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Identification of novel FLT3 kinase inhibitors

Daphnie Pauwels [a,b], Hugo Klaassen [c], Idoya Lahortiga [a,b], Amuri Kilonda [c], Kris Jacobs [a,b], Bram Sweron [a,b], Romuald Corbau [c], Patrick Chaltin [c,d], Arnaud Marchand [c], Jan Cools [a,b,*]

[a] Center for the Biology of Disease, VIB, Leuven 3000, Belgium
[b] Center for Human Genetics, KU Leuven, Leuven 3000, Belgium
[c] Center for Innovation and Stimulation of Drug Discovery (CISTIM), Leuven 3000, Belgium
[d] Center for Drug Design and Development (CD3), KU Leuven R&D, Leuven 3000, Belgium

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ABSTRACT

FLT3 and PDGFR tyrosine kinases are important targets for therapy of different types of leukemia. Several FLT3/PDGFR inhibitors are currently under clinical investigation for combination with standard therapy for treatment of acute myeloid leukemia (AML), however these agents only induce partial remission and development of resistance has been reported. In this work we describe the identification of potent and novel dual FLT3/PDGFR inhibitors that resulted from our efforts to screen a library of 25,607 small molecules against the FLT3 dependent cell line MOLM-13 and the PDGFR dependent cell line EOL-1. This effort led to the identification of five compounds that were confirmed to be active on additional FLT3 dependent cell lines (cellular EC_{50} values between 35 and 700 nM), while having no significant effect on 24 other tyrosine kinases.

1. Introduction

Acute myeloid leukemia (AML) is a heterogenous disease resulting from the clonal expansion of immature hematopoietic cells by the cooperation of various mutations and chromosomal aberrations. AML represents the most frequent acute leukemia in adults with a peak of incidence around 65 year [1]. The overall survival at 4 years is around 37% for pediatric AML and 16% for adult AML [2]. Cytogenetic analysis of the leukemic cells is used to stratify the patients in different prognostic subgroups, with CBFB-MYH11 and RUNX-RUNX1T1 showing a good survival, while patients expressing MLL fusions having a bad prognosis [3–5]. In addition to the chromosomal aberrations, point mutations in *FLT3*, *ASXL1*, *DNMT3A*, *TET2*, *IDH1*, *IDH2* and *NPM1* are also recurrently observed in AML and further influence the prognosis [6]. The presence of *FLT3* mutations is associated with a poor prognosis and an increased risk of relapse [7,8], while mutations in *NPM1* gene correlate with a high rate of complete remission and a favorable overall survival [6]. However, this positive prognostic effect is lost when this mutation is found in the presence of internal tandem duplication (ITD) of *FLT3* [9,10].

FLT3 is a transmembrane receptor tyrosine kinase, which is selectively expressed on hematopoietic cells where it mediates stem cell differentiation and proliferation [11–13]. Activation of the FLT3 receptor in AML precursor cells is believed to stimulate proliferation and differentiation, and to inhibit apoptosis of leukemia cells. Internal tandem duplications in the juxtamembrane domain of *FLT3* are the most common mechanism of activation of FLT3 that is found in up to 30% of all AML cases [14,15]. Additional activating point mutations in *FLT3* occur in the activation loop, most frequently at position 835 and are identified in 5–10% of AML cases [16]. The *FLT3-ITD* and point mutations activate the FLT3 kinase and its downstream signaling pathways, including STAT5 and PI3K/AKT pathway, known to give leukemic cells a proliferation and survival advantage [17,18].

Several kinase inhibitors with activity against FLT3 (PKC412, Lestaurtinib/CEP701, Sunitinib/SU11248 and Tandutinib/MLN518) are currently under clinical investigation for combination with standard therapy of AML. However, the currently available FLT3 inhibitors are not selective, induce only partial and transient responses. In a phase IIB clinical trial with PKC412, the treatment was not sufficient to reach complete remission, and even partial remission was only obtained in 3% of the cases [19]. In a phase II

* Corresponding author. Center for the Biology of Disease, VIB, Herestraat 49, Box 602, Leuven 3000, Belgium. Tel.: +32 16 3 30082; fax: +32 16 3 30827.
E-mail address: Jan.cools@cme.vib-kuleuven.be (J. Cools).

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clinical trial with Lestaurtinib a transient reduction in peripheral blood and bone marrow blasts was observed in 3 out of 5 patients (60%) with *FLT3* mutations and in 5 out of 22 patients (23%) with wild-type *FLT3* [20].

Quizartinib (AC220), a selective FLT3 inhibitor currently in a phase II clinical trial, shows activity at low nanomolar concentrations *in vitro* and in animal models and could represent a more attractive compound for the treatment of FLT3-ITD positive patients with relapsed/refractory AML [21]. Indeed, in an initial phase I study, responses were seen in both FLT3-ITD positive and FLT3-ITD negative AML patients, with an overall response rate of 56% and 20%, respectively [22]. However, even potent selective FLT3 inhibitors are expected to face the development of resistance due to mutations in the kinase domain [23–25]. Such mutations have been identified *in vitro*, and have been observed in AML patients treated with PKC412 or AC220 [25,26]. Resistance to PKC412 can be caused by *FLT3* point mutations at amino acids N676, F691 and G697, some of which interfere with drug binding or by modifying the stability or activity of the kinase [24,27].

PDGFRα and PDGFRβ belong to the same family as the FLT3 kinase, and aberrant activation of PDGFRα or β kinase is observed in various myeloid malignancies that are now referred to as myeloid neoplasms with eosinophilia and with chromosomal aberrations involving *PDGFRA*, *PDGFRB* or *FGFR1* [28]. The *FIP1L1-PDGFRA* is the most common *PDGFRA* fusion, whereas *ETV6-PDGFRB* is the most common *PDGFRB* fusion, but a large number of other fusion genes involving *PDGFRA* or *PDGFRB* have been described as well [29,30]. Furthermore, it is well established that the inhibitor imatinib targets PDGFRα in idiopathic hypereosinophilic syndrome and PDGFRβ in several chronic myeloproliferative diseases [29,31]. Similarly as for the FLT3 inhibitors, resistance against imatinib occurs and could be overcome by PKC412 (in the case of PDGFRα) and sorafenib (in the case of PDGFRβ) [27,32].

Here, we have used a cell-based high throughput screen to identify novel FLT3/PDGFR inhibitors and we have tested their inhibitory activity on oncogenic FLT3 mutants and on the FIP1L1-PDGFRα fusion.

2. Results

2.1. Compound screening

In order to identify novel dual FLT3/PDGFR kinase inhibitors, we screened a library of 25,607 compounds for activity against the FLT3 dependent AML cell line MOLM-13 and the PDGFRα dependent AML cell line EOL-1 [33,34]. These cells were treated for 72 h with an average compound concentration of 10 μM (fixed weight). As a positive control we used 10 μM of Ara-C, a known nucleoside analog marketed for the treatment of various leukemia [35]. At the end of the 72 h, we calculated the cell number differences between the drug-treated and DMSO-treated cells. As a negative control we tested the activity of the compounds against HEL cells, an AML cell line known to be independent of FLT3/PDGFR signaling for its proliferation. The compounds were classified as hits when they inhibited the growth of EOL-1 and MOLM-13 cells by more than 50% and inhibited the growth of HEL cells by less than 20%. Using these criteria we selected 56 compounds for further analysis.

Subsequently, the selected compounds were tested against two additional FLT3 dependent cell lines: a human AML cell line MV4-11 and the mouse Ba/F3 cells transformed by FLT3-ITD. The FLT3 independent cell lines KG-1 and parental Ba/F3 cells were used as extra controls. Ba/F3 cells transformed by FLT3-ITD are an interesting cell line for this type of experiments, as they are only dependent on FLT3-ITD for their proliferation and survival, and can be compared to Ba/F3 cells grown in the presence of IL-3, which are independent of FLT3 activity [36]. The EC_{50} values of the 56 selected compounds are listed in Supplementary Table 1. By using a cut-off value of an EC_{50} <1 μM on the FLT3 dependent Ba/F3 cells and an EC_{50} of >10 μM on the wild type Ba/F3 cells, we selected the five most potent compounds for further analysis. Concerning the chemical structures, there was only a structural relationship between compounds **1**, **2** and **3** (all aminopyrazole structures), while the chemical structure of all other identified hits were not related.

The cell-based EC_{50} values of these five identified hits were determined for all the cell lines used during the screening and are listed in Table 1. Compounds **1**, **2** and **3** were already known as kinase inhibitors [37], whereas compound **5** belongs to a family of compounds with described effects on stem cell differentiation [38]. The structures of these compounds are shown in Fig. 1. Compounds **1**, **2** and **3** belong to the same family and contain an aminopyrazole structure. More specifically, compound **1** is a 4-methoxy-*N*-(3-phenyl-1*H*-pyrazol-5-yl)benzamide, compound **2** is a 4-fluoro-*N*-(3-(*p*-tolyl)-1*H*-pyrazol-5-yl)benzamide and compound **3** is a *N*-(3-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-3-methylbenzamide. Compounds **4** and **5** belong to a different class of molecules with compound **4** being a 4-(3-(3-chloro-4-fluorophenyl)-1*H*-pyrazol-4-yl)pyridine and compound **5** a 4-methyl-*N*-(1-(*p*-tolyl)-1*H*-benzo[*d*]imidazol-5-yl)phthalazin-1-amine.

2.2. Chemical synthesis of compounds *1*, *2* and *3*

For the confirmation assays, we reordered compounds **4** and **5** and synthesized compounds **1**, **2** and **3**, because they were no longer commercially available. The synthesis was performed as followed: To a suspension of 5-phenyl-1*H*-pyrazol-3-amine derivative **4a**–**c** (1.0 mmol) in dichloromethane (5 mL) at 0 °C were added benzoyl chloride derivative **5a**–**c** (1.05 mmol) and triethylamine (1.20 mmol). The reaction mixture was stirred at 0 °C for 5 min and at room temperature for 1 h. It was then diluted with dichloromethane, washed with water and brine, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel using a gradient of methanol in dichloromethane to give compounds **1**–**3** as a white solid (Fig. 2). ^{1}H and ^{13}H spectra are available in Supplementary material. Confirmatory ^{1}H NMR, TOF-MS and LC-MS analyses were performed for compounds **1** to **3**. Analytical data for compounds **4** and **5** were obtained from the commercial suppliers Asinex (Moscow, Russia) and ChemDiv (San Diego, CA, USA) respectively. Analytical

Table 1
EC_{50} values (μM) of the top five compounds (Cpd) in the AML and Ba/F3 cell lines used during the screening.

Compound	Cell line						
	Ba/F3-FLT3-ITD no IL-3	Ba/F3-FLT3-ITD + IL-3	MOLM-13	MV4-11	EOL-1	HEL	KG-1
1	0.036	18	0.292	0.142	0.066	47	15
2	0.138	23	0.612	2.821	0.502	1000	28
3	0.296	21	0.63	1.779	0.202	62	29
4	0.405	16	0.258	2.755	0.382	1000	24
5	0.717	19	0.332	3.414	0.889	14	20

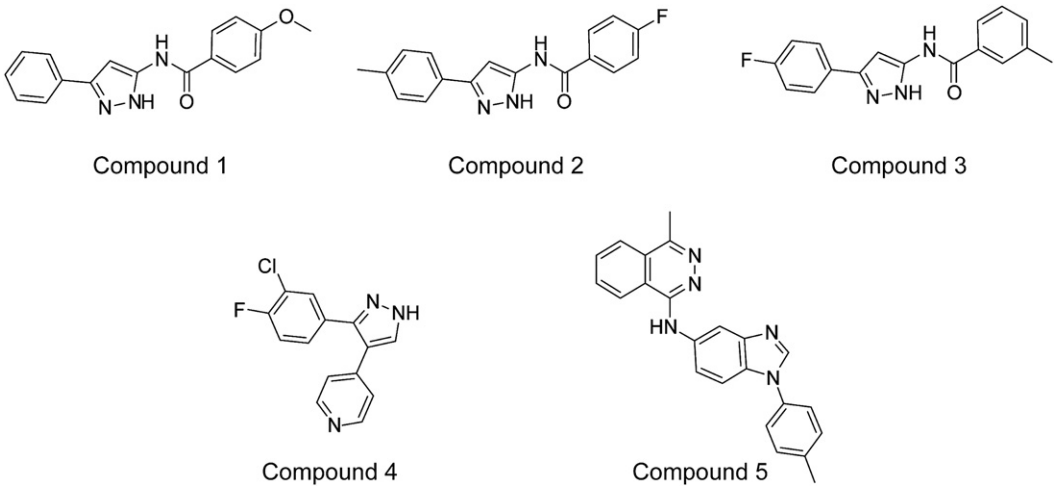

Fig. 1. Chemical structures of the identified FLT3 inhibitors. Compound **1**: 4-methoxy-*N*-(3-phenyl-1*H*-pyrazol-5-yl)benzamide; compound **2**: 4-fluoro-*N*-(3-(*p*-tolyl)-1*H*-pyrazol-5-yl)benzamide; compound **3**: *N*-(3-(4-fluorophenyl)-1*H*-pyrazol-5-yl)-3-methylbenzamide; compound **4**: 4-(3-(3-chloro-4-fluorophenyl)-1*H*-pyrazol-4-yl)pyridine; compound **5**: 4-methyl-*N*-(1-(*p*-tolyl)-1*H*-benzo[*d*]imidazol-5-yl)phthalazin-1-amine.

data of these compounds confirmed not only the exact structure of the compounds used in the biological testing but also their high level of purity. All the information and NMR spectra as a string for the different compounds are available in the Supplementary material.

2.2.1. 4-Methoxy-N-(3-phenyl-1H-pyrazol-5-yl)benzamide (compound **1**)

Prepared by reaction of 5-phenyl-1*H*-pyrazol-3-amine (**4a**) and 4-methoxybenzoyl chloride (**5a**). Purified using a gradient of methanol (1–10%) in dichloromethane. Yield 62%; white solid; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.75 (s, 1H), 8.04 (d, J = 8.8 Hz, 2H), 7.77 (dd, J = 8.4, 1.2 Hz, 2H), 7.46 (t, J = 8.4 Hz, 2H), 7.35 (tt, J = 8.4, 1.2 Hz, 1H), 7.05 (d, J = 8.9 Hz, 2H), 6.97 (s, 1H), 3.84 (s, 3H); ^{13}C NMR (151 MHz, DMSO): δ 164.36 (HN–*C*=O), 162.34 (C), 147.55 (C), 143.25 (C), 130.42 (C), 130.09 (CH), 129.37 (CH), 128.42 (CH), 126.45 (C), 125.37 (CH), 113.99 (CH), 94.65 (CH), 55.82 (OCH_3); ESI/APCI (+) m/z 294 (M + H); ESI/APCI (−) m/z 292 (M − H).

2.2.2. 4-Fluoro-N-(3-(p-tolyl)-1H-pyrazol-5-yl)benzamide (compound **2**)

Prepared by reaction of 5-(*p*-tolyl)-1*H*-pyrazol-3-amine (**4b**) and 4-fluorobenzoyl chloride (**5b**). Purified using a gradient of methanol (1–7%) in dichloromethane. Yield 23%; white solid; ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.88 (s, 1H), 10.88 (s, 1H), 8.11 (dd, J = 8.7, 5.6 Hz, 2H), 7.65 (d, J = 8.2 Hz, 2H), 7.34 (t, J = 8.7 Hz, 2H), 7.27 (d, J = 7.8 Hz, 2H), 7.00 (s, 1H), 2.34 (s, 3H); ^{13}C NMR (151 MHz, DMSO): δ 164.50 (*C*–F), 163.84 (HN–*C*=O), 148.61 (C), 142.30 (C), 137.96 (C), 130.99 (C), 130.90 (CH), 129.95 (CH), 127.03 (C), 125.30 (CH), 115.68 (CH), 94.76 (CH), 21.24 (CH_3); ESI/APCI (+) m/z 296 (M + H); ESI/APCI (−) 294 m/z (M − H).

2.2.3. N-(3-(4-Fluorophenyl)-1H-pyrazol-5-yl)-3-methylbenzamide (compound **3**)

Prepared by reaction of 5-(4-fluorophenyl)-1*H*-pyrazol-3-amine (**4c**) and 3-methylbenzoyl chloride (**5c**). Purified using a gradient of methanol (1–7%) in dichloromethane. Yield 48%; white solid; ^{1}H NMR (600 MHz, DMSO-d_6): δ 12.91 (s, 1H), 10.80 (s, 1H), 7.88–7.78 (m, 4H), 7.39 (d, J = 4.5 Hz, 2H), 7.30 (t, J = 8.7 Hz, 2H), 7.02 (s, 1H), 2.39 (s, 3H); ^{13}C NMR (151 MHz, DMSO): δ 165.03 (HN–*C*=O), 162.20 (=*C*–F), 148.81 (C), 141.30 (C), 138.04 (C), 134.37 (C), 132.61 (*C*H) 128.71 (*C*H), 128.65 (CH), 127.49 (*C*H), 126.49 (C), 125.28 (*C*H), 116.32 (*C*H), 95.21 (*C*H), 21.36 (CH_3); ESI/APCI (+) m/z 296 (M + H); ESI/APCI (−) m/z 294 (M − H).

2.3. Confirmation assays

We confirmed the growth inhibitory effects of the five selected compounds against EOL-1, MOLM-13 and MV4-11 cell lines. Dose–response curves are depicted in Fig. 3A. From these data it is clear that compound **1** is the most potent molecule with EC_{50} of 70–300 nM. All the compounds showed a similar selectivity toward FLT3-ITD (MOLM-13 and MV4-11) and PDGFRα (EOL-1).

We also determined the EC_{50} values in Ba/F3 cells transformed by FLT3-D835Y, an activated mutant form of FLT3 with a point mutation in the activation loop of the kinase domain. In addition, we also tested FLT3-ITD with additional mutations (F691I and G697R) in its kinase domain. These mutations (D835Y, F691I and

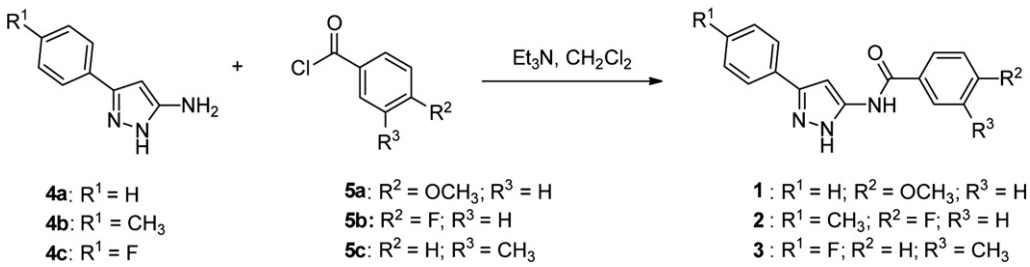

Fig. 2. Overview of the chemical synthesis of compounds **1**, **2** and **3**. 5-Phenyl-1*H*-pyrazol-3-amine derivative **4a**–**c** were added to benzoyl chloride derivative **5a**–**c** to create compounds **1**, **2** and **3**.

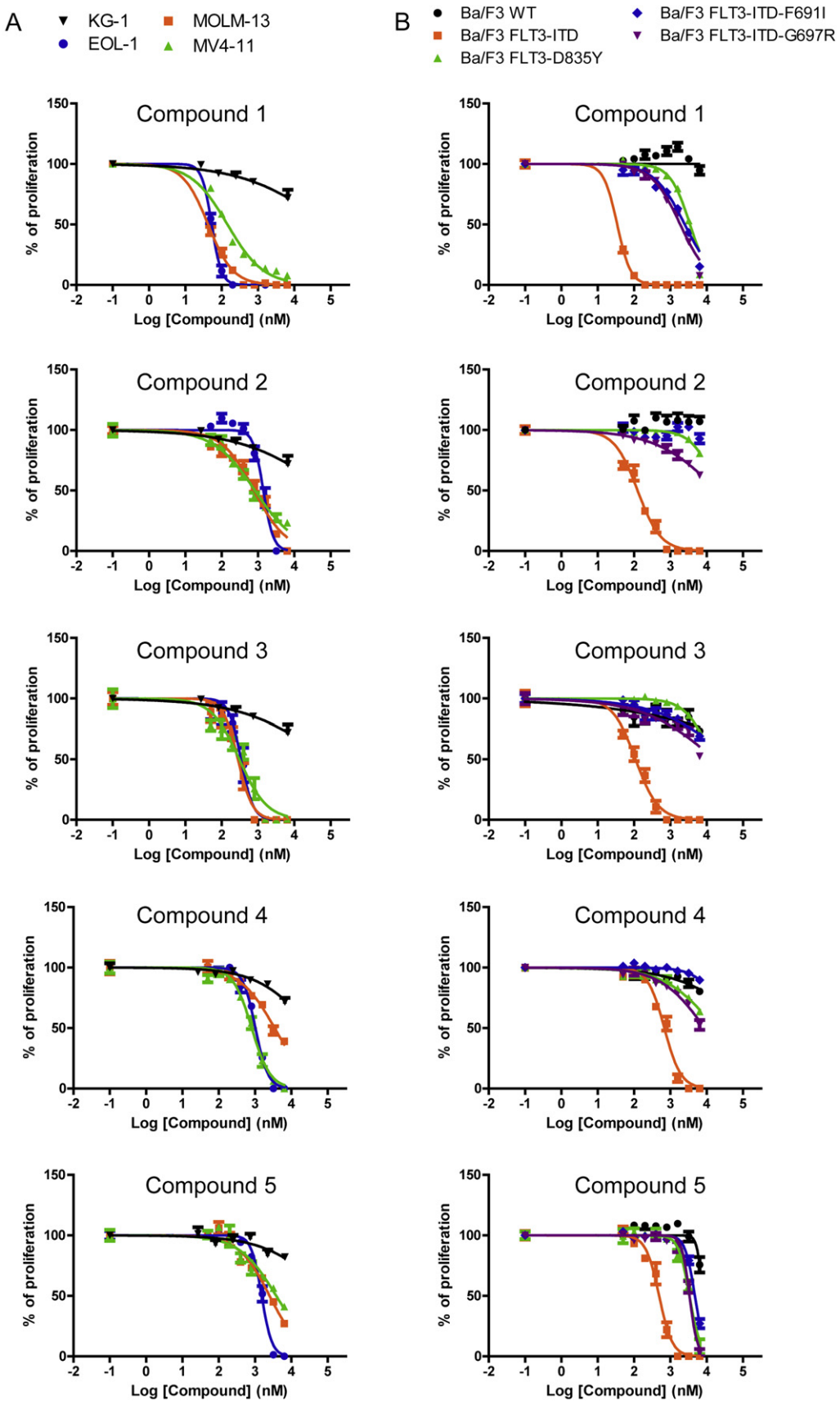

Fig. 3. Dose–response curves for the AML cell lines (A) and the Ba/F3 cell lines (B). A) The AML cell lines EOL-1 (PDGFR dependent), MOLM-13 (FLT3 dependent) and MV4-11 (FLT3 dependent) were treated with the five different hit compounds with concentrations ranging between 35 and 700 nM. The AML cell line KG-1 (FGFR1 dependent) served as a control. B) Same experiments were performed on the Ba/F3 cell lines (FLT3-ITD, FLT3-ITD-F691I, FLT3-ITD-G697R and FLT3-D835Y). Ba/F3 wild type cells (Ba/F3 WT) were used as a control. Data represent mean +/− SEM.

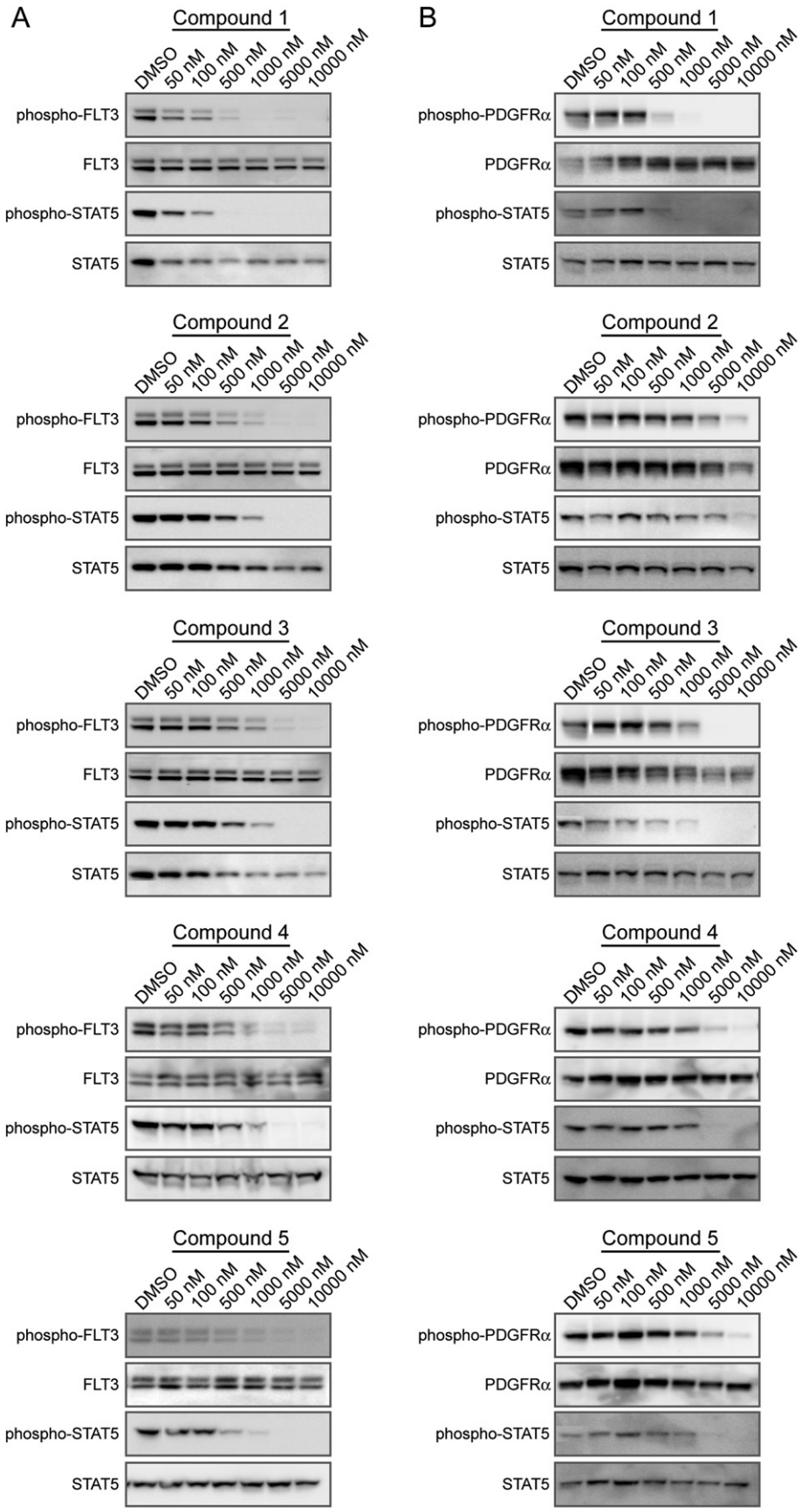

Fig. 4. Western blot analysis of the effects on phosphorylation. A) Ba/F3-FLT3-ITD cells were treated with different concentrations of the hit compounds. Phosphorylation of FLT3 and STAT5 was measured. FLT3 and STAT5 protein levels were used as loading controls. B) For the EOL-1 cells we determined phosphorylation of PDGFRα and STAT5. PDGFRα and STAT5 protein levels were used as loading controls.

G697R) are known to confer resistance to various FLT3 kinase inhibitors [25,27,32]. We tested the activity of the five compounds against these different FLT3 mutants and the resulting dose–response curves are shown in Fig. 3B, pointing out mild activities against some of the mutant FLT3 cell lines.

To confirm that the compounds had a direct effect on FLT3/PDGFR we analyzed the effects of the compounds on the auto-phosphorylation of FLT3-ITD and FIP1L1-PDGFRα in the Ba/F3-FLT3-ITD cells and EOL-1 cells, respectively. Furthermore, we determined the effect of treatment with the inhibitors on the phosphorylation of STAT5, a downstream effector of FLT3/PDGFRα. Western blot analysis confirmed the inhibiting effect of the five compounds on FLT3 auto-phosphorylation. These data also confirmed that compound **1** is the most potent inhibitor with an inhibition of FLT3 auto-phosphorylation starting at a concentration as low as 50 nM (Fig. 4). The other compounds blocked the auto-phosphorylation of FLT3 with similar potency at a concentration of 500 nM. Compound **2** showed a remarkable lower inhibition of the auto-phosphorylation of PDGFRα, indicating that compound **2** is a more specific for FLT3 than for PDGFRα.

2.4. Specificity of the FLT3/PDGFR inhibitors

To determine the specificity of these compounds for FLT3/PDGFR, we next tested their effect on 24 other tyrosine kinases by using the Ba/F3 cell system. Each of the 24 respective Ba/F3 cell lines expresses a specific activated tyrosine kinase and is dependent on this kinase activity for its proliferation and survival. Interestingly, compounds **1**, **2**, **3** and **5** did not show any activity against the 24 other kinases (Fig. 5). For compound **4** we observed an inhibiting effect on cells dependent on ABL2, EPHA5, EPHA8, ROS1 and SRC at 5 μM (Fig. 5). For these kinases we performed an additional dose–response experiment, but no inhibitory effect of compound **4** below 1 μM was seen, indicating the specificity for FLT3/PDGFR at lower concentrations (Fig. 6).

3. Discussion

AML is the most frequent acute leukemia in adults and has a low survival rate of around 37% for pediatric AML and 16% for adult AML [2]. The chemotherapeutic treatment of AML has been improved over the last years, but there remains a strong need for the introduction of targeted therapies that are more potent and less toxic than the current chemotherapy. The high incidence rate of *FLT3* mutations in AML patients and its correlation with a poor prognosis makes FLT3 a very interesting target for therapy [5,10]. FLT3 is mutated in approximately 30% of AML cases and is known to be important for the proliferation and survival of the leukemia cells.

In the past ten years, a number of FLT3 kinase inhibitors, such as PKC412, Lestaurtinib, Semaxanib, Sunitinib and Tandutinib have been developed, but the clinical results so far have only been modest, especially in monotherapy setting where these compounds seem to fail to induce a long lasting remission. In addition, we can expect that similarly to the development of resistance to BCR-ABL1 inhibitors in blast crisis CML, resistance to FLT3 inhibitors is likely to develop during therapy with FLT3 inhibitors. Different resistance mechanisms have been described in tyrosine kinases of which the most important mechanism is the acquisition of mutations in the kinase domain [24]. Indeed, in an ongoing clinical trial with the FLT3 inhibitor AC220 the development of resistance was reported, due to the acquisition of specific resistance mutations in the FLT3 kinase domain [25].

Another potential issue is the poly-pharmacology associated with the existing FLT3 inhibitors that are responsible for the unwanted side effects of the drugs. While some kinase inhibitors are

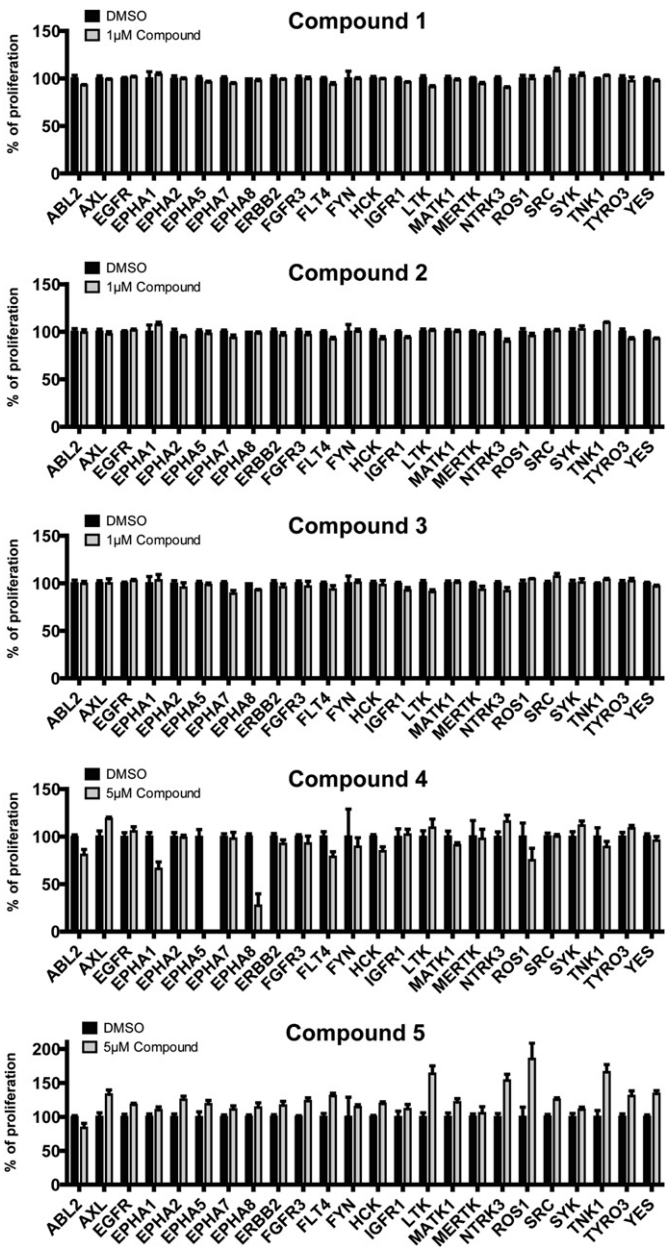

Fig. 5. Specificity testing on 24 other kinases. Each hit compound (1 or 5 μM) was tested on Ba/F3 cells transformed by one of the 24 kinases. These Ba/F3 cells are specifically dependent on the activity of the indicated tyrosine kinase. Percentage of growth was measured after 24 h. Black bars represent DMSO and gray bars represent the compound. Data represent mean +/− SEM.

very specific, many are targeting multiple kinases (both tyrosine and serine/threonine kinases), which may be beneficial to target cancer cells, but also a disadvantage with respect to side effects.

All these current limitations of the existing FLT3 inhibitors indicate the need to identify novel potent and specific FLT3 inhibitors. To this end, we screened a library of 25,607 small molecules on a FLT3 dependent AML cell line (MOLM-13) and a PDGFRα dependent AML cell line (EOL-1). Using this screening strategy, we identified five potentially interesting FLT3/PDFGR inhibitors (Table 1). The most potent compounds (compounds **1**–**3**) belong to the same chemical class (3-aminopyrazole) that were previously described as PDGFR kinase inhibitors in one patent application [37]. Our data now demonstrates that these compounds are selective inhibitors of the FLT3/PDGFR kinases, since we observed potent

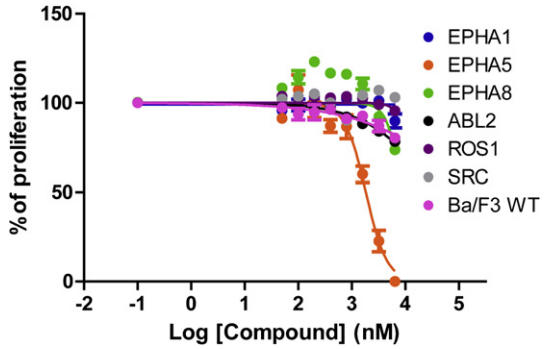

Fig. 6. Dose–response curves of compound **4** for the Ba/F3 cells transformed with ABL2, EPHA1, EPHA5, EPHA8, ROS1 or SRC. Ba/F3 wild type cells (Ba/F3 WT) were used as a control. Data represent mean +/− SEM.

inhibition of FLT3 and PDGFR, with no activity against 24 additional tyrosine kinases (Figs. 5 and 6). Based on both the cellular and the auto-phosphorylation data, compound **1** was recognized as the most potent inhibitor, while compound **2** was the most specific FLT3 inhibitor (Figs. 3 and 4). Compound **4** was found to show some activity against additional tyrosine kinases, but still the activity against FLT3 was the only inhibitory activity below 1 μM. It is interesting to note that this compound also showed good activity against EPHA5. Compound **5**, a benzimidazole-based compound, is patented for its effect on stem cell differentiation [38], which could be attributed to its inhibitory effect on FLT3 kinase, a protein important for hematopoietic stem cells. Furthermore, compounds **1** and **5** retained some inhibitory activity against the D835Y activating FLT3 mutant and the F691I/G697R resistant mutants, but none of the other compounds showed an inhibitory effect against these FLT3 mutants (Fig. 3B).

In conclusion, we identified five FLT3/PDGFR inhibitors from a library of 25,607 small molecules. All compounds had similar activity toward both FLT3 and PDGFRα, although compound **1** was clearly the most potent one, while compound **2** was the most specific FLT3 inhibitor. Finally, all five compounds were found to be selective FLT3/PDGFR inhibitors, as no effect was observed on 24 other tyrosine kinases.

4. Conclusion

Even though a large number of FLT3/PDGFR kinase inhibitors have already been identified, most of these show poor activity *in vivo* and the development of resistance has been described. Therefore, it is important to develop novel FLT3/PDGFR kinase inhibitors to overcome these current problems. We performed a cell-based high throughput screening to identify inhibitors that show cellular activity against FLT3/PDGFR. The top five compounds were further characterized and we confirmed their selective activity against FLT3/PDGFR. These compounds could be considered for further modification and optimization to obtain more potent and selective compounds.

5. Experimental

5.1. Cell culture

The human AML cell lines EOL-1, MOLM-13, MV4-11, HEL and KG-1 (DSMZ, Braunschweig, Germany) and the mouse Ba/F3 wild type, Ba/F3-FLT3-ITD, Ba/F3-FLT3-D835Y, Ba/F3-FLT3-ITD-F691I and Ba/F3-FLT3-ITD-G697R (developed in house) were cultured in RPMI 1640 medium (without phenol red) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2-incubator. The Ba/F3 cells were cultured in the presence of mouse IL-3 (1 mg/ml) (Peprotech Inc., Rocky Hill, NJ, USA). Ba/F3 cells transformed by activated kinases do not require IL-3 for their growth.

5.2. Chemical compounds

The compounds were selected by the Center for Drug Design and Discovery (CD3). The collection tested consisted of 25,607 small molecules (Mw < 500 g/mol), which were selected based on (i) chemical diversity, (ii) druglike properties (Lipinski rule of five compliant) and purchased from multiple commercial suppliers. As a positive control we used the known FLT3 inhibitor tandutinib (Selleck Chemicals LLC, Houston, TX, USA) [39].

5.3. Chemical synthesis

Reagents and anhydrous solvents were purchased from commercial suppliers (Acros, Sigma–Aldrich and Alfa Aesar) and used without further purification. Flash chromatography was performed with flash chromatography cartridges Biotage SNAP 10 and 25G either on Biotage Isolera or Biotage SP4 flash chromatography instrument. ^{1}H NMR and ^{13}C NMR were recorded at 600 and 151 MHz, respectively, on a Bruker Avance II-600. Chemical shifts are reported relative to tetramethylsilane at 0.00 ppm. Mass spectral data were obtained using a Brucker Esquire 6000 (equipped with a multimode source, ESI/APCI). Mass spectra were acquired in positive and negative modes. The chemical purity of the target compounds was determined by LC-MS. LC-MS analysis were performed on a Dionex Ultimate 3000 HPLC system (equipped with a PDA detector) connected to a mass spectrometer Brucker Esquire 6000 (equipped with a multimode source, ESI/APCI). The purity of each compound was ≥95% in either analysis.

Confirmatory ^{1}H NMR, TOF-MS and LC-MS analyses were performed for compounds **1** to **3**. Analytical data for compounds **4** and **5** were obtained from the commercial suppliers Asinex (Moscow, Russia) and ChemDiv (San Diego, CA, USA) respectively (Supplementary material).

5.4. Compound treatment

Cells (100,000 cells/ml) were seeded in 384-well plates (library screening) or 96-well plates (dose–response experiments) with the cell dispenser Fluid Xrd-384 (FluidX, Cheshire, UK). Compounds were added to the wells with the TECAN pipetting robot (TECAN Schweiz AG, Switzerland). For the library screening we used a final concentration of 10 μM compound with dimethyl sulfoxide (DMSO) as a negative control and 10 μM 1-β-D-Arabinofuranosylcytosine (Ara-C) (Merck KGaA, Darmstadt, Germany) as a positive control. For dose–response experiments we used 8 different concentrations (50, 100, 200, 400, 800, 1600, 3200 and 6400 nM) each in triplicate with DMSO as a negative control. The number of viable cells was counted after a treatment of 72 h (library screening) and 24 h (dose–response experiments) with ATP-lite 1step reagent (PerkinElmer, Boston, MA, USA). Luminescence was measured with the multilabel plate readers Envision and Victor X4 (PerkinElmer, Boston, MA, USA). EC_{50} values were calculated with GraphPad Prism. The specificity of the hit compounds was tested on Ba/F3 cells transformed with ABL2, AXL, EGFR, EPHA1, EPHA2, EPHA5, EPHA7, EPHA8, ERBB2, FGFR3, FLT4, FYN, HCK, IGFR1, LTK, MATK1, MERTK, NTRK3, ROS1, SRC, SYK, TNK1, TYRO3 and YES. The cells were incubated for 24 h with 1 μM (compounds **1**, **2** and **3**) and 5 μM (compounds **4** and **5**). Each sample was set up in triplicate. DMSO was used as a negative control.

5.5. Western blotting

The cell lines Ba/F3-FLT3-ITD and EOL-1 were treated during 90 min with increasing doses of the drugs. Cells were harvested and lysed using lysis buffer (Cell signaling, Danvers, MA, USA) supplemented with 1 mM $NaVO_4$ and protease inhibitors (Complete, Roche Applied Science, Penzberg, Germany). Samples were reduced and gel electrophoresis was performed using NuPage Bis-Tris 4–12% gels (Invitrogen). Antibodies used were rabbit polyclonal anti-FLT3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-phosphoFLT3 (Cell Signaling), rabbit polyclonal anti-PDGFRα (Santa Cruz Biotechnology), rabbit polyclonal anti-phosphoPDGFRα (Santa Cruz Biotechnology), rabbit polyclonal anti-STAT5α (Santa Cruz Biotechnology) and rabbit monoclonal anti-phosphoSTAT5 (Cell Signaling). The LAS-3000 Imaging System (Fujifilm Global) was used for protein blot analysis.

5.6. Statistical analysis

The students *t*-test was performed to determine significant differences between two groups. Normality test was applied to test the normal distribution.

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Appendix ASupplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.024.

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