Discovery of small molecules inhibitors of EphA4

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Chapter 7

General conclusions and Perspectives

Due to their involvement in a large number of pathologies, protein kinases present an important pharmacological interest. Among this family, EphA4 has been recognized to be differentially expressed in various human tumors. The aim of the research project that forms the subject this thesis was to develop potent, specific inhibitors of EphA4 kinase activity using both computational and experimental approaches.

Target Immobilized NMR screen applied to the EphA4 kinase domain

As described in **Chapter 2**, nuclear magnetic resonance spectroscopy (NMR) is a powerful tool that can provide important information at each and every step of drug development. From ligand screening to hit validation, NMR is commonly used for characterizing the structure and molecular dynamics of target or ligand molecules. During structure-based lead optimization, NMR gives insight into the structural and dynamic properties of the target-ligand complex. NMR screening methods range from the detection of signal from protein observed to ligand-observed methods. Recently, we developed a screening method called target immobilized NMR screening (TINS), in which a target and a reference protein are immobilized on solid media and binding of fragments to the immobilized proteins is monitored by NMR. Among the advantages of this method is the small amount of protein required as TINS uses a single sample of the target to screen an entire fragment library.

In **Chapter 3**, a fragment library was initially screened against the Kinase Domain (KD) of EphA4 using the TINS technology. In order to insure the physiological relevance of ligands discovered using TINS, it was important to retain full enzymatic activity of

EphA4 upon immobilization. Therefore a number of immobilizations chemistries were investigated and we determined that oriented immobilization mediated by an affinity tag was required in order to obtain a functional immobilization of EphA4 KD. Subsequently, TINS was applied to the SNAP fusion KD of EphA4 using two different T_2 relaxation periods of 80 ms and 2 ms. These screens resulted in a 6.8% and 3.9 % hit rate respectively, with a final set of 180 fragments which were selected for further characterization using a kinase inhibition assay.

Subsequent SPR studies described in **Chapter 6** yielded an alternative means of immobilization via interaction between enzymatically biotinylatedEphA4 and streptavidin covalently bound to a bead. Since the biotin mediated immobilization yielded more biochemically active enzyme, the same technique was employed to perform an additional TINS screen. The results from this second screen were compared to those from the SNAP-KD screen. Despite the fact that TINS screens typically exhibit good reproducibility, little correlation was observed between the two screens and the TINS profiles were different. At this point it was not clear where the variability came from. Moreover, the protein was successfully crystallized using different protein batches and yielded several structures as described in **Chapters 4 and 5** and below. Thus variation in the quality of the protein preparation was not a likely source of the unexpected results. As a functional SPR assay was developed, the technique was employed to investigate the source of this variability.

In **Chapter 6,** two compounds (compounds **7** and **44)**, each originating from a different TINS screen, were characterized with SPR and shown to bind to the ATP binding site. The structure of compound **7** was found to be similar to compound **27** for

which the crystal structure was determined bound to EphA4 KD **(Chapter 5)**. Based on the structural similarity of compounds **7** and **27**, it seems that the same chemical scaffold has been found both computationally and experimentally, suggesting that both approaches can be used to generate EphA4 KD inhibitors.

Crystallography

Many strategies are available for obtaining crystals of protein-ligand complexes. The most resource-effective method of obtaining the structure of a protein–ligand complex is by soaking the ligand of interest into apo protein crystals. Therefore, the first step was to obtain diffracting crystals of apo-EphA4. **(Chapter 4)** Crystallographic experiments were undertaken with the support of the Nederlands Kanker Instituut (NKI-AVL) in Amsterdam and the structure of the native EphA4 kinase domain was elucidated (PDB ID: 2Y6M). In addition, the crystal structure of EphA4 in complex with dasatinib was solved (PDB ID: 2Y6O) and revealed a binding mode closely related to the one exhibited by Src family members, c-Src and Lyn. Analysis of the two structures revealed a hydrophobic back-pocket in the ATP-binding site of EphA4 which was unknown before. The access to this hydrophobic pocket is governed by the gatekeeper residue.[172] It is likely that targeting this hydrophobic pocket during drug design could lead to inhibitor specificity.

Discovery of a series of potent and specific inhibitor

In parallel, a computational approach toward EphA4 was performed by the Ph.D. candidate Oscar van Linden in the group of Dr. de Esch in Amsterdam (**Chapter 5**). Using the structural information derived from crystal structures of two ligands bound to related kinases, a mixed pharmacophore model for the binding site of EphA4 was constructed. An *in silico* screening procedure led to the identification of a 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*]isoquinolin-1-amine fragment. Optimization of this scaffold by growing into the kinase hydrophobic back-pocket discovered in the apo structure of EphA4, resulted in the identification of compound **27.** Soaking experiments were undertaken to obtain structural information and yielded a 2.11Å X-ray structure of the EphA4 – inhibitor complex. The binding mode observed in the crystal structure was in concordance with the binding mode of the scaffold as proposed by the initial *in silico* work (PBD ID: 2XYU). This compound was further biophysically characterized using Surface Plasmon Resonance (SPR) technology in **Chapter 6** and the binding affinity was estimated to be 2 μM. The kinetics for compound **27** binding to immobilized EphA4-KD were fitted to a 1:1 binding model yielding kinetic constants k_a =2.155 x 10^5 $M^{-1}s^{-1}$ and k_d=0.497 s^{-1}, and a K_D ~ 1-2 μM. This binding affinity is comparable with the affinity measured by biochemical assay (IC_{50}= 4.5 μM, **Chapter 5**).

Development of a functional SPR assay for characterization of small molecules binding to EphA4 KD

SPR was employed to provide biophysical characterization of the compounds binding to EphA4. The SPR characterization was conducted with 2 different recombinant proteins (6His fusion KD and biotinylated KD) using 2 different mechanisms of protein immobilization (via Ni-NTA surface and NeutrAvidin immobilized on a CM5 surface). Binding of compound **27** was characterized by SPR using the two different immobilization procedures and was found to be optimal with the biotinylated protein. Immobilization of biotinylated KD had several advantages when compared to immobilization of the 6His fusion EphA4 KD: (i) the level of immobilization is significantly higher yielding a greater window to study compound binding, (ii) 95% of the calculated binding capacity is retained and (iii) immobilization via the biotin NeutrAvidin interaction is more stable.

Biochemical and biophysical characterization of the TINS fragments hits

The hits obtained with TINS were biochemically characterized using an enzyme inhibition assay. Surprisingly, most of the compounds did not exhibit any inhibition of EphA4 activity while only 15% of the fragments exhibited biological activity. Among the fragments that were characterized as inhibitory, most presented peculiar dose response curves with a steep Hill slope and the curves appeared to level at less than 100% inhibition. One possible explanation for the biochemical data was either aggregation or

precipitation of the compounds. However, these explanations were deemed unlikely as the library used for the screen was carefully designed and tested for solubility. However, a moderate number of compounds were found that exhibited some inhibition and presented an interesting chemical scaffold were therefore selected for crystallographic structure determination.

As the soaking experiments successfully yielded a structure of compound **27** in complex with EphA4, compounds originating from TINS were submitted to the same procedure. However, none of the fragments yielded a structure. Most of the crystals that had been soaked with a fragment did not diffract to high resolution and when they did, the experimental electron density map that could be obtained revealed no density for the ligand. As there is a pH difference between the crystallographic conditions and the biochemical assay, this could negatively influence binding of the fragments. Thus protein observed NMR was employed in **Chapter 6** to investigate whether the pH difference could be the cause for the lack of crystal structures. [^{15}N,^{1}H]-HSQC experiments on EphA4-KD were conducted and indicated that the change in pH does not preclude compound binding for the 2 fragments assessed. To gain further insight into the possible sources of the difficulties to obtain protein-ligand structures, SPR characterization of the fragments was performed.

Compounds selected as hits from the TINS screen were characterized by SPR using both Ni-NTA and biotinylated KD mediated immobilization. For simply detecting binding, a good correlation was observed between the results from the TINS screen and the SPR analysis: 55% of the hits from the TINS screen of the SNAP-KD protein and 82% of TINS hits from the screen of the biotinylated KD were validated. Moreover,

compounds with weaker affinity than 4 mM could have been characterized as false negatives in SPR due the dynamic range limitations of SPR **(Chapter 6).** The binding affinities of several fragments could be estimated and ranged from 0.4 to 3 mM. However, for a majority of the compounds the binding affinity could not be obtained. Many factors likely contributed to the inability to determine the affinity including: the fragments may bind too weakly and thus saturation could not be reached, compounds may have exhibited promiscuous binding via a concentration-dependent aggregation mechanism and it may also have been possible that the stringent curation of the dose–response curves may have resulted in some false negatives.

SPR competition experiments using ATP were performed on two compounds presenting the highest affinity. The two compounds, **7** and **44,** were originally selected from the two different TINS screen of EphA4 KD and were shown to bind to the ATP binding site. Compounds **7** and **44** had reasonable ligand efficiencies of 0.3 and 0.25 (ΔG/# of heavy atoms) respectively. These compounds constitute starting points for the generation of more potent EphA4 inhibitors.

Perspectives

Generally, fragments are identified using a biophysical screening method, supported by a biochemical assay to validate their biological relevance. Using only affinity to guide drug discovery has been successful in numerous HTS campaigns in which potent hits are uncovered.[17] However, in our case, most of the fragments did not display biological activity in the biochemical assay even if their binding was demonstrated with both TINS and SPR approaches. It is likely that the biochemical assay used in this project was not sensitive enough for weak inhibitors. As a biological assay is am important tool in a drug discovery project, it is important to carefully develop and select a relevant methodology.

Once hits are identified, their binding needs to be confirmed and the three dimensional structure of the protein-fragment complex should be determined, ideally by X-ray crystallography. Among the approaches available to obtain protein-ligand structures, soaking fragments into existing crystals was chosen in this study. The soaking approach yielded a protein ligand structure for compound **27** but was unsuccessful with the compounds originating from the TINS screen. It is possible that the protein conformation was not optimal for ligand binding of some ligands, or the fragments were not sufficiently ordered to yield observable electron density. As most of the crystals did not diffract to high resolution, it would be interesting to investigate post-crystallization treatments for improving diffraction quality of protein crystals.[217] Furthermore, another approach for obtaining structures of ligand-target complexes, cocrystallization, has been used in drug discovery programs with success.[207] However, cocrystallization is far more

resource intensive and therefore not realistic for the large numbers of fragments assayed in this project. However, in the few cases where the diffraction quality of the fragment soaked crystals was low, it would be interesting to perform co-crystallizations experiments on EphA4 KD with confirmed hits from TINS.

In this study, the *in silico* approach led to the identification of compound **27**, a 6,7,8,9-tetrahydro-3*H*-pyrazolo[3,4-*c*]isoquinolin-1-amine fragment. This compound was demonstrated to be reasonably potent and the binding mode was elucidated via crystallography. Selectivity profiling against 124 protein kinases[199] revealed that this compound appeared to be a reasonably selective kinase inhibitor. Moreover, this scaffold was recently disclosed in a patent and publication by the pharmaceutical company Merck [200, 201] thus confirming the potential of this scaffold for generating EphA4 inhibitors. It is interesting to note that a small academic collaboration discovered the same chemical scaffold as a large pharmaceutical company with, one assumes, considerably less resources. Considering the selectivity of this compound, even though moderate, and the ligand efficiency of 0.35, it represents an interesting starting point to design specific inhibitors. Further improvements of this scaffold could lead to a new drug for the treatment of cancer and neuronal injuries by inhibition of the EphA4 receptor tyrosine kinase.