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Steering Target Selectivity and Potency by Fragment-Based De Novo Drug Design**

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Computer-aided approaches help to mitigate attrition rates of drug candidates and suggest new chemical entities for sustained drug discovery.[1–3] In this study we followed a dual strategy of ligand-based de novo design and fragment grafting for generating innovative and readily synthesizable compounds, which we morphed into a highly potent and selective kinase inhibitor. We disclose the discovery of a ligand-efficient[4] (ligand efficiency (LE) = 0.35) inhibitor presenting an IC_{50} value of 64 nM against vascular endothelial growth factor receptor-2 (VEGFR-2) kinase. This lead compound exhibits the highest kinase selectivity profile known to date among VEGFR-2 kinase inhibitors (Gini index[5] = 0.87), with the essential selectivity feature having been generated by de novo design. Further profiling fully corroborated VEGFR-2-selective effects on a cellular level. Our findings validate fragment-based de novo design as a premier method for rapid lead-structure prototyping, thereby offering a compelling solution to finding tailored bioactive compounds for chemical biology and drug discovery.

Tyrosine kinase VEGFR-2 is a drug target for antiangiogenic therapy and known to dimerize upon VEGF-A stimulation, which is especially implicated in tumor angiogenesis.[6] We used type-II VEGFR-1/2 inhibitor AMG-706[7] ($IC_{50} = 2.3 \pm 0.6$ nM against VEGFR-2) as template for automated de novo design (Figure 1), since it featured the best selectivity profile amongst published kinase inhibitors primarily targeting VEGFR-2.[8] With recent phase-III clinical trials of AMG-706 showing no apparent benefit for overall survival of patients,[9] there is an urgent need for innovative successor drugs. A recent structure-based design study reports compounds with a pyrazole core as VEGFR-2 inhibitors,[10] but the fully automated design of structurally novel and kinase-subtype-selective chemotypes with comparable properties to AMG-706 may well be envisaged as an extremely challenging task.

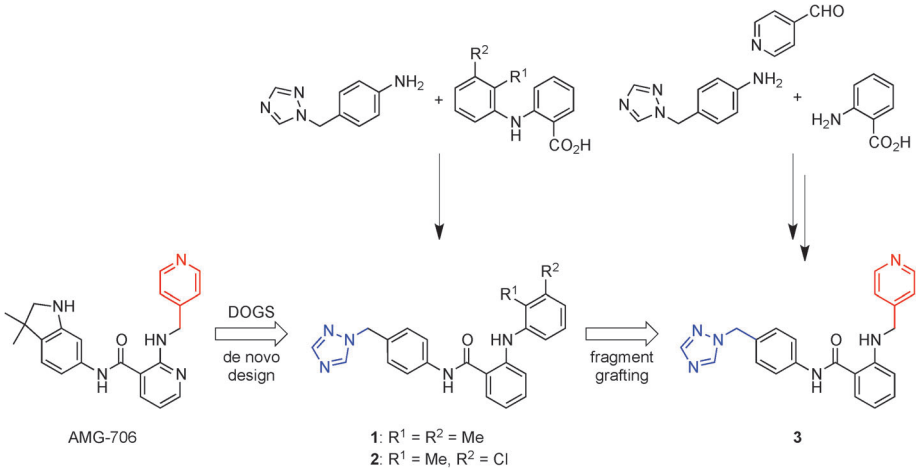

Figure 1. Computer-generated synthesis scheme for compounds **1** and **2**, which were designed by the software DOGS as mimetics of the template AMG-706, and design strategy for compound **3**. The red moiety is supposed to bind to the kinase hinge region, and the blue fragment determines selectivity.

Here, we utilized the software DOGS (design of genuine structures)[11,12] to computationally generate candidate compounds. DOGS implements reaction-based molecule construction, requiring only a template drug for generating new molecules. The algorithm suggested a total of 3368 hypothetical VEGFR-2 inhibitors combined with potential synthetic pathways. We analyzed the designs for underlying molecular scaffolds (the Supporting Information Table S1 and Figure S1) and found remarkable structural variety: Approximately every second design featured a different scaffold. This observation reflects the scaffold-hopping capabilities of the

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DOGS algorithm. We selected compounds **1** and **2** for synthesis and testing, since they explored contiguous chemical space to AMG-706, yet apparently remained free of intellectual property (SciFinder; https://scifinder.cas.org). Their structural similarity to AMG-706, expressed as Jaccard–Tanimoto index and computed from substructure keys (MACCS, Accelrys), is 0.60 (**1**) and 0.66 (**2**), respectively. Hence, they may be considered a different chemotype than the template.[13] Both compounds were synthesized as suggested by the design algorithm.

We then tested their inhibitory activity against VEGFR-1/2. In a direct binding assay both molecules inhibited VEGFR-2 in a concentration-dependent manner (**1**: $IC_{50} = 23\ \mu M$, **2**: 14 μM; Figure 2b). Profiling them against a panel of 48 human kinases revealed negligible potency against other enzymes, as expressed by Gini index[5] values of 0.71 for **1** and **2**, and 0.72 for AMG-706 (the Supporting Information Table S2). Apparently, the computer-designed compounds inherited overall kinase selectivity from the template AMG-706. Quite remarkably, unlike AMG-706 ($IC_{50} = 34 \pm 15\ nM$, Figure 2a), **1** and **2** were devoid of anti-VEGFR-1 activity ($IC_{50} > 100\ \mu M$, Figure 2a). Thus, these compounds possess crucial features to obtain an innovative generation of kinase inhibitors that differentiate between VEGFR-1/2. Most notably, the key feature for selectivity was generated de novo, and bioactive compounds were readily obtained with minimalist synthetic effort.

AMG-706 potently inhibits the receptor tyrosine kinases VEGFR-1/2/3, Kit, and PDGFR. Despite being thought that inhibition of several tyrosine kinases might result in stronger inhibition of tumor angiogenesis and delayed incidence of resistance,[7] AMG-706 failed to show significant benefits in a phase-III clinical trial.[9] In another study AMG-706 led to blood serum increase of placental growth factor (PlGF) in patients.[14] PlGF has proangiogenic activity,[15] is an important mediator of resistance to antiangiogenic therapy,[16] and binds selectively to VEGFR-1.[17] Specific blockade of VEGFR-1 induced angiogenesis while blocking VEGFR-2 inhibited angiogenesis in a murine tumor model.[18] Moreover, mutant- and cancer-selective irreversible inhibitors of the epidermal growth factor receptor (EGFR) highlight the benefit of new chemotypes for selective inhibition of neovascularization-related targets.[19]

Consequently, we studied the effects of **1** and **2** on VEGF-sensitive cells. Treatment of human blood vascular endothelial cells (BVEC) resulted in concentration-dependent inhibition of cell proliferation, with minimal effective concentrations of 20 μM (**1**) and 10 μM (**2**; Figure 2c,d). In the presence of recombinant human VEGF-A, we found minimal inhibitory concentrations of 20 μM for compound **1** and 5 μM for compound **2**. We next investigated whether **1** and **2** inhibit phosphorylation of VEGFR-2. Treatment of BVEC with VEGF-A for 15 min potently induced VEGFR-2 phosphorylation (Figure 2e,f). Preincubation with **1** (50 μM) for five hours completely inhibited VEGF-A-induced phosphorylation of VEGFR-2 (Figure 2e). Compound **2** presented an even stronger effect, with an identical outcome at 20 μM (Figure 2f). These results establish that the de novo designed compounds **1** and **2** potently inhibit phosphorylation of VEGFR-2 after stimulation by VEGF-A, and are in perfect agreement with the inhibition of VEGF-A-induced BVEC proliferation.

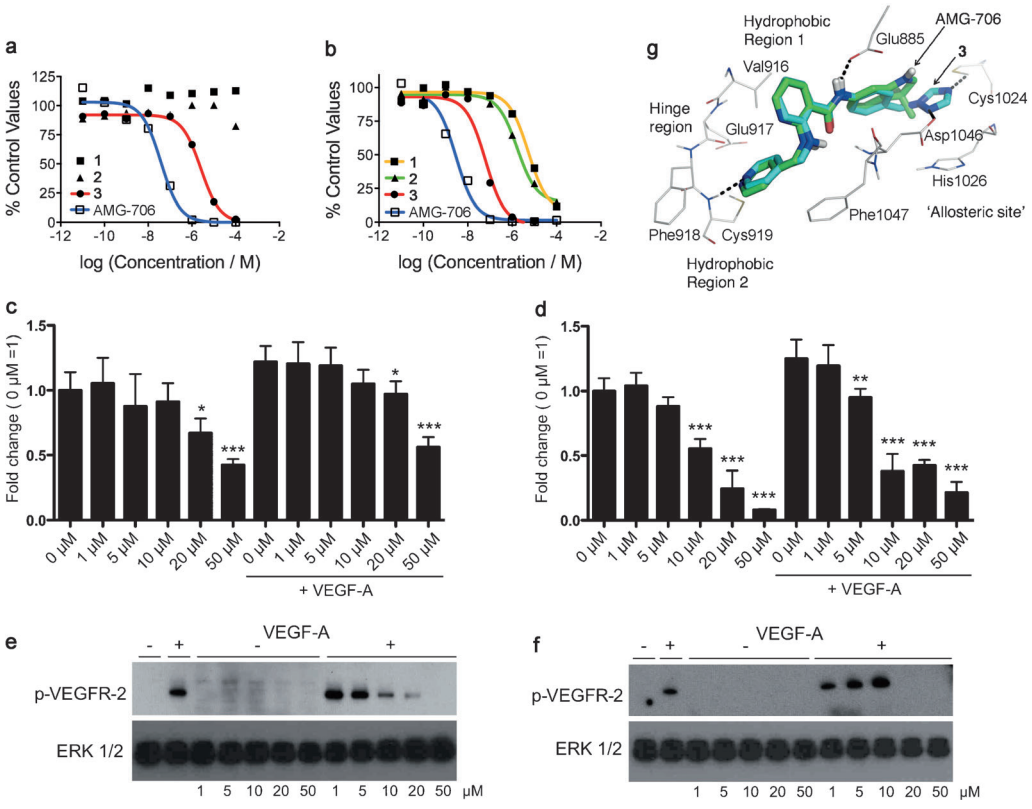

Figure 2. a,b) Direct in vitro inhibitory effect of compounds **1**–**3** and AMG-706 against VEGFR-1 (a) and VEGFR-2 (b). c,d) Compounds **1** (c) and **2** (d) inhibit BVEC proliferation in vitro (5-methylumbelliferyl heptanoate (MUH) assay). Data are representative of three independent experiments (mean ± σ; $n = 5$ per group; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). e,f) VEGF-A-induced phosphorylation of VEGFR-2 is inhibited by compounds **1** (e) and **2** (f); ERK 1/2 expression served as a loading control; p-VEGFR-2 = phosphorylated VEGFR-2. g) Crystal structure of AMG-706 bound to VEGFR-2 (PDB ID: 3efl) and docking pose of compound **3**. Potential interactions are shown as dotted lines. The image was created with PyMOL (http://www.pymol.org).

Inhibition of baseline proliferation in the absence of exogenous recombinant VEGF-A corroborates the reported dependency of in vitro endothelial cell growth on VEGFR-2 signaling.[20]

Mediocre VEGFR-2 inhibition by compound **2** suggested the lack of a hinge-binding motif. Therefore, we envisaged that grafting a suitable fragment would improve potency of the resulting chimeric molecule (Figure 1), while maintaining full kinase selectivity. Hybrid compound **3** was synthesized and tested. It sustained high VEGFR-2 selectivity ($IC_{50} = 2400 \pm 300$ nM and 64 ± 19 nM against VEGFR-1/2) and showed remarkably low activity in a panel of 48 kinases. In fact, compound **3** is the most selective VEGFR-2 inhibitor known to date (Gini index = 0.87), to our knowledge. Its high lipophilic ligand efficiency (LLE) of 5.03 (ALOGPS 2.1; http://www.vcclab.com) suggests appropriate properties for drug development. To rationalize the potency and selectivity of **3**, we docked the ligand in the ATP-binding site of VEGFR-2 using the software GOLD[21] and compared it to an AMG-706–VEGFR-2 complex (PDB ID: 3efl [Tasker&Patel, unpublished]). The model pose of **3** indicates crucial preserved interactions, for example, hydrogen bridges to Cys^{919}/Glu^{885} (Figure 2 g). Its outstanding selectivity may be explained by polar and π–π interactions in the "allosteric site", which are not seen for AMG-706. Keeping in mind the pitfalls of automated molecular docking,[22] the model offers an explanation for VEGFR-2-selective inhibition.

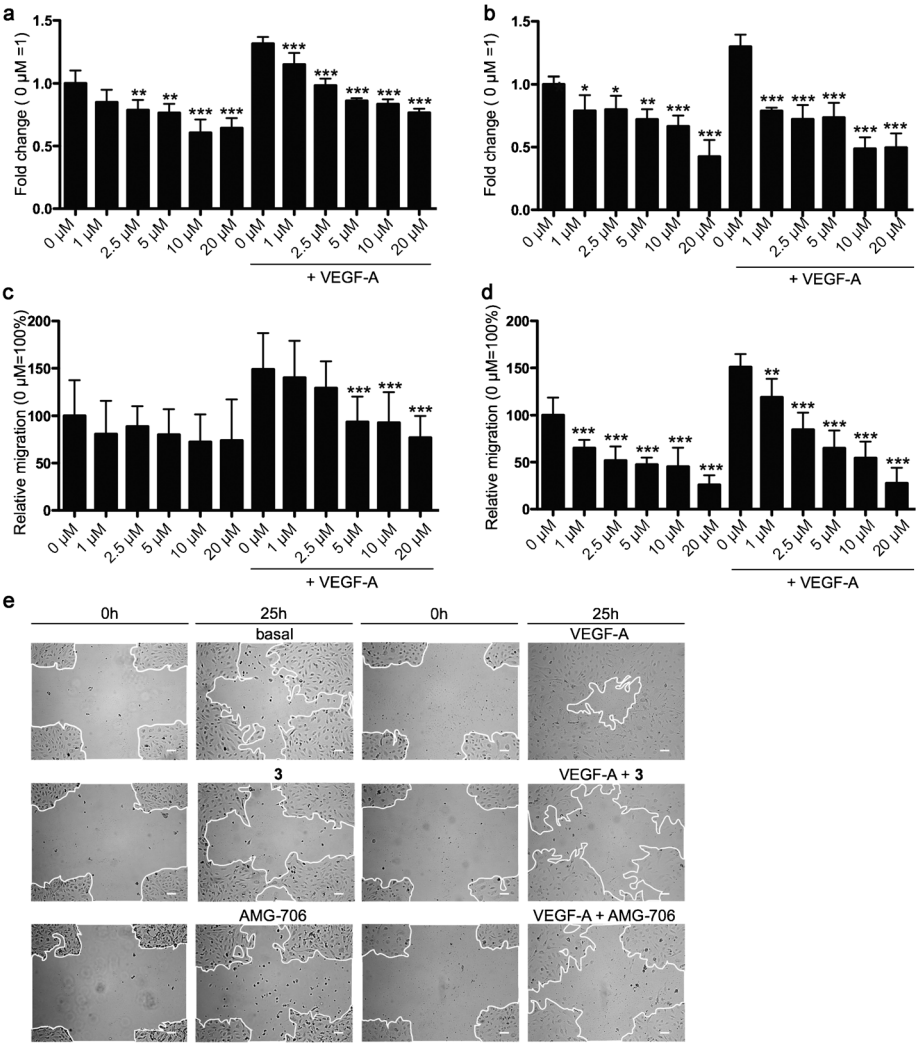

Figure 3. a, b) Compound **3** (a) and AMG-706 (b) inhibit proliferation of BVECs in vitro (MUH assay). Data are representative of four independent experiments (mean ± σ; $n = 5$ per group). c, d) Compound **3** (c) and AMG-706 (d) inhibit BVEC migration in vitro. Cell migration was measured using the TScratch software. Data are representative of two independent experiments (mean ± σ; $n = 4$ per group). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. e) Representative images of scratches before addition of compound in the absence or presence of VEGF-A and corresponding images of the scratch closures after 25 h. Scale bars: 100 µm.

In vitro effects of **3** revealed concentration-dependent inhibition of BVEC proliferation, with minimal effective concentrations of 2.5 µM for **3** and 1 µM for AMG-706. In the presence of recombinant human VEGF-A, both compounds showed minimal inhibitory concentrations of 1 µM (Figure 3 a, b). In wound healing scratch assays, compound **3** did not affect BVEC migration, while AMG-706 yielded a minimal inhibitory concentration of 1 µM. Incubation of both compounds with recombinant human VEGF-A inhibited cell migration with minimal inhibitory concentrations of 5 µM (**3**) and 1 µM (AMG-706; Figure 3 c,d). Impeded basal cell migration by AMG-706 can be explained by blocking multiple kinases such as EphA2,[23] EphB4,[24] and p38alpha,[25] whereas our data suggest selectivity of **3**, even on a cellular level. Since the VEGF-A–VEGFR-2 axis is considered to represent the major mediator of pathological angiogenesis, including tumor growth and chronic inflammation, we investigated the effect of **3** on MCF7 breast, HeLa-S3 cervical, LU-1205 melanoma, and A549 lung cancer cell lines. Compound **3** showed no effect on cell viability (the Supporting Information Figure S2). Apparently, VEGFR-2 expression appears to be irrelevant in those cell lines, which would be in line with failure of AMG-706 in clinical trials. Altogether, the results not only provide a prime lead for antiangiogenic therapy in several diseases, including VEGFR-2-responsive cancer, but also a valuable tool for chemical biology and molecular medicine.

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