

COMPARATIVE VEGF RECEPTOR TYROSINE KINASE MODELING FOR THE DEVELOPMENT OF HIGHLY SPECIFIC INHIBITORS OF TUMOR ANGIOGENESIS

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The Vascular Endothelial Growth Factor receptors (VEGF-Rs) play a significant role in tumor development and tumor angiogenesis and are therefore interesting targets in cancer therapy. Targeting the VEGF-R is of special importance as the feed of the tumor has to be reduced. In general, this can be carried out by inhibiting the tyrosine kinase function of the VEGF-R. Nevertheless, there arise some problems with the specificity of known kinase inhibitors: they bind to the ATP-binding site and inhibit a number of kinases, moreover the so far most specific inhibitors act at least on these three major types of VEGF-Rs: Flt-1, Flk-1/KDR, Flt-4. The goal is a selective VEGF-R2 (Flk-1/KDR) inhibitor, because this receptor triggers rather unspecific signals from VEGF-A, -C, -D and -E. Here, we describe a protocol starting from an established inhibitor (Vatalanib) with 2D-/3D-searching and property filtering of the *in silico* screening hits and the “negative docking approach”. With this approach we were able to identify a compound, which shows a fourfold higher reduction of the proliferation rate of endothelial cells compared to the reduction effect of the lead structure.

Keywords: VEGF; cancer; tumor angiogenesis; homology modeling; *in silico* screening; docking

1. Introduction

Angiogenesis, the formation of new blood vessels, normally occurs moderately in adults, e.g. during wound healing and during the menstrual cycle. The process of angiogenesis is regulated by activators and inhibitors [1].

Tumor angiogenesis is the formation of networks of blood vessels supplying the tumor with oxygen and nutrients. Tumor cells induce this process by releasing signaling proteins to the surrounding normal tissue. The most important signaling proteins, which are also released by most of the cancer cells, are the vascular endothelial growth factors (VEGFs). The VEGF family consists of the following secreted glyco-proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and the placental growth factors (PlGF-1 and -2) [2-4]. The VEGFs bind

to VEGF receptor (VEGF-R) proteins on the endothelial cell surface with different binding affinities for each of the VEGF-Rs.

Expression of VEGF-Rs varies in specific endothelial cell layers. The VEGF-R-2 is located on almost all endothelial cells; however, the VEGF-R-1 and -3 are alternatively located on endothelial cells in distinct vascular layers [5].

Since angiogenesis was found to be necessary for tumor growth [6], the inhibition of pathological angiogenesis is a main goal in cancer therapy. Particularly, the VEGF/VEGF-R pathway plays a significant role in the development of angiogenesis and therefore represents a point of interference for therapy in oncology [5].

Different strategies to inhibit tumor angiogenesis exist: It is possible to interfere with angiogenesis from the extracellular as well as from the intracellular site. In the extracellular region, for example, antibodies and soluble receptors can avoid binding of the VEGF to the binding site of the receptor [6]. Moreover, VEGF antagonists block the ligand binding site of the VEGF-R on the extracellular site. Another way is the inhibition of the VEGF-R in the intracellular region by blocking the ATP-binding-site of the tyrosine kinase [7].

However, there arise some problems concerning the specificity of known tyrosine kinase inhibitors: they bind into the ATP binding site and inhibit a number of kinases. So far the most specific inhibitors act on the VEGF receptors. The goal would be to find a selective inhibitor for the VEGF-R-2 (KDR), because it is expressed on almost all endothelial cells and the majority of the effects in angiogenesis, including cell proliferation, micro-vascular permeability [8], invasion, migration, and survival [9, 10], are mediated by VEGF-R-2.

To find new compounds by using structure-based drug design, structural information about the target is needed. But today, no complete crystal structures of the VEGF-Rs are available.

Here, we describe a protocol to find novel potential VEGF-R inhibitors starting from an established inhibitor (Vatalanib, see Figure 1) [11]. A known inhibitor was used as lead structure for an *in silico* two- and three-dimensional searching in an "Inhouse" database to identify novel potential VEGF-R tyrosine kinase inhibitors. Moreover, the structures of the ATP-binding site of three VEGF-Rs were modeled, starting from an incomplete crystal structure of the VEGF-R-2. These homology models were then used for comparative docking as qualitative evaluation of the *in silico* screening results.

2. Methods

The *in silico* searching protocol consists of several steps, which are described in this section. In Figure 1 the procedure is schematically depicted.

2.1. Compound database

To search for new potential VEGF-Rs inhibitors we used our Inhouse database which contains about four million compounds and more than 140 million conformers, which were pre-calculated by using the MedChemExplorer of Accelrys [12, 13]. Around 95% of the

compounds stored in the Inhouse database are commercially available for experimental validation.

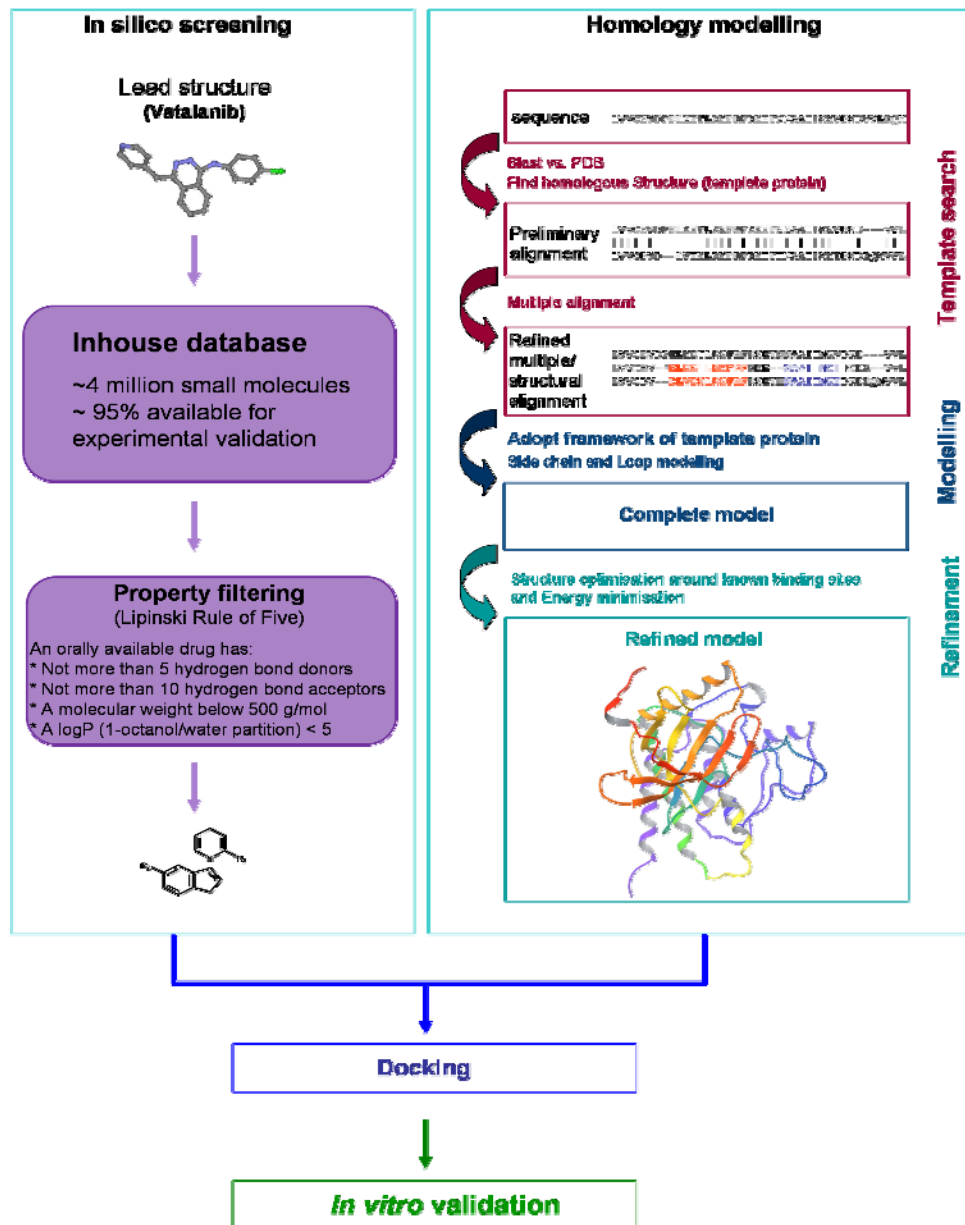


Figure 1. Scheme of the *in silico* and *in vitro* screening protocol.

2.2. Two-dimensional searching

To search for similar structures in our Inhouse database we pursued 2D-searching. The screening is based on the chemical similarity between two molecules according to the similar property principle of Johnson and Maggiora [14].

A structural fingerprint [15], a binary string encoding for the chemical characteristics of a compound, was calculated for the lead structure as well as for the database compounds. To screen the database, the fingerprint of the lead structure was compared to the fingerprints of the database entries by using the Tanimoto coefficient [16]. The Tanimoto coefficient is defined as:

$$T = \frac{N_{ab}}{N_a + N_b - N_{ab}}$$

N_a describes the number of bits, which were set 1 in the fingerprint of compound a , N_b stands for the number of bits, which were set to 1 for compound b and N_{ab} is the number of bits, which have compound a and compound b set to 1 in common.

A molecule with a similarity greater than 85% (≥ 0.85) to an active compound is assumed to be biologically active itself [17]. Therefore, only compounds with a similarity greater than 85% to the lead structure were considered.

2.3. Three-dimensional searching

A 3D-similarity search was applied to identify potential scaffold hoppers. For this purpose, the lead structure was compared to the conformers of drug-like compounds stored in our database. A plane representing the moment of inertia was put into all structures. For a comparison of two structures, the long and short sides of the planes were superimposed, which resulted in four different superimposition possibilities. The superimpositions were evaluated by using a scoring function, which includes the number of superimposed atoms and the Root Mean Square Deviation (RMSD). This scoring function is defined as:

$$\text{score} = (\text{percentage of superimposed atoms}) \cdot e^{-\text{RMSD}}$$

2.4. Homology Modeling

For homology modeling of the three VEGF-Rs several steps were necessary and were performed with the aid of the Swiss-PDBViewer [18].

A crystal structure of the VEGF-R-2 (PDB-code: 1YWN) was obtained from the Protein Data Bank (PDB). This structure is not complete; two gaps are located in and near the ATP-binding site. The ATP-binding pocket was completed by using the SuperLooper web server

[19]. Loops were extracted from the LIP database [20] and inserted into the structure via the web service. Furthermore, the completed model of the VEGF-R-2 was used as template structure for the VEGF-R-1 and VEGF-R-3.

Finally, the models were subjected to an energy minimization using the respective function of the Swiss-PDB Viewer.

2.5. Property filtering

To estimate the drug-likeness of the 2D/3D-searching results the compounds were filtered according to their molecular properties by using the “Lipinski rule of five”. There are four empirical rules, which say, that an orally available drug has:

- not more than 5 hydrogen bond donors
- not more than 10 hydrogen bond acceptors
- a molecular weight below 500 g/mol and
- a logP (water/n-octanol partition) < 5.

If a compound breaks more than one rule, it does not promise to become a drug [21]. Therefore, only compounds with no or at most one violation of the Lipinski rules were considered. The properties were calculated with the Accord for Excel Add-On [22].

2.6. Docking

To evaluate the remaining drug-like candidates, they were docked into the ATP-binding site of the modeled VEGF-Rs by using the docking program Glide from Schrödinger [23]. The Glide scoring function (Glide SP score) was used to rank the docking results.

The docking scores and the visual inspection of the docked ligand-protein complexes were used as qualitative evaluation of the candidates and resulted in a ranking of those compounds. The best molecules were used for further *in vitro* screening.

2.7. In vitro screening

A kinase assay was used to test the drug candidates for their inhibitory effect on VEGF-Rs. The potential of inhibition is expressed by the IC₅₀ value (the concentration where kinase activity is reduced to 50%).

Cytotoxicity was measured using a LDH-assay. The ability of cell proliferation inhibition was tested on different cell lines (endothelial cell line EA-HY 926) for each of the potential angiogenesis inhibitors.

3. Results and Discussion

3.1. Sequence alignment and homology modeling

The sequence alignment of the VEGF-Rs, as shown in Figure 2, is the basis of homology modeling. In a second step the non-identical amino acids of the template structure were exchanged according to the VEGF-R sequences. Only gaps in the ATP-binding pocket were filled in.

VEGFR-3	827	QCEVLSYDASQW	EFPRERLHLGRV	LG	GAFGKVV	EASAFGIHKGSS	CDTV
VEGFR-2	816	HCERLPYDASKW	EFPRDRLKLGKPL	GR	GAFGQVIEAD	AFGIDKTAT	CRTV
VEGFR-1	809	QCERLPYDASKW	EFAERLKLKGLS	LR	GAFGKVVQAS	AFGIKKSPT	CRTV
VEGFR-3	877	AVKMLKEGATA	SEHRALMSELKIL	IHI	GNHLNVNLL	GACTKPG	GPLMVI
VEGFR-2	866	AVKMLKEGATH	SEHRALMSELKIL	IHI	GHHLNVNLL	GACTKPG	GPLMVI
VEGFR-1	859	AVKMLKEGATA	SEYKALMTELKIL	ITHI	GHHLNVNLL	GACTKQG	GPLMVI
VEGFR-3	927	VEFCKVGNLSN	NFLRAKRDAFS			975	LTMEDLVCYSFQVAR
VEGFR-2	916	VEFCKFGNLS	TYLRSKRNEFV			965	LTLEHLICYSFQVAK
VEGFR-1	909	VEYCKVGNLSN	NYLKSQRDLFF			959	ITMEDLISYSFQVAR
VEGFR-3	1024	GMEFLASRKC	IHRDLAARNILL	SESD	VVKICDFGL	ARDIYKDP	DYVRKGS
VEGFR-2	1015	GMEFLASRKC	IHRDLAARNILL	SEK	NVVKICDFGL	ARDIYKDP	DYVRKGD
VEGFR-1	1009	GMEFLSSRKC	IHRDLAARNILL	SENN	VVKICDFGL	ARDIYKNP	DYVRKGD
VEGFR-3	1074	ARLPLKWMAP	ESIFDKVYTTQ	SDVVS	FVLLWEIF	SLGASPY	PGVQINEE
VEGFR-2	1065	ARLPLKWMAP	ETIFDRVYTI	QSDVVS	FVLLWEIF	SLGASPY	PGVKIDEE
VEGFR-1	1059	TRLPLKWMAP	ESIFDKIYSTK	SDVVS	FVLLWEIF	SLGGS	PGVQIMDEE
VEGFR-3	1124	FCQRLRDG	TRMRAPELAT	PAIR	RLMLNCW	SGDPKAR	PAFSELVEILG
VEGFR-2	1115	FCRRLKEG	TRMRAPDYTT	PEMYQ	TMLDCW	HGEP	SQRPTFSELVEHL
VEGFR-1	1109	FCSRLREG	MRRAPDYST	PEIYQ	TMLDCW	HRDPKER	PRFAELVEKLG
VEGFR-3	1174	QGRGLQE					
VEGFR-2	1165	QANAQQD					
VEGFR-1	1159	QANVQQD					

Figure 2. Sequence alignment of the three VEGF-Rs (amino acid differences highlighted) after the homology modeling.

Figure 3 shows a superimposition of the ATP-binding sites of all three homology modeled VEGF-Rs. Different amino acid residues in the ATP-binding site are shown in stick representation.

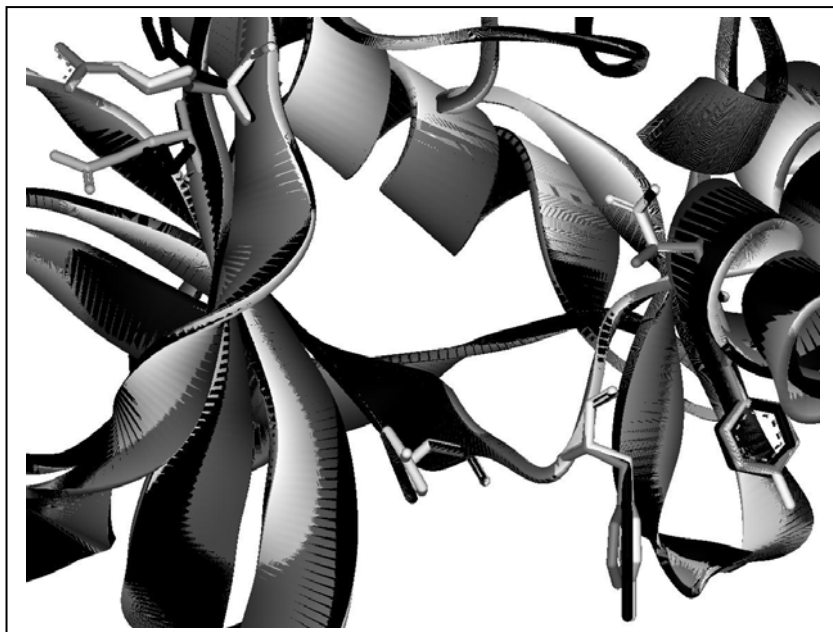


Figure 3. Superimposition of the homology models of the VEGF-R-1 (light grey), VEGF-R-2 (dark grey) and VEGF-R-3 (black). Different amino acid residues are shown in stick representation.

3.1. *In silico* screening

The 2D-/3D-similarity screening of the Inhouse database for chemically and structurally similar compounds resulted in about 60 compounds which resemble the lead structure (with a Tanimoto ≥ 0.85). The number of potential candidates could be reduced to 21 drug-like compounds by applying the Lipinski rule of five as molecular property filter.

3.2. Docking

The remaining 21 structures were docked into the ATP-binding site of the VEGF-Rs. The docking scores and the visual inspection of the docked ligand-receptor complexes were combined as qualitative evaluation of the *in silico* screening results. The docked structures of the lead compound Vatalanib and compound 10 to VEGF-R-1, -2 and -3 are exemplarily shown in Figure 4a-c) and Figure 4d-f), respectively.

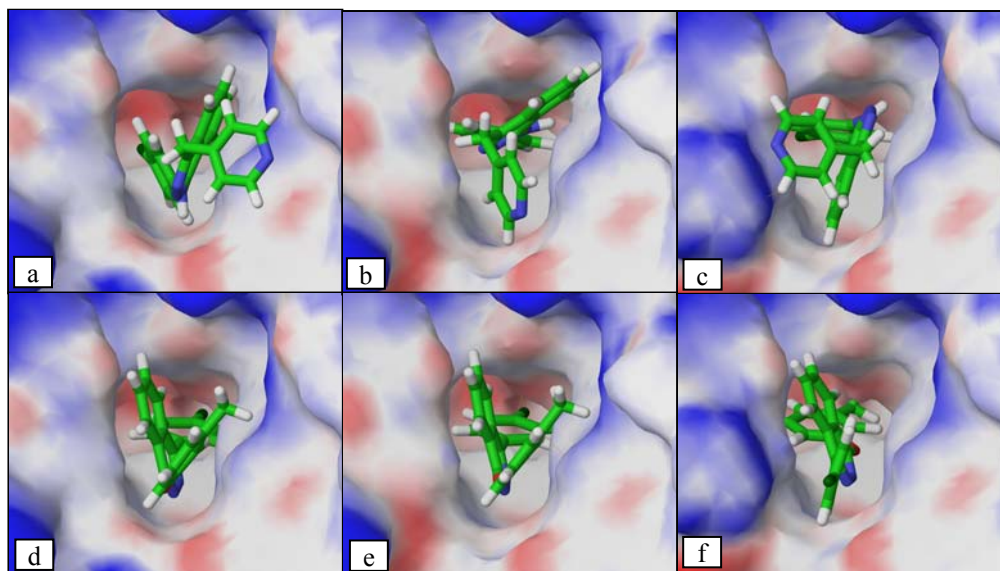


Figure 4. Ligand docked into the ATP-binding site (surface representation of the VEGF-Rs)
Lead structure (Vatalanib) : a) in VEGF-R-1 b) in VEGF-R-2 and c) in VEGF-R-3
Compound 10: d) in VEGF-R-1 e) in VEGF-R-2 and f) in VEGF-R-3

In Table 1 the docking scores for Vatalanib and compound 10 are listed. The evaluation of the docking results reveals better scores for compound 10 as for the lead structure. This suggests that compound 10 should have similar or even better biological activity.

Therefore, compound 10 was one of the 21 substances selected for experimental validation.

Table 1: Docking scores (Glide Score SP)

	Lead (Vatalanib)	Compound 10
VEGF-R-1	-4.51	-5.01
VEGF-R-2	-4.27	-4.86
VEGF-R-3	-4.92	-5.15

3.3. Experimental validation

The twelve compounds were tested *in vitro* for VEGF-R kinase activity inhibition, cell proliferation, migration inhibition and cytotoxicity. In Figure 5 the result of a cell proliferation assay on the endothelial cell line EA-HY 926 for compound 10 compared to the lead structure Vatalanib is exemplarily shown.

It can be concluded that compound 10, at a concentration of 10 μM , reduces cell proliferation by ~40% (light grey) whereas the cell proliferation decreases about 8% when treated with the lead compound. The results shown here confirm the *in silico* screening results.

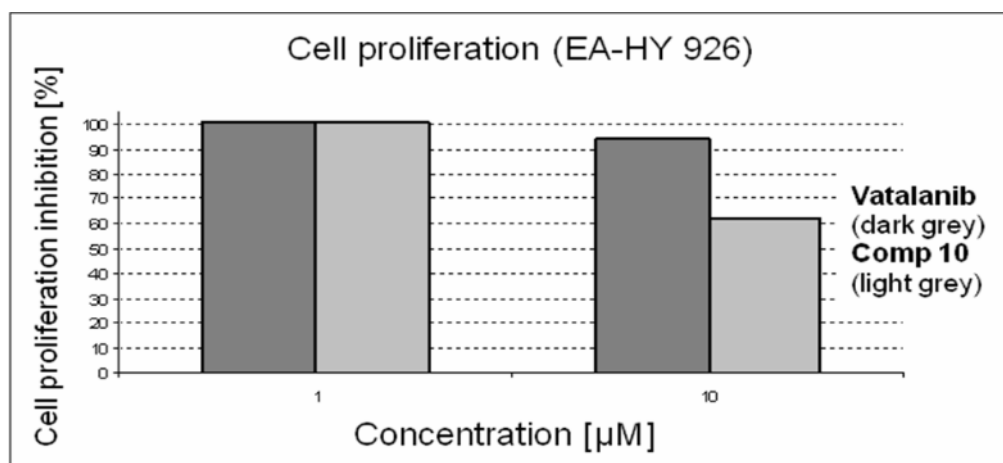


Figure 5. Cell proliferation assay (endothelial cell line EA-HY 926).

4. Conclusion and future work

Using this approach, we were able to identify new potential VEGF-receptor tyrosine kinase inhibitors. One of the hits was found to have a better effect on the inhibition of the cell proliferation than the lead structure. Therefore, we reason that the compound is a specific inhibitor of tumor angiogenesis. This compound will undergo further *in vitro* and *in vivo* experiments and will be starting point for further refinement cycles.

5. Acknowledgements

This work was supported by the International Research Training Group Boston-Kyoto-Berlin, funded by the DFG.

6. References

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