, **4b** and **4f** were the most potent *versus* both cancer cell lines having mutant and wild type EGFR.

Concerning the inhibitory activity on the recombinant wild type EGFR enzyme, it was generally observed that the potent activity was accompanied by di-substitution on the 4-aniline ring, either with dihalo or alkyl halo groups as in **4a**, **4b** and **4e**. In addition, this is the first report that replacing the usual aniline derivatives with a cyclohexyl amine as in compound **4o** resulted in an active and potent compound towards the wild type EGFR.

It has also been found that *ortho* substitution on the 4-phenyl ring with fluorine is tolerable as in **4b** and **4e** which are the most potent compounds. Bulkier groups like "Br" or "Me" at the *ortho* position, as in **4a** and **4c**, are also still tolerable while the potency decreased by further increasing the chain length like with the ethyl or methoxy groups, as in **4h** and **4g**. In addition, extended substituents at the *para* position like ethyl, methoxy, sulfonamide or substituted sulfonamide generally lead to a decrease in activity. This indicates that steric hindrance is a limiting factor to substituents at the *ortho* or *para* positions. Similarly, compounds with a sole ethyl substitution at the *meta* position gave a more potent compound than at the *para* or *ortho* position.

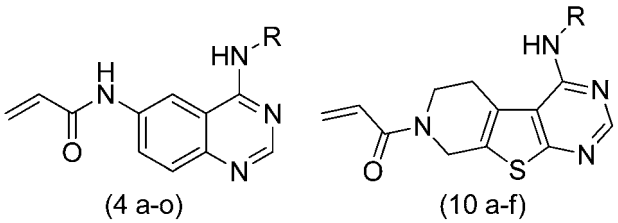
Polar substituents such as the sulfonamide group were found to significantly decrease the activity, but when substituted with heterocylic rings such as pyridine, the activity increased and resulted in highly potent compounds. Furthermore, replacing the quinazoline nucleus with the tetrahydropyridothieno[2,3-*d*]pyrimidine nucleus resulted in less potent compounds.

Concerning the activity on the mutant EGFR, several substituents significantly enhanced the activity such as dihalo in **4a** and **4b**, fluoro methyl in **4e**, bromo methoxy in **4f** and *m*-ethyl in **4i**. Some other substituents were found to affect the mutant EGFR potency and should be avoided. This includes substituents such as sulfonamide or substituted sulfonamide anilines as well as the cyclohexylamine which destroy the activity, while bulky substituents at the *para* or *ortho* positions such as 2,4-dimethoxy, *p*-ethyl or *o*-ethyl as well as the tetrahydropyridothieno[2,3-*d*]pyrimidine derivatives significantly decrease the activity towards the mutant EGFR.

Generally, concerning the cell growth inhibitory activity, it was found that the dihalo substituted anilines at position 4 as **4a** and **4b** are the most potent compounds. Also it was clear that replacing the methyl group in **4d** by a methoxy group in **4f** enhanced the activity at the cellular level against both cell lines. The 3-ethyl group in **4i** was also optimum in producing a potent compound towards the mutant EGFR-expressing cell line.

Docking of the most active compounds **4a**, **4b**, **4e** together with gefitinib and compound **I** was done to give a better

Table 1 IC_{50} for the inhibition of recombinant EGFR (active) kinase, cell growth inhibitory activity, EGFR autophosphorylation inhibition in the mutant EGFR-expressing cell line[a]



Compound	IC_{50} (nM) Recombinant EGFR kinase	IC_{50} (μM) growth inhibition SKBR3 cells	IC_{50} (μM) growth inhibition H1975 cells	IC_{50} (μM) autophosphorylation inhibition Mutant EGFR(H1975)
4a	2.2	0.23	0.26	ND
4b	2.1	0.51	0.28	0.036
4c	2.2	0.63	1.86	ND
4d	2.3	1.42	1.82	ND
4e	1.5	1.86	0.39	111.0
4f	2.5	0.36	0.40	ND
4g	53.6	6.89	13.87	0.931
4h	18.9	7.70	15.96	2.0
4i	2.7	2.82	0.68	0.275
4j	3.2	1.14	15.69	ND
4k	76.5	2.50	>40	ND
4l	53.3	>40	>40	ND
4m	43.7	4.00	>40	ND
4n	9.8	0.39	>40	4.39
4o	3.4	0.40	>40	2.8
10a	3.95	1.4	33.8	0.28
10b	3.71	2.3	>40	ND
10c	4.40	>40	>40	ND
10d	8.73	3.2	23.8	ND
10e	7.38	6.2	15.2	0.13
10f	>150	>40	>40	>5.0
Gefitinib	4	5.36	11.39	13.98
I	3.5	0.20	0.44	0.028

[a] SE ≤ 5%, ND: not determined.

understanding of their binding modes in the ATP binding site of the double mutated and wild type EGFR. Fig. 2 clearly demonstrates that gefitinib as well as the most active compounds exhibit a similar binding mode as the co-crystallized ligand **I** towards the wild type EGFR. The 4-anilino substituent of all compounds accommodates the deep hydrophobic pocket of the ATP-binding site. The Michael acceptor groups at position 6 of **4a**, **4b**, **4e** and **I** form a covalent interaction with Cys797, while the side chain of gefitinib extends towards the surface of the pocket.

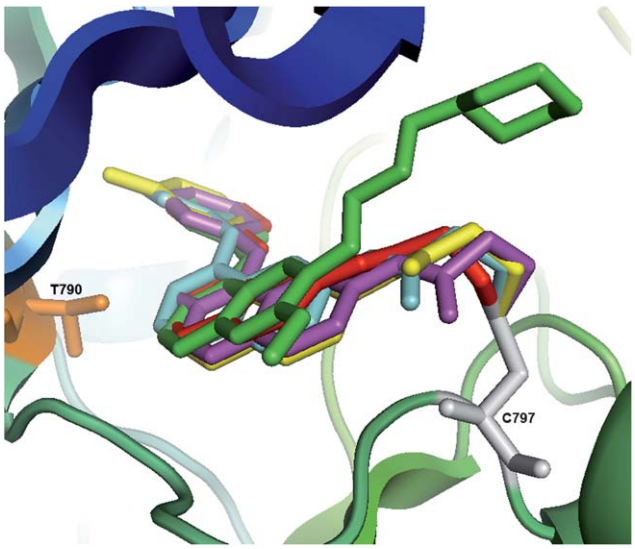


Fig. 2 Docked pose of compounds **4a** "cyan", **4b** "magenta", **4e** "yellow", gefitinib "green" and the co-crystallized ligand **I** "red" in the ATP binding site of wild type EGFR (PDB entry 2J5F). All compounds exhibit a similar binding mode as the co-crystallized ligand **I**. The 4-anilino moiety of all compounds accommodates the deep hydrophobic pocket of the ATP-binding site of wild type EGFR. The position 6 side chain of compounds **4a**, **4b**, **4e** and **I** forms a covalent interaction with residue Cys797 "grey" while that of gefitinib extends to the surface of the pocket.

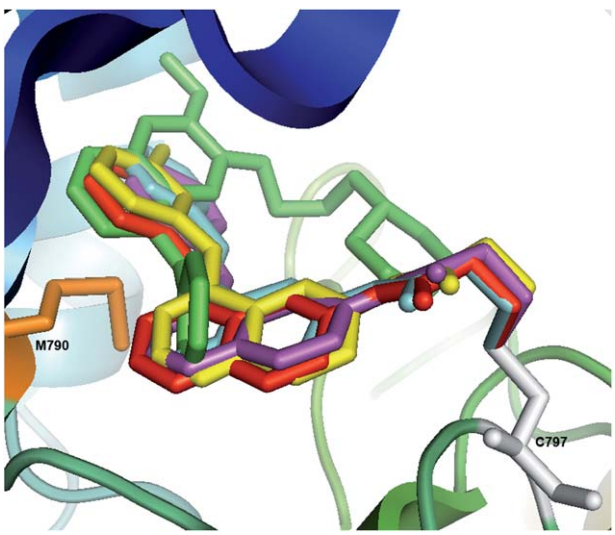


Fig. 3 Docked pose of compounds **4a** "cyan", **4b** "magenta", **4e** "yellow", gefitinib "green" and the co-crystallized ligand **I** "red" in the ATP binding site of the double mutated EGFR (PDB entry 3W2P). All compounds with a Michael acceptor group **4a**, **4b**, **4e**, and **I** potentially form a covalent interaction with Cys797 "grey" exhibit a similar binding mode while gefitinib exhibits a totally different binding mode. The 4-anilino moiety of all Michael acceptor group containing compounds accommodates the deep hydrophobic pocket of the ATP-binding site of the double mutated EGFR, while this did not take place in the case of gefitinib.

Fig. 3 shows that compounds **4a**, **4b**, **4e** and **I**, having a Michael acceptor group that can potentially form a covalent interaction with Cys797, exhibit a similar binding mode while gefitinib exhibits a totally different binding mode which could explain its much lower activity towards the double mutated EGFR. The figure also demonstrates that in the presence of a covalent interaction the 4-anilino substituent can still accommodate the back hydrophobic pocket of the mutated EGFR which was not the case with gefitinib.

Conclusions

A series of 6-acrylamide-4-substituted quinazoline derivatives and a series of 7-acrylamide-4-substituted tetrahydropyridothieno[2,3-*d*]pyrimidine derivatives have been synthesized. Several potent compounds were obtained and were able to overcome the mutation associated drug resistance. Compounds **4a**, **4b** and **4f** were the best compromise showing potent growth inhibitory activities towards cancer cells with mutant or wild type EGFR kinase. Although it is clear that the presence of a potential covalent interaction is the limiting factor and is responsible for retaining the activity towards the mutant EGFR, the modifications in the substituents at position 4 still have a significant influence towards this inhibitory activity which should be taken into consideration to achieve highly potent compounds. Several substituents showed potent inhibitory activity against both mutant and wild type EGFR containing cancer cell lines, while other substituents lead to more potent compounds towards either cell lines such as *m*-ethyl in **4i**, or fluoro methyl in **4e** were more potent towards mutant EGFR expressing cell line. Among the new findings is that substituents like the cyclohexyl amine in **4o** as well as the pyridyl sulfonamide aniline in **4n** resulted in active and potent compounds towards the wild type EGFR while they were not active towards the mutant EGFR. The quinazoline nucleus still remains among the best scaffolds since replacing it with a tetrahydropyridothieno[2,3-*d*]pyrimidine scaffold did not seem to be beneficial towards the EGFR inhibitory activity.

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