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NVP-BHG712: Effects of regioisomers on the affinity and selectivity towards the EPHrin family

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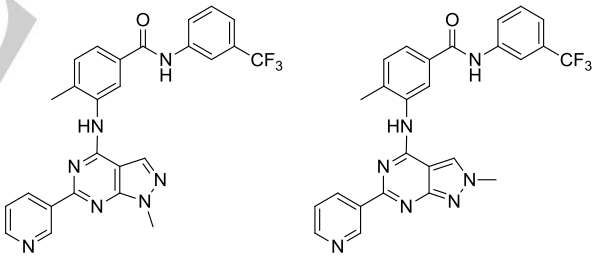
Abstract: EPH receptors are transmembrane receptor tyrosine kinases. Their extracellular domains bind specifically to ephrin A/B ligands, and this binding modulates the intracellular kinase activity. EPHs are key players in bidirectional intercellular signaling, controlling cell morphology, adhesion and migration. They are increasingly recognized as cancer drug targets. We analyzed the binding of the *Novartis* inhibitor NVP-BHG712 (NVP) to EPHA2 and EPHB4. Unexpectedly, all tested commercially available NVP samples turned out to be a regioisomer (NVPiso) of the inhibitor, initially described in a *Novartis* patent application. They only differ by the localization of a single methylation on either one of two adjacent nitrogen atoms. The two compounds of identical mass revealed different binding modes. Further, both *in vitro* and *in vivo* experiments showed that the isomers differ in their kinase affinity and selectivity.

Erythropoietin-producing hepatocellular (EPH) receptors represent the largest family of receptor tyrosine kinases (RTKs). There are two EPH subfamilies based on sequence conservation that exhibit different ephrin A/B ligand affinities (EPHA1-10, EPHB1-6).[1] 14 out of 16 EPH receptors can be found in humans (EPHA1-8 & 10; EPHB1-4 & 6).[2] Upon interaction with the corresponding ephrin ligands, these RTKs act as key elements in bidirectional intercellular signal transduction pathways, controlling cell morphology, adhesion and migration.[3] Moreover, EPHs are involved in cancer development: As a result of different signaling modalities, several partly opposing effects (promotion and suppression of tumorigenicity) can be observed.[4,5] Within the EPH family, particularly EPHA2 and EPHB4 are overexpressed in cancer cells. Downregulation of their expression can inhibit tumorigenicity, suggesting an important role in tumor cancer biology.[4] For example EPHA2 is overexpressed in head and neck,[6] breast,[7] or non-small cell lung cancer[8] and associated with poor patient prognosis.[9,10] To further evaluate the role of EPHA and EPHB receptors as therapeutic targets, specific inhibitors for these kinases are in demand.[11–14]

Recently, we investigated the off-target binding and activity of 235 clinically evaluated kinase inhibitors on EPHA2 to identify promising pharmacophores for further inhibitor development using the so-called Kinobeads technology.[15] 24 inhibitors were shown to bind EPHA2 with submicromolar affinity. We solved structures of the EPHA2 kinase domain (D596-G900) with nine different bound inhibitors.

In the context of this off-target screening with Kinobeads, we could identify NVP-BHG712 (NVP), a nanomolar EPHB4 inhibitor claimed by a *Novartis* patent application in 2007,[16,17] to inhibit EPHA2 with similar affinity than EPHB4. NVP consists of two parts, a methylated *N*-phenylbenzamide moiety and the adenine-related pyrazolo[3,4-*d*]pyrimidine moiety (Scheme 1).



NVP-BHG712 (NVP) **NVPiso**

Scheme 1: Constitution of NVP-BHG712 (NVP) and its isomer (NVPiso).

Based on previous structural data, we anticipated that the *N*-phenylbenzamide moiety would lead to a binding of NVP to the DFG-out conformation of EPHA2, comprising a conserved Asp-Phe-Gly (DFG, in EPHA2: D757, F758, G759) motif at the proximal end of the activation loop of kinases.[18,19] This prediction was verified by $^{1}H,^{15}N$ correlation spectra of selectively ^{15}N-phenylalanine labeled EPHA2 kinase domain expressed in *Sf9* cells.[20] The spectra showed a dynamic behavior of the DFG-motif in the apo state of the kinase.[20,21] Addition of NVP gave rise to a single additional amide signal at $\delta(^{15}N)$ = 124.29 ppm, $\delta(^{1}H)$ = 7.95 ppm, which could be assigned to F758. This resonance is characteristic for EPHA2 locked in a single, DFG-out conformation (see Figure S6 in the SI).[20] We next co-crystallized the EPHA2 kinase with the commercially available NVP compound.

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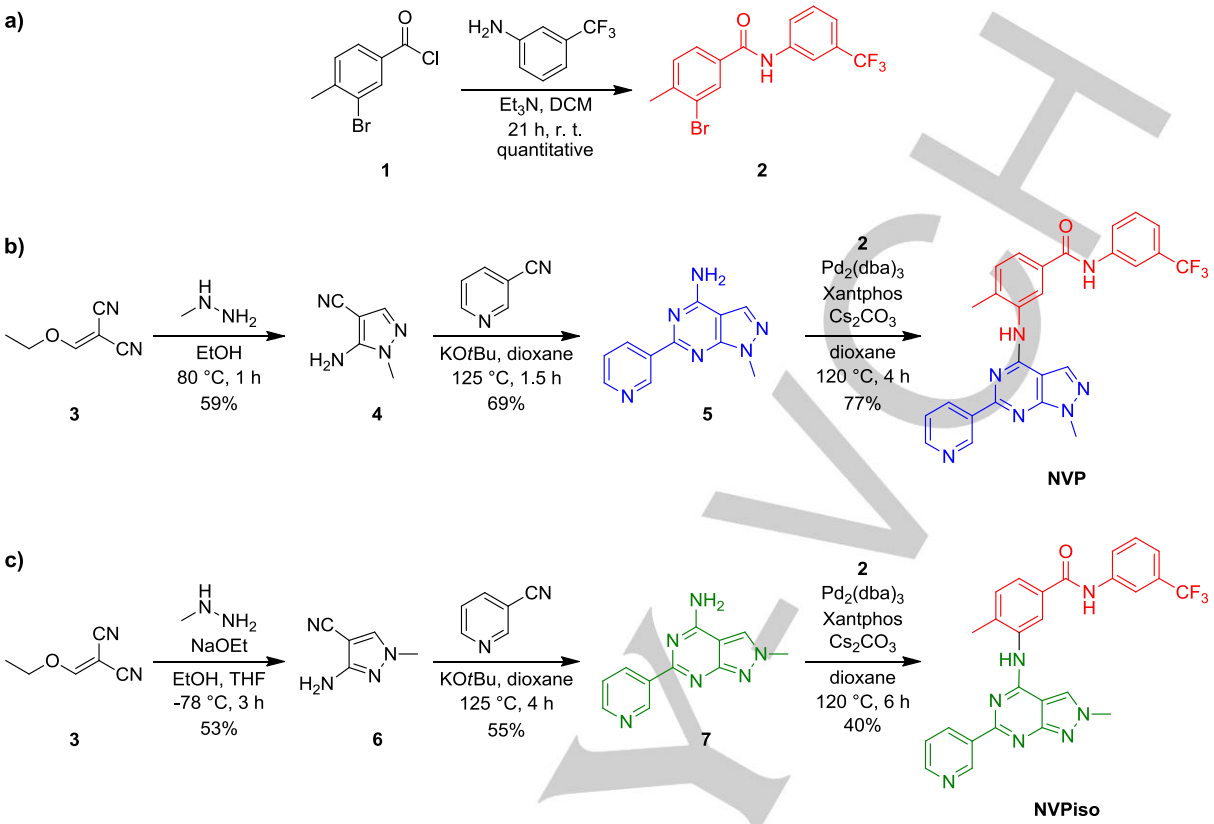

Scheme 2: Synthesis of NVP-BHG712 (4 steps) and its structural isomer NVPiso using the new improved synthesis strategy. (a) Reaction to the *N*-phenylbenzamide fragment; b) Synthesis of the patented *Novartis* compound NVP-BHG712; c) Synthesis route of the structural isomer NVPiso).

Crystallographic data at a resolution of 1.04 Å confirmed the conformational arrest of the activation loop and the DFG-out binding mode of the inhibitor·EPHA2 complex. Unexpectedly, however, the data revealed that the commercially available inhibitor possessed a wrong constitution. It was only possible to fit a regioisomer of NVP (NVPiso, Scheme 1) of identical mass with shifted position of a single methyl group into the electron density. Further NMR analysis of inhibitor samples from six different suppliers identified all of those as the same NVPiso regioisomer (see Figure S4 in the SI). Therefore, we decided to synthesize and analyze the original compound described by *Novartis* and its structural isomer in-house.

The *Novartis* patent application describes a seven step synthesis for NVP. An aniline synthon, (compound **S3**, Scheme S1 in the SI, depicted in red, obtained in two steps) is coupled to a nitrogen-rich chloro-heterocycle (pyrazolo[3,4-*d*]pyrimidine **S6,** Scheme S1 in the SI, depicted in blue, obtained in four steps), to yield the final product.[17] In our synthesis, we relied on *Buchwald-Hartwig* chemistry for the final step in order to shorten the synthetic route (Scheme 2). Instead of an aniline, we prepared a bromoaryl derivative (compound **2**) and moved the amine substituent to the pyrazolo[3,4-*d*]pyrimidine building block (compound **5**), which could be obtained in two steps. Overall, this new synthesis route reduced the number of reaction steps from seven to four and avoided pyrophoric (Raney-Ni) and highly corrosive ($POCl_3$) reagents. Our synthesized NVP batch was characterized by standard analytical techniques (MS, ^{1}H-, ^{13}C- and ^{19}F-NMR). Additionally, its molecular structure was unambiguously confirmed by small molecule X-ray crystal structure analysis (see SI for details).

The regioisomer NVPiso, which is the compound that one can purchase from six vendors, can be synthesized applying a minor modification in the synthesis (Scheme 2). Under neutral reaction conditions, the 5-aminopyrazole derivative **4** is formed predominantly, whereas the addition of a base yields the 3-aminopyrazole derivative **6** as the main product.[22] Both structural isomers can be distinguished by ROESY NMR spectroscopy: A close distance was observed between CH_3 and NH_2 in **4**, the derivative **6** showed however a cross peak between CH_3 and CH_2 (see Figure S12 and Figure S17 in the SI). The two subsequent steps were identical to the synthesis of NVP (small molecule X-ray crystal structure of NVPiso available in the SI). Due to the different substituent pattern, both isomers show different UV/Vis absorption spectra (Figure S34 in the SI). This offers the opportunity to differentiate both compounds, without access to more sophisticated analytical techniques.
Thus, a small modification in the reaction conditions led to the two different regioisomers. By starting the synthesis of pyrazole **4** from hydrazine hydrochloride as described by *Novartis*, the use of an excess of base could easily lead to the isomeric form **6** instead of the desired product.

Crystal structures of EPHA2 and EPHB4 with NVP and NVPiso revealed very deep binding in the ATP pocket and its adjacent region (see Figure 1) for both compounds. The regioisomers show conserved non-bonded binding to EPHA2 and EPHB4 and

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feature typical characteristics of kinase inhibitors: hydrogen bonds to the gate keeper T692 (EPHB4: T693), to D757 (D758) of the activation loop (DFG-motif) and to M695 (M696) of the hinge region, either to nitrogen atoms of the NVP pyrazole or the NVPiso pyridine moiety. Both regioisomers arrest the DFG-loop in an inactive out conformation. Interestingly, the salt bridge between E663 (E664) and K646 (K647), which is usually characteristic for an active kinase state, is maintained, which stabilizes the αC-helix and preserves a pseudo-active kinase conformation. Another 13 (15) hydrophobic contacts and further water-mediated interactions stabilize the binding of both regioisomers.

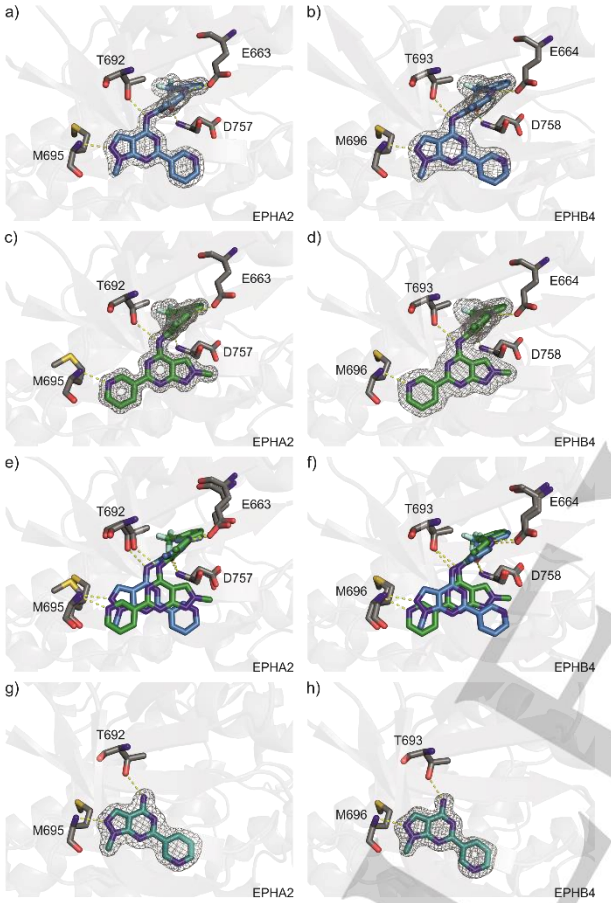

Figure 1: Crystal structures of NVP, NVPiso and the fragment **5** in complexes with EPHA2 and EPHB4. a & b) Crystal structures of NVP·EPHA2 (a), PDB ID: 6FNF) and NVP·EPHB4 (b), PDB ID: 6FNI); the nitrogen of the pyrazole ring is forming the hydrogen bond towards M695 or M696. c & d) Crystal structures NVPiso·EPHA2 (c), PDB ID: 6FNG) and NVPiso·EPHB4 (d), PDB ID: 6FNJ); the hydrogen bond towards the M695 and M696 is formed by the nitrogen atom in the pyridine substituent. e & f) Comparison of the binding mode of both isomers in EPHA2 (e) and EPHB4 (f); the pyrazolo[3,4-*d*]pyrimidine moiety shows a rotation around 180°. g & h) Crystal structures of the fragment **5**·EPHA2 (g), PDB ID: 6FNH) and **5**·EPHB4 (h), PDB ID: 6FNK); the fragments bind in comparable poses as the pyrazolo[3,4-*d*]pyrimidine moiety of NVP to the kinases, which here adopt a DFG-in conformation.

Although the interaction network for both regioisomers is conserved, the binding mode is surprisingly not. While the *N*-phenylbenzamide moiety of both compounds extends deep into the hydrophobic pocket between activation loop and αC-helix, for NVPiso the pyrazolo[3,4-*d*]pyrimidine moiety rotates by 180° (see Figure 1). The methyl group at the NVPiso pyrazole ring prevents the formation of a hydrogen bond to the hinge region backbone. Instead the planar ring system flips and enables the formation of an alternative hydrogen bond between M695 (M696) and the nitrogen atom of the pyridine ring. Although the flip does not induce major changes of the interaction pattern, a detailed interaction surface analysis (PISA analysis in the SI) revealed a reduction of overlapping areas between the isomer and M695 by more than 50%.

To further characterize the preferred binding mode of the pyrazolo[3,4-*d*]pyrimidine moiety we were able to co-crystallize the NVP fragment **5**. Indeed, the binding mode of **5** is highly similar to the one of NVP, the interactions with T692 (EPHB4: T693) and M695 (M696) are conserved (Figure 1, g+h). Due to the absence of the *N*-phenylbenzamide moiety, the kinases adopt the DFG-in conformation.
By NMR, we could characterize relevant interactions of the fragments of NVP and NVPiso using waterLOGSY NMR experiments (Figure S7 in the SI). Briefly, fragment **5** and the *N*-phenylbenzamide fragment (typically present in DFG-out ligands) displace the ATP analogue AMP-PNP. In addition, both fragments bind simultaneously to the kinase, most likely because they can occupy two different binding sites. Whereas fragment **5** strongly displaces AMP-PNP from EPHA2, the "DGF-out-*N*-phenylbenzamide fragment" displaces AMP-PNP only partially. The more detailed account, including binding to EPHB4, is given in the SI (Figure S8 and Figure S9).

The affinities of NVP, NVPiso and of the DFG-in inhibitor dasatinib as control were then investigated by microscale thermophoresis (MST),[23] Kinobeads[24–26] and NanoBRET assays[27]. MST uses the fluorophore-labeled kinase domain to determine *in vitro* changes of the diffusion constant in an inhibitor concentration-dependent manner. The Kinobeads assay investigates the competition of inhibitors to binding of broad-band, ATP-competitive unspecific immobilized tool compounds within native cellular lysates. For the cellular NanoBRET assays, EPH receptors are fused to luciferase and a bioluminescence resonance energy transfer (BRET) signal is induced upon binding of an energy transfer probe. ATP-competitive kinase inhibitors prevent binding of the BRET probe in the live cell and lead to an inhibitor concentration-dependent signal decrease.

Using MST, we determined dissociation constants of NVP and dasatinib binding to EPHA2 in the low nanomolar range but a sixfold lower binding affinity to NVPiso (Table 1). This trend of lower EPHA2 affinity for NVPiso was confirmed by the Kinobeads and NanoBRET assays, indicating low affinity binding also in a cellular context. However, we also observe differences between the two cellular assays that could be explained by different experimental setups of the assays. The Kinobeads assay is performed with cell lysates from several cancer cell lines containing the endogenous full-length kinase receptor. The life cell NanoBRET assay targets the ectopically expressed full-length receptor fused to an intracellular luciferase reporter in intact HEK293T cells. Higher dissociation constants as in the Kinobeads assays are often observed in lysate-based binding assays[28] where the full-length target proteins are present in different activation states, PTM (posttranslational modifications) signatures and protein complexes. Nevertheless, both assays agree in that NVPiso has a different profile of targeting the two EPH receptors compared to NVP.

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We then expanded the NanoBRET assay to probe for differential binding affinities of NVP over NVPiso to other members of the EPH receptor family. In fact, NVP targeted the majority of all tested EPH receptors with excellent affinities between 0.3 nM (EPHA3) and 303 nM (EPHA1); only EPHA7 and the pseudokinase EPHB6 were not or only slightly bound by this inhibitor. In comparison, NVPiso affinities ranged from 50 nM (EPHA3) to 630 nM (EPHB2) and no or low inhibitory effects were determined for half of the tested EPH receptors (EPHA1, EPHA4, EPHA6, EPHA7, EPHB4, EPHB6). NanoBRET data further showed highest difference in binding affinity of NVP over NVPiso for EPHA4 (~1300) and EPHB4 (~550) (see Table S1 in the SI). Using the Kinobeads assay, we could not reach a similar coverage of the different members of the receptor class, but could gain additional information about the proteome-wide selectivity profiles of both isomers (see Table S1 and Figure S5 in the SI). While NVP primarily targets EPH family members, the main target of NVPiso is the cancer relevant receptor tyrosine kinase DDR1.

Table 1: Comparison of affinities for NVP and the isomer NVPiso toward EPHA2 and EPHB4 using different techniques (MST, NanoBRET and Kinobeads assay). All values are denoted in nM.

	NVP-BHG712 (NVP)			NVPiso			Selectivity factor Sf
	K_D MST	IC_{50} NanoBRET	K_D^{app} Kinobeads	K_D MST	IC_{50} NanoBRET	K_D^{app} Kinobeads	IC_{50} (NVPiso)/ IC_{50} (NVP)
EPHA2	13	3.3	240	73	163	2546	49.4
EPHB4	5.7	3.0	695	142	1660	1113	553

Several chemical vendors currently provide the incorrectly synthesized and DDR1-targeting regioisomer. Unfortunately, such a lack of quality control is not uncommon in the literature.[29,30] For example, an isomer of bosutinib was commercially available over a period of several years until the false identity was discovered in 2012.[29,31,32] NVP has been used in 15 different published studies since 2010. Four of the studies used the compound provided by *Novartis*, while eleven used the purchased compound likely having the wrong configuration (Table S2 in the SI).

In summary, this study provides analytical, structural and cellular data for the kinase inhibitor NVP-BHG712 and its incorrectly synthesized regioisomer NVPiso. By serendipity, we observed in our EPHA2 and EPHB4 crystal structures that small changes of the inhibitor's molecular structure can induce strong effects on its binding mode. We observed a 180° flip of the pyrazolo[3,4-*d*]pyrimidine moiety as the main structural difference between both regioisomers. Results of biochemical assays proved that alternate binding modes correlate with changes in target affinity and selectivity. This observation could potentially be utilized as a starting point for the development of more selective EPH kinase inhibitors. For example, designing bulky compounds occupying both areas could lead to more inhibitor specificity which is urgently required for the understanding of the role of EPH receptor signaling in pathological conditions. Such efforts are ongoing in our laboratory.

Experimental Section

All experimental details are described in the Supporting Information.

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Keywords: Medicinal Chemistry • Structural Biology • Eph receptors • Xray • NMR spectroscopy

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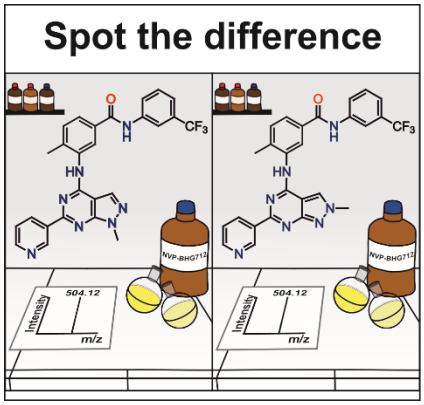

A small change can make a big difference: A modification in the substitution pattern of the published kinase inhibitor NVP-BHG712 from *Novartis* leads to different binding modes as well as binding affinities towards EPH receptor tyrosine kinases. Additionally also the selectivity profiles of the two compounds for binding to kinases are substantially changed.

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