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Virtual Screening and in Vitro Activity Evaluation of Novel VEGFR-2 Inhibitors Designed Based on Scaffold Hopping Strategy

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Abstract
Tumor angiogenesis is a core process in the growth and metastasis of solid tumors. Vascular endothelial growth factor receptor 2 (VEGFR-2), as a key target regulating this process, has become an important direction for the research and development of antitumor drugs. In this study, tivozanib, a highly selective VEGFR-2 inhibitor, was used as the lead compound, and structural modification and optimization were performed via the scaffold hopping strategy. A virtual compound library containing 250 derivatives was constructed using SeeSAR software. After preliminary drug-likeness screening by Discovery Studio 2019, high-precision molecular docking was carried out using the XP mode of the Glide module in the Schrödinger suite, combined with ADMET property prediction via QikProp. Three representative candidate compounds (Y1, Y2, Y3) were obtained through multi-dimensional screening.The in vitro kinase activity assay showed that compound Y1 exhibited excellent VEGFR-2 inhibitory activity, with inhibitory rates against KDR kinase of 103.56% and 71.01% at concentrations of 10 μmol/L and 1 μmol/L, respectively, presenting a significant dose-dependent manner. In contrast, the inhibitory activities of Y2 and Y3 decreased markedly, suggesting that the amide linkage, 6-methyl substitution, and the spatial structure of the hydrophobic tail are crucial for maintaining target-binding activity. The candidate compound Y1 identified in this study possesses favorable druggability potential, providing a novel lead molecule and research strategy for the development of novel highly selective VEGFR-2 inhibitors.
Keywords: *VEGFR-2; tivozanib; scaffold hopping; virtual screening; in vitro kinase activity; antitumor drugs*

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I. Introduction

Tumor angiogenesis plays a critical role in the occurrence and progression of cancer. When a tumor grows beyond 1-2 mm^3 in volume, it relies on the formation of new blood vessels to supply sufficient oxygen and nutrients[1]. Vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) constitute one of the most important signaling pathways regulating tumor angiogenesis. Among them, VEGFR-2 (also known as KDR), as the primary transducer of VEGF signaling, is highly expressed in vascular endothelial cells and mediates key processes including endothelial cell proliferation, migration, survival, and increased vascular permeability[2].

VEGFR-2 belongs to the receptor tyrosine kinase family, and activation of its intracellular kinase domain is crucial for initiating downstream signaling cascades. Upon binding of VEGF to the extracellular domain of VEGFR-2, receptor dimerization and autophosphorylation are induced, which in turn activate multiple signaling pathways including Ras/Raf/MEK/ERK and PI3K/Akt, ultimately promoting endothelial cell proliferation and neovascularization (Figure 1)[3]. Since tumor angiogenesis is a prerequisite for the growth and metastasis of solid tumors, VEGFR-2 has become an important target for antitumor drug development. Inhibiting the kinase activity of VEGFR-2 can effectively block tumor angiogenesis, thereby achieving the therapeutic goal of suppressing tumor growth.

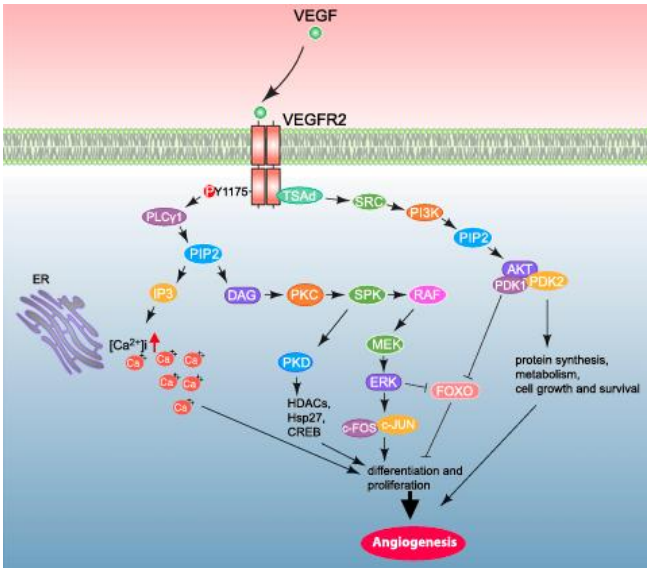
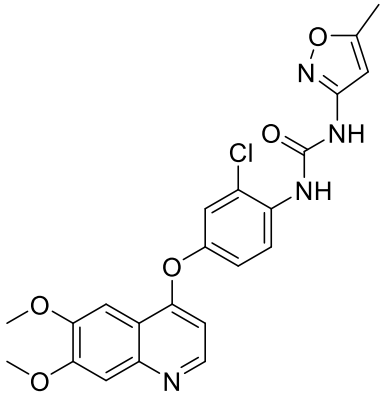


Figure 1 VEGFR-2 signaling pathway.

In recent years, several small-molecule tyrosine kinase inhibitors targeting VEGFR-2 have been approved for marketing successively and have played important roles in clinical cancer therapy. First-generation multi-target inhibitors such as sorafenib, sunitinib, and pazopanib exert dual effects of anti-angiogenesis and direct anti-proliferation by inhibiting multiple kinase targets including VEGFR-2. However, the multi-target nature of these agents also leads to relatively broad side effects, such as hand-foot skin reaction, myelosuppression, and liver dysfunction[4].

Tivozanib (Figure 2), a new-generation VEGFR inhibitor, was approved by the U.S. FDA in March 2021 for the treatment of recurrent or refractory advanced renal cell carcinoma[5]. Unlike first-generation inhibitors, tivozanib exhibits high selectivity and potent inhibitory activity against VEGFR-1, VEGFR-2, and VEGFR-3, while showing significantly lower affinity for other receptor tyrosine kinases[3]. This high selectivity contributed to a superior progression-free survival (11.9 months vs. 9.1 months) compared with sorafenib in the Phase III clinical trial (TIVO-1), together with a manageable toxicity profile. Tivozanib has a long elimination half-life, enabling more sustained inhibition of VEGFRs.



Tivozanib

Figure 2 Structure of tivozanib.

Despite the remarkable clinical efficacy of VEGFR-2 inhibitors, several urgent challenges remain. First, the development of drug resistance is a major factor limiting their long-term efficacy. Tumor cells can evade the anti-angiogenic effects of VEGFR inhibitors through various mechanisms, such as activating alternative angiogenic pathways, enhancing invasiveness, or upregulating other receptor tyrosine kinases. Second, side effects of existing inhibitors remain prominent. The most common adverse reaction of tivozanib is hypertension (45% incidence, including 22% of grade ⩾3 events), along with fatigue, diarrhea, dysphonia, hypothyroidism, and others. Although most of these side effects are manageable, they still impair patients' quality of life and treatment compliance[6]. Therefore, the development of VEGFR-2 inhibitors with novel structural scaffolds, capable of overcoming drug resistance and exhibiting reduced side effects, remains a hot topic in current drug

research.

In the present study, with VEGFR-2 as the target, novel derivatives were designed using the scaffold hopping strategy based on the structural characteristics of the highly selective inhibitor tivozanib. A virtual compound library was constructed using SeeSAR software, and multi-stage molecular docking screening was performed using the Glide module in the Schrödinger suite to obtain candidate compounds with excellent docking scores. QikProp was applied to predict their druggability, laying a foundation for subsequent chemical synthesis and biological activity evaluation. This study provides a feasible research strategy for the discovery of novel VEGFR-2 inhibitors and establishes a basis for overcoming the limitations of existing inhibitors and obtaining candidate compounds with independent intellectual property rights.

II. Results and Discussion

2.1 Overall Design Strategy

In this study, tivozanib, a highly selective VEGFR-2 inhibitor, was used as the lead compound. Structural modification was performed via the scaffold hopping strategy based on its co-crystal structure with the target protein (PDB ID: 4ASE)[7]. First, using the scaffold hopping module of SeeSAR software, the nitrogen atom linked to the hydrophobic tail in the urea core of tivozanib was removed to form an amide moiety (-CONH-), thereby preserving key hydrogen-bonding interactions[8]. Meanwhile, the benzene ring in the original hydrophobic tail was replaced with nitrogen-containing heterocycles (e.g., pyridine, pyrimidine, pyrazole) or five-membered aromatic heterocycles (e.g., thiazole, oxazole), establishing a virtual compound library containing 250 derivatives[9].

Subsequently, drug-likeness screening (based on Lipinski's Rule of Five and preliminary ADMET properties) was performed on these compounds using Discovery Studio 2019, yielding 200 compounds with favorable drug-likeness. These 200 compounds were imported into the Schrödinger suite, and high-precision molecular docking was carried out using the XP mode of the Glide module. With a binding free energy (XP Gscore) < -7 kcal/mol as the threshold, 125 candidate molecules were selected[10, 11]. ADMET properties were further predicted using QikProp, and molecules with potential toxicity or pharmacokinetic defects were excluded, giving 102 qualified compounds[12, 13]. Finally, through visual inspection, considering the novelty of binding modes, synthetic feasibility, and structural diversity, three representative compounds (coded Y1, Y2, Y3) were selected for chemical synthesis and in vitro activity validation (Figure 3).

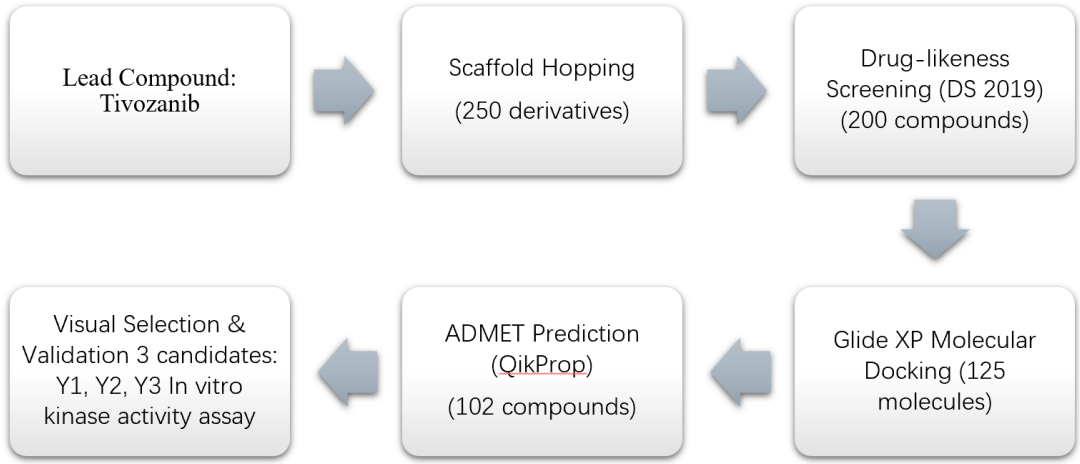


Figure 3 Screening process of small molecule inhibitors of VEGFR-2

2.2 Molecular Docking Screening and Binding Mode Analysis

To evaluate the binding ability of scaffold-hopped derivatives to VEGFR-2, high-precision molecular docking was performed on the 200 compounds retained after drug-likeness screening in Discovery Studio, using the Extra Precision (XP) mode of the Glide module in the Schrödinger suite. The XP mode enables more accurate discrimination between active and inactive compounds via more comprehensive energy scoring and conformational searching. Its scoring function includes van der Waals interactions, electrostatic interactions, hydrogen-bond contributions, desolvation effects, π-π stacking, and other energy components. The resulting XP Gscore (an estimate of binding free energy) effectively reflects the binding affinity between ligand and receptor.

In this study, XP Gscore < -7 kcal/mol was used as the preliminary screening threshold, which was set based on experience and known active compounds (e.g., XP Gscore of tivozanib = −10.892 kcal/mol). This threshold enriches potential highly active molecules while avoiding the loss of structurally novel weakly active compounds due to an excessively strict cutoff. Finally, 125 candidate molecules meeting the energy criteria were

obtained from the 200 compounds.

ADMET properties of these 125 compounds were then predicted using the QikProp module, focusing on key pharmacokinetic parameters including oral absorption, CYP450 inhibition risk, blood–brain barrier penetration, and compliance with Lipinski's Rule of Five. Molecules with obvious toxicity risks or pharmacokinetic disadvantages (e.g., predicted oral absorption < 80%, strong CYP2D6 inhibition, or high blood–brain barrier penetration) were eliminated, leaving 102 compounds with favorable overall properties.

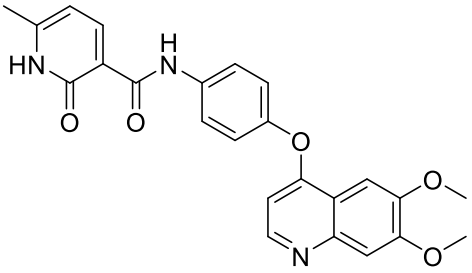
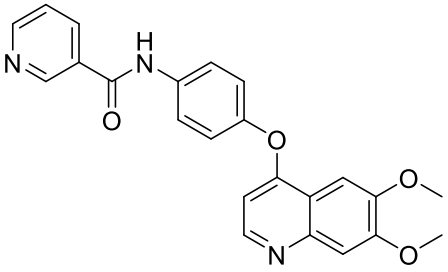
To further focus on the most promising molecules, detailed visual analysis was conducted on these 102 compounds. Within the Maestro visualization interface of Schrödinger, the binding modes of each molecule in the VEGFR-2 active site were examined one by one. Key attention was paid to: whether a critical hydrogen bond was formed with the backbone carbonyl of Cys919 in the hinge region (the core interaction for maintaining VEGFR-2 inhibitory activity); whether additional hydrogen bonds were formed with the side chain of Glu885 and residue Asp1046 to enhance binding specificity; whether the hydrophobic tail could effectively insert into the hydrophobic pocket formed by Leu840, Val848, Val916, and other residues, providing favorable π-alkyl or hydrophobic contacts.

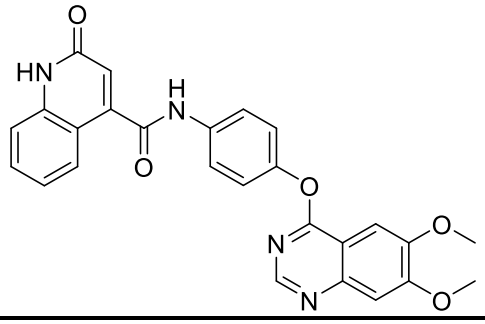
Meanwhile, synthetic feasibility (e.g., presence of overly complex fused rings or chiral centers, availability of known synthetic routes) and potential for subsequent structural modification were evaluated. Through the above multi-dimensional screening, three representative compounds with novel structures, clear interaction patterns, and moderate synthetic difficulty were ultimately chosen from the 102 compounds, designated Y1, Y2, and Y3, for subsequent chemical synthesis and in vitro activity validation.

2.3 In Vitro Kinase Activity Validation

After chemical synthesis of Y1, Y2, and Y3, their in vitro inhibitory activities were evaluated using a KDR (VEGFR-2) kinase activity assay kit. The results showed that Y1 exhibited favorable inhibitory activity against VEGFR-2. At a test concentration of 10 μmol/L, the inhibition rate of compound Y1 on KDR kinase reached 103.56%. When the concentration was reduced to 1 μmol/L, the inhibitory activity remained at 71.01%, showing obvious dose dependence, indicating high binding affinity between the compound and the target (Table 1).

Table 1 Inhibition Rate of Compounds Against KDR Protein

Compound	Structure	KDR inhibition(%)	
		10 μ m	1 μ m
Y1		103.56	71.01
Y2		69.50	15.05

Y3		19.57	4.97

In contrast, compound Y2 (with the 6-methyl group removed and simplified to a nicotinamide scaffold) exhibited inhibition rates of 69.50% at 10 μmol/L and 15.05% at 1 μmol/L, respectively, representing a significant decrease in activity compared with Y1. Compound Y3, in which the parent core was replaced with a bulkier quinolinone amide structure, showed an inhibition rate of only 19.57% at 10 μmol/L, almost completely losing effective inhibitory capacity against the KDR protein

III. Materials and Methods

3.1 Computer Hardware, Software and Databases

In this study, the Schrödinger Suite 2022-4 software package (Schrödinger, LLC, USA) was used for protein structure preparation, molecular docking, and ADMET property prediction, including the Protein Preparation Wizard, LigPrep, Glide, and QikProp modules. Scaffold hopping and virtual compound library construction were performed using SeeSAR software (BioSolveIT, Germany). Preliminary drug-likeness screening was carried out using Discovery Studio 2019 (Dassault Systèmes, France). Molecular visualization and binding mode analysis were performed in the Maestro 12.8 interface.

The three-dimensional crystal structure of the VEGFR-2 kinase domain was obtained from the RCSB Protein Data Bank (PDB ID: 4ASE), with a resolution of 2.5 Å, corresponding to the co-crystal complex of tivozanib bound to VEGFR-2.

3.2 Protein Structure Preparation

The 4ASE crystal structure was preprocessed using the Protein Preparation Wizard module: all crystal water molecules were removed (except those that might form hydrogen bonds with the active site and participate in ligand interactions), missing hydrogen atoms and side-chain atoms were added, the hydrogen-bond network was optimized, and energy minimization was performed. Energy minimization was conducted using the OPLS4 force field, with a convergence criterion of root-mean-square deviation (RMSD) of non-hydrogen atoms reaching 0.30 Å. The processed protein structure was used for molecular docking studies.

3.3 Compound Library Construction

Using tivozanib as the lead compound, structural modifications were performed via the scaffold hopping module in SeeSAR. The modification strategies included:(1) Removing the nitrogen atom linked to the hydrophobic tail in the urea core to form an amide moiety (-CONH-), thereby preserving key hydrogen-bonding interactions;(2) Replacing the benzene ring in the original hydrophobic tail of tivozanib with nitrogen-containing heterocycles (pyridine, pyrimidine, pyrazole, etc.) or five-membered aromatic heterocycles (thiazole, oxazole, etc.).

Based on the above principles, an initial library containing 250 virtual compounds was constructed using the combinatorial library generation function of SeeSAR.

The 250 constructed compounds were imported into the LigPrep module. Possible ionization states were generated at pH 7.0±2.0, and energy optimization was performed using the OPLS4 force field to generate three-dimensional structures for subsequent screening.

3.4 Preliminary Drug-Likeness Screening

The 250 compounds processed by LigPrep were imported into Discovery Studio 2019. Drug-likeness evaluation was performed based on Lipinski's Rule of Five (molecular weight ≤ 500, number of hydrogen-bond donors ≤ 5, number of hydrogen-bond acceptors ≤ 10, logP ≤ 5) and preliminary ADMET parameters (including oral absorption, blood-brain barrier penetration, CYP450 inhibition potential, etc.). A total of 200 compounds meeting the drug-likeness criteria were selected for molecular docking studies.

3.5 Molecular Docking

Grid generation: A docking grid was generated in the active site region centered on the co-crystal ligand tivozanib, with a grid size of 10 Å × 10 Å × 10 Å, covering key residues including Cys919, Glu885, Asp1046, Phe1047, Leu840, Val848, and Val916.

Docking parameters: High-precision molecular docking was performed using the Extra Precision (XP) mode of the Glide module. Ligand conformational flexibility was considered during docking; up to 5 conformations were generated per ligand, and only the optimal conformation was retained for scoring. All other parameters were set to default.

Docking screening procedure: The 200 compounds screened by Discovery Studio were imported into Glide XP for docking. Using a threshold of XP Gscore < −7 kcal/mol, 125 candidate compounds were selected.

Docking reliability validation: The co-crystal ligand tivozanib was extracted from the original complex and re-docked into the original binding site. The RMSD between the re-docked conformation and the co-crystal conformation was 0.85 Å (less than 2.0 Å), indicating that the selected docking parameters could well reproduce the experimental binding mode.

3.6 ADMET Property Prediction

The 125 compounds obtained from XP docking were imported into the QikProp module for pharmacokinetic property prediction. Evaluated parameters included molecular weight, logP, number of hydrogen-bond donors, number of hydrogen-bond acceptors, predicted oral absorption, CYP2D6 and CYP3A4 inhibition risk, blood-brain barrier penetration, etc. Molecules with potential defects such as predicted oral absorption < 80%, strong CYP2D6 inhibition, or high blood-brain barrier penetration were eliminated, leaving 102 compounds with favorable overall properties.

3.7 Candidate Compound Selection

Detailed visual inspection was performed on the 102 compounds retained after ADMET prediction in the Maestro visualization interface. The screening criteria included:(1) Formation of a key hydrogen bond with the backbone carbonyl of Cys919 in the hinge region;(2) Formation of additional hydrogen bonds with residues including Glu885 and Asp1046 to enhance binding specificity;(3) Effective insertion of the hydrophobic tail into the hydrophobic pocket formed by Leu840, Val848, Val916, and other residues to form favorable hydrophobic or π-alkyl interactions;(4) Synthetic feasibility and potential for further structural modification.

Based on the above criteria, three representative compounds were finally selected and designated Y1, Y2, and Y3.

3.8 Chemical Synthesis

Compounds Y1, Y2, and Y3 were synthesized by conventional organic synthetic methods (outsourced or prepared in our laboratory). The synthetic routes used corresponding substituted aromatic amines or heterocyclic amines as starting materials, and the target scaffolds were constructed via amide condensation, cyclization, and other steps. All target compounds were purified by silica gel column chromatography. Their structures were confirmed by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry (HRMS), and their purities determined by high-performance liquid chromatography (HPLC) were all above 95%.

3.9 In Vitro Kinase Activity Assay

The inhibitory activities of the compounds against KDR (VEGFR-2) kinase were determined using the homogeneous time-resolved fluorescence (HTRF) assay. Experiments were performed in 384-well plates. The reaction system consisted of kinase reaction buffer, KDR kinase (purchased from Carna Biosciences), biotin-labeled substrate polypeptide, ATP (concentration near the Km value), and serially diluted test compounds[14]. After incubation at room temperature for 30 min, detection reagent (containing Eu-labeled anti-phosphotyrosine antibody and streptavidin-XL665) was added to terminate the reaction. After further incubation for 1 h, the HTRF signal was recorded[15].

Each compound was tested at two concentrations: 10 μmol/L and 1 μmol/L. DMSO was used as the negative control, and tivozanib as the positive control. The inhibition rate was calculated using the following formula:

$$Inhibition\ \% = \frac{OD_{control} - OD_{sample}}{OD_{control} - OD_{blank}} \times 100\%$$

Triplicate wells were set for each concentration, and results were expressed as mean ± standard deviation.

IV. Conclusions

In this study, structural modification of tivozanib was carried out based on the scaffold hopping strategy. Three structurally novel candidate VEGFR-2 inhibitors, namely Y1, Y2 and Y3, were obtained from 200 candidate

compounds via multi-stage virtual screening, including drug-likeness screening in Discovery Studio, high-precision Glide XP molecular docking, QikProp-based ADMET property prediction and visual inspection analysis.

The in vitro kinase activity assay results demonstrated that compound Y1 exerted excellent inhibitory activity against KDR protein, with an inhibition rate of 103.56% at a concentration of 10 μmol/L, and still retained 71.01% inhibitory activity even at the low concentration of 1 μmol/L, showing remarkable dose-dependent manner and high target affinity. These activity results were consistent with its outstanding docking score (-11.578 kcal/mol) and favorable ADMET prediction properties. In contrast, the inhibitory activities of Y2 (with a simplified nicotinamide structure) and Y3 (with an extended quinolinone scaffold) decreased significantly, indicating that the 6-methyl group and amide linkage mode are crucial for maintaining the VEGFR-2 inhibitory activity, and an overlarge hydrophobic tail volume may hinder effective occupation of the target active pocket.

Combining the results of virtual screening and in vitro activity validation, compound Y1 possesses the optimal in vitro inhibitory activity and druggability potential, and can be used as a preferred lead compound for novel VEGFR-2 inhibitors. It is worthy of further in vivo efficacy evaluation and in-depth mechanism of action research to lay a foundation for the development of high-selectivity antitumor drugs targeting VEGFR-2.

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