Pyrrolo[3,2-b]quinoxaline Derivatives as Types I\textsubscript{1/2} and II Eph Tyrosine Kinase Inhibitors: Structure-Based Design, Synthesis, and \textit{in Vivo} Validation

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\textsuperscript{§}Supporting Information

ABSTRACT: The X-ray crystal structures of the catalytic domain of the EphA3 tyrosine kinase in complex with two type I inhibitors previously discovered \textit{in silico} (compounds A and B) were used to design type I\textsubscript{1/2} and II inhibitors. Chemical synthesis of about 25 derivatives culminated in the discovery of compounds 11d (type I\textsubscript{1/2}), 7b, and 7g (both of type II), which have low-nanomolar affinity for Eph kinases \textit{in vitro} and a good selectivity profile on a panel of 453 human kinases (395 nonmutant). Surface plasmon resonance measurements show a very slow unbinding rate (1/115 min) for inhibitor 7m. Slow dissociation is consistent with a type II binding mode in which the hydrophobic moiety (trifluoromethyl-benzene) of the inhibitor is deeply buried in a cavity originating from the displacement of the Phe side chain of the so-called DFG motif as observed in the crystal structure of compound 7m. The inhibitor 11d displayed good \textit{in vivo} efficacy in a human breast cancer xenograft.

I. INTRODUCTION

Several protein kinases are relevant targets for the treatment of diseases ranging from cancer, inflammation, and cardiovascular conditions to immune related disorders. Over the past decade, more than 13 small-molecule kinase inhibitors have been approved by the FDA as therapeutics for various human pathologies. \textsuperscript{3}−\textsuperscript{6} In this context, receptor tyrosine kinases play a prominent role, as they are involved in a number of biologically relevant processes for cancer development including oncogenic regulation, cell signal transduction, proliferation, and survival among many others. \textsuperscript{7,8} Although irreversible inhibitors that form covalent bonds with cysteine or other nucleophilic residues in the ATP-binding pocket have been recently explored,\textsuperscript{9,10} ATP competitive, noncovalent inhibitors are much more abundant and, depending on the binding mode with their protein target, are classified as type I−IV.\textsuperscript{11} Most kinase inhibitor drugs are of type I, i.e., they are direct competitors of ATP within the catalytic site of the phosphorylated active conformation of the protein.\textsuperscript{12} However, because of the strong similarities between the ATP binding pocket of all human kinases, alternative approaches providing selective binders have been sought.\textsuperscript{13}−\textsuperscript{18} Small molecules forming additional interactions with hydrophobic regions adjacent to the ATP binding site are termed type I\textsubscript{1/2} inhibitors. Alternatively, type II inhibitors target the kinase catalytic site but bind to the inactive conformation of the protein, thus exploring a pocket generated upon displacement of the phenylalanine side chains of the DFG motif.\textsuperscript{4} Type III inhibitors, also known as allosteric inhibitors, target areas of the kinase not related to the catalytic domain, whereas type IV do so without competing with ATP. Higher degrees of selectivity are to be expected with the latter two inhibitor types.\textsuperscript{19}

Recently, our groups have focused on the \textit{in silico} design, synthesis, and computational-aided optimization of potent and selective receptor tyrosine kinase inhibitors. Successful campaigns have yielded single-digit nanomolar EphB4 inhibitors whose potential antiproliferative activities have been characterized by cellular assays.\textsuperscript{20}−\textsuperscript{22} Furthermore, the predicted binding mode could also be confirmed by X-ray diffraction analysis of their complexes with EphA3. Given the
critical role of Eph receptors and (Eph)—ephrin signaling in tumor growth and progression, a subset of these compounds are currently being pursued toward preclinical development.

Here we describe a multidisciplinary campaign toward the design of novel and potent, type I/II and II tyrosine kinase inhibitors based on the crystal structure of two type I inhibitors. The parent pyrrolo[3,2-b]quinazoline scaffold was decorated with characteristic functional groups present in previously successful type II binders, thus speeding up the hit to lead optimization campaign. The binding kinetics of the low-nanomolar derivatives 11d (type II/2) and 7m (type II) were characterized by surface plasmon resonance (SPR) measurements. Extensive profiling by biochemical (competition binding) and cellular assays, together with pharmacokinetic measurements in mice resulted in the prioritization of inhibitor 11d for final validation in vivo by a human breast cancer xenograft.

II. CRYSTAL STRUCTURES OF TYPE I INHIBITORS A AND B WITH EPHA3

II.1. Docking Validation by X-ray Diffraction Analysis of Binding Complex. Recently, we reported the discovery of two type I EphB4 inhibitors A and B by automated docking. The in silico predicted binding mode of these molecules is confirmed here by X-ray diffraction analysis of the catalytic domain of EphA3 in complex with both A and B (Figure 1).

Figure 1. Crystal structures of the catalytic domain of the tyrosine kinase EphA3 in complex with the high-nanomolar inhibitors A (left, pdb code 4P4C) and B (right, pdb code 4P5Q). The ATP binding site of the EphA3 kinase is shown in magenta ribbons, while the side chains mentioned in the text and the inhibitors are shown by sticks (with carbon atoms in magenta and green, respectively).

The pyrrolo[3,2-b]quinazoline scaffold occupies the ATP binding site with the phenyl substituent nestled into the so-called hydrophobic pocket. The amino substituent at position 2 of the pyrrole ring is involved in a bifurcated hydrogen bond with the side chain hydroxyl of the Thr693 gatekeeper and the backbone carbonyl of Glu694. Furthermore, in the structure with inhibitor A, the amide substituent at position 3 of the pyrrole ring is optimally oriented for two hydrogen bonds with the backbone polar groups of Met696 so that A forms a total of three hydrogen bonds with the backbone of the hinge region. Only two hydrogen bonds with the same region are observed for inhibitor B due to the ethoxy-propyl substitution at the nitrogen of the amide whose trans configuration prevents it from acting as donor to the carbonyl group of Met696. The lack of this hydrogen bond is consistent with the about 10-fold weaker affinity of inhibitor B with respect to A (IC50 of 300 nM for EphB4), which might also originate, at least in part, from the different substituents of the phenyl ring in the hydrophobic pocket, i.e., −OCH3 and −Cl in A and B, respectively.

II.2. Design of Type I/II and Type II Derivatives Based on the X-ray Crystal Structure of the Type I Inhibitors A and B. On the basis of our previous experience and earlier reports toward the synthesis of potent type I kinase inhibitors, several modifications within the phenyl ring were designed in order to fine-tune the interactions of the quinoxaline inhibitors with the threonine gatekeeper residue (Thr693) of EphB4. Because of the rather limited space around the phenyl group revealed by the binding modes of A and B, the introduction of small substituents was envisioned, including the incorporation of a methyl and a hydroxyl group at positions 2 and 5, a moiety present in some type II kinase inhibitors, in analogy to the side chain hydroxyl of the Thr693 gatekeeper and the backbone carbonyl of Glu694. Furthermore, in the structure with inhibitor A, the amide substituent at position 3 of the pyrrole ring is optimally oriented for two hydrogen bonds with the backbone polar groups of Met696 so that A forms a total of three hydrogen bonds with the backbone of the hinge region. Only two hydrogen bonds with the same region are observed for inhibitor B due to the ethoxy-propyl substitution at the nitrogen of the amide whose trans configuration prevents it from acting as donor to the carbonyl group of Met696. The lack of this hydrogen bond is consistent with the about 10-fold weaker affinity of inhibitor B with respect to A (IC50 of 300 nM for EphB4), which might also originate, at least in part, from the different substituents of the phenyl ring in the hydrophobic pocket, i.e., −OCH3 and −Cl in A and B, respectively.

III. CHARACTERIZATION OF NEW TYPE I/II AND II INHIBITORS

III.1. Synthesis. The synthesis of 1H-pyrrolo[2,3-b]-quinazoline derivatives 6 and 7 is shown in Scheme 1. Compound 1 was prepared according to previously reported procedures by condensation of commercially available 2,3-dichloroquinazoline with malononitrile in the presence of sodium hydride. The substitution of the chlorine at position 3 with commercially available anilines 2a−i followed by cyclization afforded intermediates 4a−i. The reaction with synthetically prepared anilines 3a−n delivered tricyclic intermediates 5a−n (Scheme 1).
The preparation of the noncommercially available anilines 3a–m used in the above-mentioned condensation reaction has been summarized in Scheme 2. Anilines 3a–e, bearing a N-amide group in meta-relative position, were obtained by

Table 1. EphA3/EphB4 Inhibition Data for the Synthesized Quinoxaline Derivatives

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<th>Compound</th>
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<th>( R_1 )</th>
<th>( R_2 )</th>
<th>( R_3 )</th>
<th>( R_4 )</th>
<th>Thermal shift (degrees)</th>
<th>FRET enzymatic assay (% of inhibition at 1μM)</th>
<th>Cellular IC₅₀ (nM)</th>
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*Average values of triplicate measurements. The standard deviation is smaller than 0.5 degrees. †FRET-based enzymatic assay was carried out using the Z-LYTE Kinase Assay Kit–Tyr 1 Peptide (Invitrogen) following the vendor instructions. ‡Cell IC₅₀ values were measured in a cellular phosphorylation assay using MEF cells overexpressing EphB4 at Proqinase.
condensation of 3-nitro-substituted anilines 8 with the corresponding readily available benzoyl chlorides (R3COCl) or benzoic acids (R3COOH)38 using different protocols (Conditions a, Methods I–IV). Reduction of the nitro group with either SnCl2 or Pd/C and H2 (Conditions b, Supporting Information S12) furnished the desired intermediates. Anilines 3f–j were prepared by condensation of the 3-nitro-substituted benzoic acid or benzoyl chlorides 9 with the corresponding anilines39 under reaction conditions c. Reduction of the nitro group with SnCl2 or Pd/C and H2 delivered the corresponding anilines in 30–77% overall yields. Finally, anilines 3k–m, bearing urea moieties at meta- and para-relative positions, were prepared by condensation of aniline 8 with the corresponding isocyanate (for 3k–l) or by condensation of the isocyanate 10 with the respective commercially available aniline under reaction conditions d. Reduction of the nitro group under the conditions described above furnished the corresponding aniline-containing ureas 3k, 3l, and 3m in 42, 85, and 41% yields, respectively. Aniline 3n was prepared according to a previously reported procedure.40

Hydrolysis of the cyano group under strong acidic conditions furnished the desired type I and type II inhibitors 7a–n, respectively. The presence of a cyano group in compounds 4 and 5 was confirmed by the presence of a characteristic IR band at around 2200 cm−1 and a 13C NMR signal at 115–116 ppm, whereas the amino group appeared as a broad signal at 8 to 8.5 ppm in 1H NMR.67

Two more inhibitors were prepared, first by demethylation of 6d in the presence of BBr3 under reflux to give the corresponding phenol derivative 11d, and a second one by condensation of the aniline 7n with m-CF3-phenyl isothiocyanate, to give the corresponding thiourea 12n (Scheme 3).

**III.2. Biophysical Characterization.** The thermodynamics and kinetics of binding of the designed quinoxaline inhibitors were investigated by an array of biophysical techniques including differential scanning fluorimetry, fluorescence resonance energy transfer (FRET) based enzymatic assays, and surface plasmon resonance (SPR). Differential scanning fluorimetry is a high throughput technique in which the increase in thermal stability of a folded protein upon ligand binding is detected by a fluorescent dye while measuring its melting temperature during denaturation.

In order to allow the binding of type II inhibitors to the inactive form of the kinase, the protein (EphA3) was incubated in the presence of the compounds for 1 h. The results (shown in Table 1) highlight the differences in binding between type I, I1/2, and II compounds, with type I being the weakest binders (1.5–4.1 °C) and type II the most potent (with up to 16 °C thermal shifts).

For type I inhibitors, the largest thermal shifts (ca. 4 °C) were obtained for ortho-methyl (6a) and ortho-chlorine (6f)
substituted quinoxalines, which is in agreement with previously reported kinase inhibitors20,42–47 and could be a consequence of restricting the accessible conformations of the phenyl ring as we have previously reported.20 However, the ortho-fluoro substituted inhibitor 6g or bis-ortho substituted 6b and 6c caused a lower stabilization of the protein probably due to the small size of fluorine or the introduction of an extra steric bulk, respectively.

The transition from type I to type I1/2 by the presence of a hydrogen bond donor at position R1, and therefore the formation of hydrogen bonds with Glu664 and Asp758, resulted in a remarkable increase in thermal shift for compound 6h (7.2 °C), which became more pronounced upon introduction of a methyl group in the ortho-relative position (following the trend observed in type I inhibitors) to yield 11d with a thermal shift of 11.2 °C.

Type II compounds bearing an amide linker followed by a m-CF3-phenyl group caused a similar stabilization effect in the protein as the type I1/2 inhibitor 11d. As expected from previous results with type I and I1/2 compounds, the addition of a methyl (7c and 7h) or fluorine (7d and 7i) substituent in R1 lead to higher thermal shifts. Interestingly, 3-amides (7g–i) triggered a higher stabilization of the kinase than 1-amides (7b–d), which could indicate the formation of a more favorable hydrogen bond with Glu664. The presence of imidazoles within the allosteric binding site led to compounds 7e and 7j, which showed the most promising thermal shifts (16 °C). Urea (7m) or thiourea (12n) linkers located in para-relative position retained or even enhanced (in the case of urea 7m) the binding affinity, whereas compound 7l, bearing the urea in meta-relative position barely presented any thermal shift, suggesting a disruption or a nonfavorable hydrogen bond interaction with Glu664. The replacement of the m-CF3-phenyl group by a cyclopropyl ring proved to be detrimental in all cases, and no thermal shift was observed for products 7a, 7f, and 7k.

The inhibitory activities of type I and type I1/2 inhibitors were further evaluated on an enzymatic assay based on fluorescence resonance transfer (FRET) at a single concentration (1 μM, Table 1, column 8). The results were in line with the thermal shifts described above. Compounds 6a and 6f showed inhibitory activities higher than 66% at 1 μM (Table 1). However, substitution at R1 by a smaller fluorine atom yielded compound 6g with lower binding affinity (30%). Along the same lines, bis-ortho substitutions with either methyl or chlorine (6b,c) decreased the binding affinity (36 and 45% inhibitory activities, respectively) probably due to unfavorable steric effects. The presence of a hydrogen bond donor at R3 (type I1/2 inhibitors) either as a phenol (6h and 11d) or methylene alcohol (6e) greatly improved the inhibitory activity of the molecules thanks to the formation of hydrogen bonds with Glu664 and Asp758 (68–105%). To our surprise, a triazole group at the same position (6i) dramatically decreased the binding affinity (7%).

The kinetics of binding of the optimized type I1/2 (11d) and type II urea inhibitor (7m) were investigated using SPR. Upon titrating 11d and 7m over immobilized dephosphorylated EphA3, dissociation constants (Kd) in the low nanomolar range were obtained (8.6 and 39.3 nM, respectively), confirming the high affinity of the compounds (Figure 2). One of the advantageous characteristics of type II inhibitors over type I or I1/2 is their slow dissociation rate from the target protein,48–50 as demonstrated by the remarkably slow koff measured for 7m (1.45 × 10^{-4} s^{-1}) in comparison to the type I1/2 11d (1.52 × 10^{-3} s^{-1}). The slow koff of 7m corresponds to a residence time of 115 min, a value that compares positively with that of marketed drugs such as imatinib (28 min for dephosphorylated ABL), nilotinib (202 and 205 min for dephosphorylated and phosphorylated ABL, respectively), and dasatinib (15 and 4 min).51 The long residence time of type II inhibitors is considered to be beneficial for drug efficacy and selectivity in vivo due to the high concentration of the drug near the
target, as described for the EGFR-specific inhibitor lapatinib.

IV. VALIDATION OF TYPE II BINDING BY X-RAY CRYSTAL STRUCTURE DETERMINATION

The crystal structure of the catalytic domain of the EphA3 kinase in complex with inhibitor \(7m\) (solved at 2.0 Å resolution, Supporting Information S34) confirms a type II binding mode, i.e., with the DFG-out conformation (Figure 3). The pyrrolo[3,2-b]quinoxaline scaffold occupies the ATP binding site and is involved in the same hydrogen bonds with the hinge region as the type I inhibitor \(A\) (Figure 1). In addition, the urea linker of inhibitor \(7m\) acts as hydrogen bond acceptor from the Ser757 side chain and the amide backbone of Asp758, and hydrogen bond donor to the side chain of Glu664. The \(m\)-CF\(_3\)-phenyl moiety is nestled in the hydrophobic pocket, which originates from the displacement of the Phe side chain of the DFG motif. Thus, the type II binding mode of compound \(7m\) validates our design based on the crystal structures of the complexes with the type I inhibitors (vide supra, section II.2).

V. SELECTIVITY AND CELLULAR ACTIVITY

V.1. Selectivity Profiles from Biochemical Assays. The selectivity profile of inhibitors \(11d\), \(7b\), and \(7g\) was determined by an in vitro competition binding assay using recombinant kinases (KINOMEScan at DiscoveRx). It is important to note that this assay reports on binding affinity and does not require ATP. The selectivity panel consisted of 453 human kinases, 58 of which were disease related mutant kinases (mainly of ABL1, EGFR, and PIK3CA). Single dose measurements were carried out at 1 μM concentration of the inhibitor.

Compounds \(11d\), \(7b\), and \(7g\) present a very similar selectivity profile (Figure 4); in particular, strong binding is only observed for tyrosine kinases with threonine as a gatekeeper residue, e.g., ABL1/2, BRAF, DDR1, EphA/B (all but EphA7, which has an Ile gatekeeper), KIT, LCK, SRC, and YES. The latter data suggest that most (or even all) tyrosine kinases with a Thr gatekeeper can assume the DFG-out conformation. Quantita-

Figure 3. Crystal structures of the catalytic domain of the tyrosine kinase EphA3 in complex with the low-nanomolar inhibitor \(7m\) (left, pdb code 4P5Z) and superposition of the three inhibitors \(A\), \(B\), and \(7m\) based on structural alignment of the C\(_{\alpha}\) atoms of the EphA3 kinase domain (right). The ATP binding site of the EphA3 kinase is shown in magenta ribbons, while the side chains mentioned in the text and the inhibitors are shown by sticks.

Figure 4. Selectivity profiles of compound \(11d\) (left), \(7b\) (center), and \(7g\) (right) tested on a panel of 395 nonmutant (top) and 58 mutant (bottom) kinases at DiscoveRx. Measurements were performed at a concentration of 1 μM of the inhibitor. The affinity is defined with respect to a DMSO control. The dendrogram was obtained from KinomeScan using the KinomeTree software.
tively, each of the three inhibitors 11d, 7b, and 7g binds with an affinity 10-fold (100-fold) higher than the DMSO negative control to only about 10% (5%) of the 395 wild-type kinases tested. Interestingly, the selectivity profiles of the type I/2 (11d) and II (7b and 7g) quinazoline-based inhibitors is very similar to the one of our previously reported type I and I/2 xanthine-based inhibitors (compounds 40 and 3, respectively, in ref 22), which is due, at least in part, to the use of an Eph tyrosine kinase (EphB4) as primary target for the in silico screening and optimization.

V.2. Cellular Assays. The most potent inhibitors obtained in the optimization campaign were further tested in cell-based assays. Cellular phosphorylation assays on MEF cells transfected with myc-tagged human EphB4 revealed a comparable tendency to the one observed in the enzymatic assay (Table 1, column 9). The type I inhibitors (6a−d) displayed cellular GI50 values in the 230−4400 nM range, with the ortho-methyl substituted derivative 6a as the most potent member of this series. The type I/2 inhibitor 11d and type II compounds bearing amide linkers and a meta-CF3-phenyl group (7b−d and 7g−i) displayed levels of inhibitory activity in the low nanomolar range (6−24 nM), thus being the most promising molecules of the optimization campaign. In agreement with thermal shift experiments, the presence of a urea (7m) and specially the thiourea linker (12n) decreased the potency of the compounds (89 and 560 nM, respectively). Interestingly, the imidazole substituted compounds (7e and 7j) proved to be the weakest type II inhibitors (170 and 270 nM, respectively) in contrast to the high thermal shifts observed (16 °C) in the differential scanning fluorimetry measurements, pointing toward potential cell permeability or efflux issues.

EphB4 overexpression has been linked to several types of cancer, including breast, 85 colon, 86 and ovarian. 87 Compounds 11d and 7m were screened against the NCI-60 cancer cell line panel (Supporting Information S3 and S4) displaying antiproliferative activities against leukemia (K-S62), lung (HOP-92), colon (HT-29), renal (A498), and breast cancer cells (MDA-MB-231 and HS 578T) in the low nanomolar range. Driven by these results, the most promising inhibitors of our optimization campaign were tested in-house against the above-mentioned NCI cancer cell lines (Table 2). The leukemia K-S62 cell line was particularly sensitive toward the optimized type II quinazoline inhibitors, especially in the case of 3-amide compounds 7h and 7i, which showed remarkably low GI50 values (36 and 81 nM, respectively). Interestingly, similar levels of potency were found for imidazole substituted compounds 7e and 7j, which seemed to be among the weakest type II binders in the cellular phosphorylation assays, possibly indicating other targets than Eph for these molecules. In addition, the potential of 11d (the most potent compound on cellular phosphorylation assays with an IC50 of 6 nM) to inhibit the growth of patient-derived tumor cell lines was studied using a propidium iodide-based proliferation assay and dasatinib as a reference (Oncotest, Table 3). Cell lines included colon, lung, kidney, pancreatic, prostate, and stomach cancer cells. Whereas dasatinib presented double-digit nanomolar activities against RXF 393NL, LFA9 983L, and PRXF DU14S, 11d exhibited low micromolar GI50 values, with RXF 393NL being the most sensitive cell line.

The implication of EphB4-eprrinB2 signaling in sprouting angiogenesis and blood vessel maturation 88 and the inhibition of vascular endothelial growth factor (VEGFR)-driven angiogenesis by the selective EphB4 inhibitor NVP-BHG712, 89 led us to examine the efficacy of 11d on human endothelial cell sprouting in a sphere based cellular angiogenesis assay (ProQinase, Supporting Information S36). 90 Compound 11d was able to successfully inhibit VEGF-A induced HUVEC (primary human umbilical vein endothelial cells) sprouting in a dose dependent manner with an IC50 value of 1.5 μM.

VI. IN VIVO DATA

Three of the most promising compounds from these series (7b, 7g, and 11d) were selected for evaluation of pharmacokinetic properties in 20−30 g male CD-1 (ICR) mice on intravenous (IV) and oral (PO) administration. Low to moderate oral bioavailability of tested compounds in mice was observed, with compounds 11d and 7g giving the highest values (Table 4). Promising cellular efficacy and pharmacokinetic properties incited the subsequent evaluation of compound 11d in a xenograft mouse model with a tumor derived from the MDA-MB-231 cell line. High compound clearance (Cl) and moderate half-life (t1/2) values determined in the pharmacokinetic study motivated a twice-daily dosing regime totaling 100 mg/kg/day of compound 11d over 21 days. Median tumor volume

| GI50 values were determined using resazurin reduction after 2−3 days of incubation with the corresponding compound. GI50 values are given in micromolar concentrations (μM) as the mean of at least three independent experiments. Variability around the mean value was <50% unless otherwise indicated by an SE value in parentheses. |

<p>| Table 3. Antiproliferative Activity against Patient Derived Tumor Cell Lines* |</p>
<table>
<thead>
<tr>
<th>compd</th>
<th>11d</th>
<th>dasatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXF 393NL</td>
<td>0.725</td>
<td>0.0217</td>
</tr>
<tr>
<td>CXF 1103L</td>
<td>3.83</td>
<td>4.36</td>
</tr>
<tr>
<td>LFA 983L</td>
<td>2.22</td>
<td>0.0565</td>
</tr>
<tr>
<td>GXF 251L</td>
<td>8.01</td>
<td>2.25</td>
</tr>
<tr>
<td>PAXF 1657L</td>
<td>2.92</td>
<td>0.121</td>
</tr>
<tr>
<td>PRXF DU14S</td>
<td>2.92</td>
<td>0.0623</td>
</tr>
</tbody>
</table>

*GI50 values were determined at Oncotest using a modified propidium iodide assay. Measurements were performed after 4 days of incubation with 11d and dasatinib. GI50 values are given in micromolar concentrations (μM).
progression over time, starting from 108 mm$^3$, of both treatment and control cohorts is given in Figure 5.

In this study, median treatment-group tumor volume remained essentially stable throughout the treatment period, achieving a median tumor volume of 126 mm$^3$ at day 19 as opposed to the control group whose median tumor reached 650 mm$^3$ in the same period. Tumor growth inhibition (%TGI) was statistically significant (Mann−Whitney $U = 0$, $P \leq 0.001$, two-tailed) and quantified at 81% relative to the control group. Mean body weight of the treatment cohort decreased up to 16.3% of the initial mean body weight of this cohort during the treatment period. Treatment with compound 11d provides a significant limitation in tumor progression over the control, suggesting that further studies of such xenograft model at lower doses of compound 11d might provide tumor volume control with lessened weight loss. The further evaluation of compounds 11d on mouse models of K-562 leukemia is underway.

VII. CONCLUSIONS

The X-ray crystal structures of the EphA3 kinase in complex with two high-nanomolar inhibitors based on the 2-amino-1-phenyl-pyrrolo[3,2-b]quinoxaline-3-carboxamide scaffold confirmed the type I binding mode obtained previously by automatic docking (Figure 1). This structural information was used to design type I$_{1/2}$ and type II derivatives by taking advantage of the existing knowledge on privileged chemical motifs, i.e., hydroxyl group in meta position of the phenyl ring (for type I$_{1/2}$) and hydrophobic moieties connected to the phenyl ring by amide or urea linkers (on type II). Chemical synthesis of ca. 25 derivatives (Table 1) culminated in several low nanomolar inhibitors with a good selectivity profile (Figure 4). The X-ray crystal structure of the EphA3 kinase in the complex with the inhibitor 7m (Figure 3) provided the final validation of the structure-based design; in particular, the DFG-out conformation confirmed the type II binding. Moreover, the slow kinetics of unbinding of compound 7m (measured by SPR, Figure 2) is congruent with the type II binding mode. Three interesting observations emerge from this study. First, it is possible to "elongate" a type I$_{1/2}$ into a type II inhibitor by introducing an amide or urea linked to a bulky hydrophobic group. These type II linkers are involved in the same hydrogen bonds as the type I$_{1/2}$ bearing a hydroxyl group in the same position, while the hydrophobic moiety occupies the pocket resulting from the displacement of the Phe side chain of the DFG motif. The similar selectivity profiles of type I$_{1/2}$ and type II inhibitors indicate that mainly the moiety in contact with the gatekeeper’s side chain and hinge region determines specificity. Finally, in vivo assays (mice xenographed with human breast cancer) confirmed the cytostatic activity of one of our inhibitors (11d), which makes this type I$_{1/2}$ compound a candidate lead for further preclinical development.

Table 4. Pharmacokinetic Properties in Mice

<table>
<thead>
<tr>
<th>compd</th>
<th>11d</th>
<th>7b</th>
<th>7g</th>
</tr>
</thead>
<tbody>
<tr>
<td>dose (mg/kg)</td>
<td>iv</td>
<td>po</td>
<td>iv</td>
</tr>
<tr>
<td>Cl (mL/min/kg)</td>
<td>42</td>
<td>32</td>
<td>31</td>
</tr>
<tr>
<td>$V_a$ (L/kg)</td>
<td>1.6</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>1.7</td>
<td>1.7</td>
<td>1.2</td>
</tr>
<tr>
<td>AUC$_{int}$ (h·ng/mL)</td>
<td>392</td>
<td>493</td>
<td>506</td>
</tr>
<tr>
<td>F (%)</td>
<td>25</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

Figure 5. In vivo antitumor activity of compound 11d in MDA-MB-231 nude mice xenografts. The mice received by gavage twice-daily 50 mg/kg of compound 11d (red) or vehicle control (blue). Each data point is the median or mean of a cohort of 9 animals. Error bars show standard deviations of the mean.
**REFERENCES**


