

## ORIGINAL ARTICLE

**A comprehensive target selectivity survey of the BCR-ABL kinase inhibitor INNO-406 by kinase profiling and chemical proteomics in chronic myeloid leukemia cells**

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**Resistance to the BCR-ABL tyrosine kinase inhibitor imatinib poses a pressing challenge in treating chronic myeloid leukemia (CML). This resistance is often caused by point mutations in the ABL kinase domain or by overexpression of LYN. The second-generation BCR-ABL inhibitor INNO-406 is known to inhibit most BCR-ABL mutants and LYN efficiently. Knowledge of its full target spectrum would provide the molecular basis for potential side effects or suggest novel therapeutic applications and possible combination therapies. We have performed an unbiased chemical proteomics native target profile of INNO-406 in CML cells combined with functional assays using 272 recombinant kinases thereby identifying several new INNO-406 targets. These include the kinases ZAK, DDR1/2 and various ephrin receptors. The oxidoreductase NQO2, inhibited by both imatinib and nilotinib, is not a relevant target of INNO-406. Overall, INNO-406 has an improved activity over imatinib but a slightly broader target profile than both imatinib and nilotinib. In contrast to dasatinib and bosutinib, INNO-406 does not inhibit all SRC kinases and most TEC family kinases and is therefore expected to elicit fewer side effects. Altogether, these properties may make INNO-406 a valuable component in the drug arsenal against CML.**

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**Keywords:** CML; INNO-406; kinase profiling; chemical proteomics; kinase inhibitor

**Introduction**

Expression of the oncogenic fusion protein BCR-ABL is the hallmark of chronic myeloid leukemia (CML) and inhibition of its tyrosine kinase activity by imatinib (Gleevec, STI-571) has become the paradigm of targeted therapy. However, resistance toward imatinib has necessitated the development of new BCR-ABL inhibitors such as dasatinib (Sprycel, BMS-354825) and nilotinib (Tasigna, AMN107) as well as bosutinib (SKI-606) and INNO-406 (NS-187, bafetinib). The newest of these, the dual ABL/LYN inhibitor INNO-406, is a structural analogue of imatinib and nilotinib, which exhibits a 25- to 55-fold increase over imatinib in *in vitro* activity against BCR-ABL (Figure 1).<sup>1</sup> Furthermore, INNO-406 inhibits several imatinib-resistant BCR-ABL mutants, with the exception of T315I, and the SRC family

kinase (SFK) LYN, the overexpression and BCR-ABL-independent activation of which has been associated with imatinib resistance.<sup>1–4</sup> In cellular assays, INNO-406 inhibited PDGFR phosphorylation comparable to imatinib, while showing a much higher IC<sub>50</sub> against wild-type c-KIT phosphorylation.<sup>1</sup> It has, however, been shown to block cell proliferation of mutant c-KIT V560G-harboring cells similar to imatinib, suggesting that INNO-406 could also be used to treat patients with gastrointestinal stromal tumors (GISTs) carrying this mutation.<sup>5</sup> Finally, in contrast to imatinib, INNO-406 has been shown to reach sufficient concentrations in the central nervous system (CNS) to treat BCR-ABL-positive CNS leukemia in a mouse model.<sup>6</sup>

It has recently been shown that some BCR-ABL kinase inhibitors, particularly dasatinib and bosutinib, are highly promiscuous.<sup>7–9</sup> Although intended to hit multiple disease-relevant targets simultaneously, that is, BCR-ABL and the SFKs, this promiscuity can also lead to side effects such as the pleural effusions often associated with dasatinib.<sup>10</sup> Identifying the complete target profile of a drug can therefore help not only with understanding the drug's molecular mechanism of action within the disease, but also of unwanted side effects. Identifying new targets could also lead to novel therapeutic applications like imatinib's use against GIST.<sup>5</sup>

Large-scale profiling of kinase inhibitors has been successful in determining the full spectrum of target proteins in, for example, cell lines representing CML (for example, K562).<sup>7</sup> In our laboratory, we emphasize identifying targets in clinically relevant primary patient cells.<sup>8,9</sup> We use a chemical proteomic approach in which we immobilize suitably modified drug analogues and expose them to cell lysates, thereby identifying the 'natural' target profile of the drugs. In this way we have identified novel kinase (for example, BTK, DDR1, CAMK2G, STE20 kinases) and nonkinase (for example, NQO2) targets for each of the CML inhibitors imatinib, nilotinib, dasatinib and bosutinib.<sup>8,9,11</sup> As recently reported with bosutinib, in parallel to the chemical proteomic approach described above, we also measure drug activity against a large-scale panel of recombinant kinases, thereby providing a global view of the targeted kinome.<sup>9</sup>

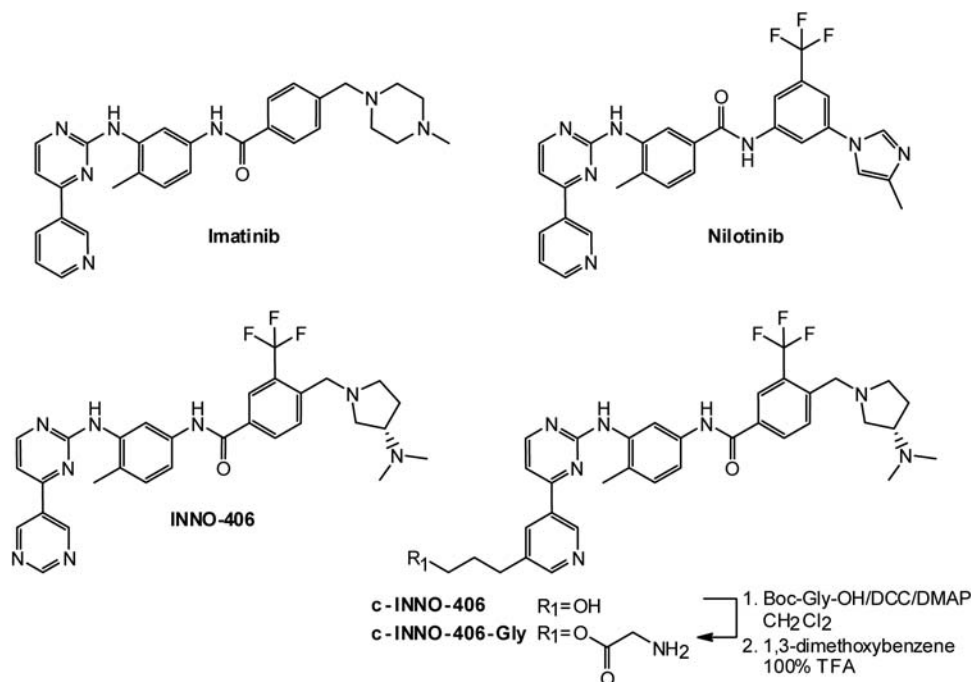
In this study, this complementary method allowed us to identify several novel kinase targets of INNO-406. These include, among others, the mixed-lineage kinase ZAK, the receptor tyrosine kinases DDR1 and DDR2, and various ephrin receptor kinases, predominantly EPHA2, EPHA5 and EPHA8. Furthermore, we observed potent activity against the PDGFR $\alpha$  V561D, but not the D842V mutant, both of which are frequently found in GIST. Surprisingly, the oxidoreductase NQO2, which was previously shown to be potently inhibited by imatinib and

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**Figure 1** Chemical structures of imatinib, nilotinib, INNO-406 and the coupleable analogue of INNO-406. The pyrimidinyl ring of INNO-406 was replaced with a 2-propylhydroxy-pyridine ring to give c-INNO-406. Esterification and deprotection afforded the directly coupleable free amine in c-INNO-406-gly.

nilotinib, is not a significant target of INNO-406. One of the most relevant differences of INNO-406 to the other second-generation BCR-ABL inhibitors, however, lies in its distinct selectivity profile about the SFK and TEC family kinase, possibly translating into a reduced potential for eliciting immune-related side effects, while retaining improved efficacy against imatinib-resistant CML cells through inhibition of LYN.

## Materials and methods

### Biological material

K562 cell pellets were generated by CilBiotech (Mons, Belgium). KU812 cells were cultured in RPMI 1640 media supplemented with 20% fetal calf serum. Unfractionated peripheral blood leukocytes (buffy coat) were collected from patients in chronic phase CML. Peripheral blood mononuclear cells (PBMCs) were obtained using Ficoll. Studies were approved by the institutional review board (Medical University of Vienna). Written informed consent in accordance with the Declaration of Helsinki Principles was obtained before blood donation.

Antibodies used include monoclonal mouse anti-ABL (21-63); polyclonal rabbit anti-DDR1 (C20), anti-LYN (44) and anti-NQO2 (H-50) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Alexa Fluor 680 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA); IRDye 800 donkey anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE, USA) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA).

### Compounds, immobilization and affinity purification

INNO-406, coupleable c-INNO-406 and imatinib were synthesized by WuXi AppTec (Shanghai, China). Following esterification and deprotection (Figure 1), c-INNO-406-gly was

immobilized on NHS-activated Sepharose 4 Fast Flow resin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as previously described.<sup>8</sup> Affinity chromatography was performed as described including the addition of Roche's Complete Protease Inhibitor Cocktail tablet (1 tablet per 50 ml lysis buffer) to our standard inhibitor mix during experiments with patients' cells.<sup>8,12</sup> For compilation of the 'frequent hitter' list, amphotericin B, daunorubicin and paroxetine (Sigma, St Louis, MO, USA), kanamycin (Roth, Karlsruhe, Germany) and ciprofloxacin (Fluka, St Louis, MO, USA) were directly immobilized (Supplementary Figure S2) on NHS-activated Sepharose 4 Fast Flow resin and the resulting matrices were exposed to the respective total cell lysates as previously described.<sup>8</sup> In competition experiments, an aliquot of each cell lysate was preincubated (30 min, 4 °C) with 20 μM free INNO-406 before being presented to the affinity matrix.

### Sample preparation, mass spectrometry (MS) and MS data processing.

See Supplementary Materials and Methods.

### Kinase inhibition analysis

INNO-406 and c-INNO-406 were assayed *in vitro* for inhibition of recombinant full-length c-ABL (Upstate Biotechnology, Lake Placid, NY, USA). Phosphorylation of 5-FAM-KKGEIYAAPFANH<sub>2</sub> was monitored using the IMAF kinase assay kit and a SpectraMax M5 plate reader (Molecular Devices, Eugene, OR, USA). *In vitro* ZAK kinase inhibition was performed based on a method modified from Wang *et al.*<sup>13</sup> Recombinant ZAK (100 ng; Invitrogen) and biotinylated myelin basic protein (2 μg; Abcam, Cambridge, MA, USA) as substrate were incubated in the presence of 50 μM ATP, 5 mM MgCl<sub>2</sub>, 50 mM HEPES (pH 7), 0.01% Triton X-100, 10% glycerol, 0.1 mg/ml bovine serum

albumin, 4 mM DTT and 1.5  $\mu\text{Ci}$  [ $^{32}\text{P}$ ]- $\gamma$ -ATP for 2 h at room temperature. The terminated reaction was transferred to a SAM2 Biotin Capture membrane (Promega, Madison, WI, USA) and treated according to the manufacturer's instructions. Protocols detailing kinase assays conducted using Millipore's KinaseProfiler can be found at <http://www.millipore.com/drugdiscovery/dd3/KinaseProfiler> (see also Supplementary Figure S1), where ATP concentration for each specific kinase assay was set within 15  $\mu\text{M}$  of the apparent  $K_m$  for ATP where determined.

#### NQO2 inhibition assay

Inhibition of recombinant NQO2 by INNO-406 and imatinib was performed as previously described using menadione and 1-carbamoylmethyl-3-carbamoyl-1,4-dihydropyrimidine as substrate and co-substrate, respectively.<sup>14</sup> MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) reduction was monitored at 590 nm using a SpectraMax M5 plate reader.

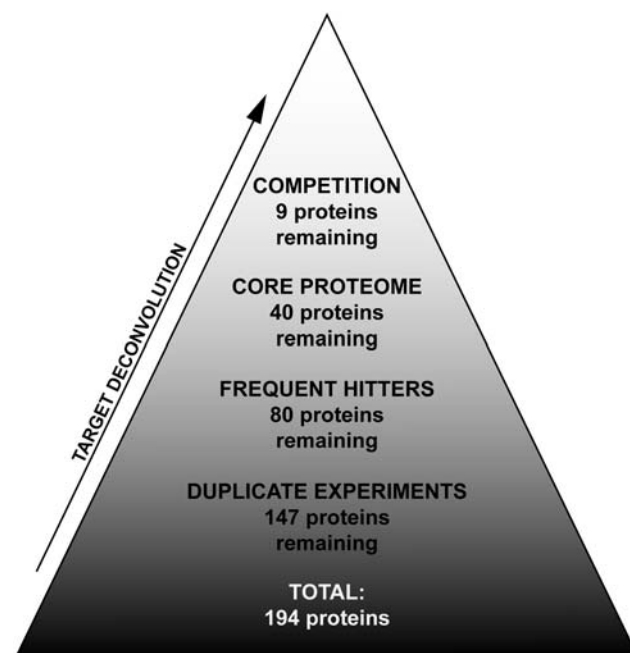
#### Cellular PDGFR $\alpha$ assays

Chinese hamster ovary (CHO) cells were transiently transfected with PDGFR $\alpha$  wt, PDGFR $\alpha$  D842V and PDGFR $\alpha$  V561D encoding plasmids, cultivated and treated with drug as described previously before immunoblot analysis of total cell extracts for phosphotyrosine-PDGFR $\alpha$  and total PDGFR $\alpha$ .<sup>15</sup>

## Results and discussion

Based on the similarity of the binding mode of INNO-406 and imatinib to the ABL kinase,<sup>16</sup> c-INNO-406 was synthesized with the pyrimidinyl ring of INNO-406 being replaced with a 2-propylhydroxy-pyridine ring. Concentration-dependent inhibition of the ABL kinase by INNO-406 and c-INNO-406 was identical, confirming that the modification did not affect inhibitory potential (data not shown). Esterification and deprotection to obtain c-INNO-406-gly provided an amino group suitable for coupling to the Sepharose resin (Figure 1). We investigated three cell populations including a pool of PBMCs from four chronic phase CML patients (patient information given in Supplementary Table 1), as well as the BCR-ABL-positive cell lines K562 and KU812, the latter retaining some granulocytic (basophilic) differentiation capacity and therefore representing an earlier disease stage than the more blast crisis-like K562 cells. K562 was used to allow comparisons between INNO-406 and other BCR-ABL inhibitors profiled in this cell line.<sup>7-9</sup>

One of the main challenges of drug affinity proteomics is target deconvolution. We have addressed this issue in several different ways (Figure 2). First, the resulting list of identified proteins (Supplementary Table S2a-c) was filtered against nonspecific or 'frequent hitter' proteins (Supplementary Table S3). This list contains all proteins identified from K562 or KU812 total cell lysates on matrices of five kinase-inhibitor-unrelated drugs serving as negative controls, namely paroxetine, kanamycin, daunorubicin, amphotericin B and ciprofloxacin (Supplementary Figure S2). Given limited primary cell material, only paroxetine was used to determine frequent hitters in the patient pool. Second, from those proteins identified in the K562 cell lysate, a previously established 'core proteome' data set made up of the most abundant/prevalent proteins in this cell line was subtracted.<sup>8,17</sup> Finally, we performed competition experiments where each cell lysate was preincubated with free INNO-406 before being presented to the c-INNO-406 affinity



**Figure 2** Target deconvolution strategy for proteins identified via chemical proteomics. Starting with the complete list of proteins obtained from incubation of the INNO-406 affinity matrix with K562 total cell lysates, nonspecific binders were eliminated based on (1) not being found in duplicate experiments, (2) being present at least once in the frequent hitter pulldowns, (3) also being present in the core proteome (when available) and (4) not being completely competed away by preincubation with free INNO-406. The number of proteins remaining at each step is shown. The pyramid is representative of the target deconvolution performed for each of the various cell lysates (see also Supplementary Table S2).

matrix. Targets completely absent ('competed') in these samples compared to the untreated samples were considered to be specific (Table 1).

In parallel to the chemical proteomic approach, INNO-406 was assayed at 1 and 10  $\mu\text{M}$  against a panel of 272 recombinant protein and lipid kinases, including several clinically relevant mutants (Table 2; Supplementary Table S4). To delineate specific differences between INNO-406 and imatinib,  $\text{IC}_{50}$  values of both drugs were subsequently determined for those kinases, which showed a reduction of activity of more than 50% at an INNO-406 concentration of 1  $\mu\text{M}$ , excluding those known not to be affected by imatinib (Tables 1 and 2).

Thus, we detected the INNO-406 cognate targets BCR-ABL, ABL1 (c-ABL), ABL2 (ARG) and LYN tyrosine kinases by chemical proteomics and found them to be inhibited in the low to mid nanomolar range (Table 1). In agreement with this, these kinases were completely competed away, as reflected in anti-ABL and anti-LYN western blot analysis of the K562 pretreated and non-pretreated lysates (Figure 3). Whereas LYN was found in all three cell types investigated, BCR-ABL (and c-ABL) peptides were only found in the lysates of K562 and KU812 cells, but not in the patient sample. This is most likely due to a combination of lower levels of expression in chronic phase CML compared to the more advanced disease K562 cells and the previously observed sensitivity of particularly BCR-ABL toward high protease activity present in primary CML patient cell lysates.<sup>8,9,12</sup>

Besides the cognate targets, also several novel INNO-406 kinase targets were identified. Most noteworthy, both the

**Table 1** Target profile of INNO-406 following deconvolution of chemical proteomics data

INNO-406	K562		KU812		Patient pool		IC <sub>50</sub> (nM)	
	-	+	-	+	-	+	INNO	IMA
Gene name	PC (SC)		PC (SC)		PC (SC)			
<i>Kinases</i>								
<i>ABL1</i>	33 (23%)	0	8 (6%)	0	0	0	9	11
<i>BCR-ABL</i>	42 (25%)	0	12 (8%)	0	0	0	11 <sup>1</sup>	280 <sup>1</sup>
<i>ABL2</i>	25 (25%)	0	11 (9%)	0	0	0	9	112
<i>DDR1</i>	16 (19%)	0	0	0	0	0		337 <sup>21</sup>
<i>LYN</i>	12 (28%)	0	12 (30%)	0	8 (21%)	0	51	>1000
<i>ZAK, isoform 1</i>	24 (36%)	0	23 (35%)	0	4 (6%)	0	73	>10000
<i>ZAK, isoform 2</i>	12 (31%)	0	14 (38%)	0	4 (11%)	0		
<i>Nonkinases</i>								
<i>LCN2</i>	0	0	0	0	4 (23%)	0		
<i>LRRFIP2</i>	5 (8%)	0	0	0	0	0		
<i>LTA4H</i>	0	0	0	0	3 (6%)	0		

Abbreviations: IMA, imatinib; PC, peptide count; SC, sequence coverage.

The average SC is based on the PC and the size of the protein. The INNO-406 ± rows indicate whether the samples were pretreated with 20 μM INNO-406. IC<sub>50</sub> values (nM) were determined for both INNO-406 (INNO) and imatinib (IMA). Published values are referenced accordingly. Proteins are sorted alphabetically.

receptor tyrosine kinase DDR1 and the mixed-lineage kinase ZAK were found to be specific binders of INNO-406 by chemical proteomics (Table 1). Other kinases found to interact with the INNO-406 affinity matrix were p38α, PYK2 and TTBK2 (Supplementary Table S2). However, these three kinases were not fully competed away by free drug in all three cell types investigated. This correlates well to the weaker inhibition seen with p38α and PYK2 in the large-scale screen (Supplementary Table S4). Three nonkinase proteins also bound reproducibly to the INNO-406 matrix and therefore could potentially represent additional targets (Table 1). These include the largely uncharacterized leucine-rich repeat flightless-interacting protein 2 (LRRFIP2), lipocalin 2 (LCN2) and leukotriene A-4 hydrolase (LTA4H), the latter two of which have been suggested to contribute to the disease.<sup>18–20</sup>

Illustrating the complementary nature of our approach, several additional kinase targets, such as the ephrin receptors, the DDR1-related DDR2 receptor tyrosine kinase, the macrophage colony-stimulating factor-1 receptor FMS, the serine-threonine kinase c-RAF, the TEC family kinase BMX and the SRC-related kinase FRK (PTK5) were revealed in the large-scale kinase inhibition assay. These proteins are probably absent or only expressed to low extent in the cell types investigated by chemical proteomics.

DDR1 and DDR2 have been shown to be inhibited by imatinib, nilotinib and dasatinib with similar respective values.<sup>7,8,21</sup> It was therefore not surprising to identify them also as targets of INNO-406. Western blot analysis further confirmed complete competition of DDR1 from the INNO-406 matrix by free INNO-406 (Figure 3). In our study, DDR2 was inhibited by INNO-406 with an IC<sub>50</sub> of 220 nM (Table 2), strongly suggesting that INNO-406 would also inhibit DDR1 in the nanomolar range and may therefore be an important therapeutic in DDR1/2-dependent conditions, including several malignant, fibrotic and inflammatory diseases.

Other novel targets of INNO-406 shared with imatinib are FMS and c-RAF. Although FMS was inhibited by INNO-406 with a similar IC<sub>50</sub> value as seen for imatinib (204 and 147 nM, respectively), the serine-threonine kinase c-RAF was inhibited more potently by INNO-406 (181 versus 482 nM) (Table 2).

c-RAF has been reported to be important in BCR-ABL-dependent growth and therefore, in addition to targeting BCR-ABL and LYN, may represent a third mode of action of INNO-406 against CML.<sup>22</sup>

Regarding c-KIT, we confirmed the increased selectivity of INNO-406 for the c-KIT V560G mutant, present in approximately 60% of all GISTs, over wild-type c-KIT,<sup>5</sup> possibly correlating to a reduced occurrence of side effects while retaining efficacy for mutation-dependent tumors. Initial analysis suggested this selectivity applying also to the GIST-related PDGFRα mutants D842V and V561D (Table 2). Thus, the IC<sub>50</sub> values of INNO-406 for these two mutants were determined and found to be 1.28 μM and 59 nM for D842V and V561D, respectively. These activities were reflected in cellular assays where INNO-406-treated CHO cells transiently transfected with either wild-type or mutant PDGFRα showed a dose-dependent decrease of PDGFRα phosphorylation congruent with the generated IC<sub>50</sub> values (Figure 4). Wild-type PDGFRα was also found to be potently inhibited by INNO-406, which, although not suggested by our initial screen, has been shown in a previous study.<sup>1</sup> Although V561D mutant is targeted by imatinib with similar potency,<sup>15</sup> the more common of the two PDGFRα mutants, D842V, which is resistant to both imatinib and nilotinib,<sup>23</sup> is probably not potently enough inhibited for providing clinical relevance.

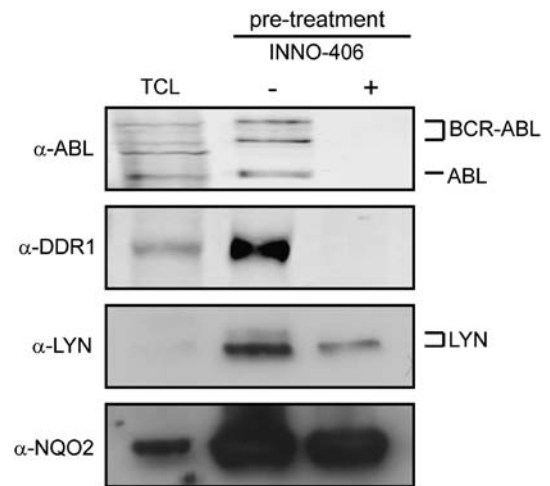
Whereas DDR1/2, c-KIT, PDGFR, FMS and c-RAF are inhibited to varying degrees by both INNO-406 and imatinib, the ephrin receptors are exclusively targeted by INNO-406. There seems to be some level of selectivity displayed by INNO-406 within the ephrin receptor family with EPHA2, EPHA5 and EPHA8 being most strongly inhibited by INNO-406 (Table 2; Supplementary Table S4). Although much work has been carried out on deciphering ephrin receptor signaling, this largest subgroup of the receptor tyrosine kinase family is complex and not well understood. It is, however, known to have implications in neuronal development, diabetes and cancer.<sup>24,25</sup> In particular, EPHA2 has been shown to act within tumor cells and also to regulate interactions between cells in the tumor stroma and vasculature, thus making it an attractive drug target in breast cancer.<sup>26</sup>

**Table 2** Target profile of INNO-406 from kinase inhibition assays

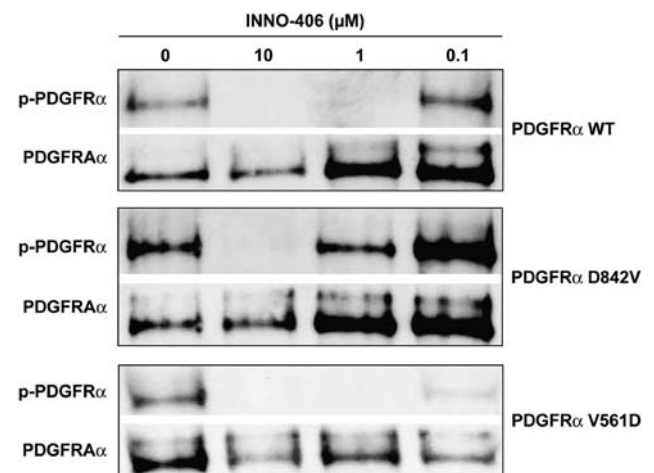
Gene name	Activity remaining (%)		IC <sub>50</sub> (nM)	
	1 μM	10 μM	INNO	IMA
<b>ABL family</b>				
ABL	2	1	9	11
ABL (H396P)	3	3		
ABL (M351T)	3	5		
ABL (Q252H)	1	1		
ABL (Y253F)	0	0		
ARG	4	1	9	112
<b>SRC family</b>				
BLK	58	10		
FGR	34	7		
FYN	13	2		
HCK	17	7		
LCK	1	1		
LYN	2	1	51	> 1000
SRC (1-530)	43	2		
SRC (full length)	56	15		
YES	53	5		
<b>KIT/PDGFR</b>				
c-KIT	98	58	840 <sup>1</sup>	210 <sup>1</sup>
c-KIT (V560G)	14	22	51 <sup>5</sup>	75 <sup>5</sup>
PDGFRα	93	54	56 <sup>1</sup>	100 <sup>1</sup>
PDGFRα (D842V)	36	3	1281	
PDGFRα (V561D)	5	11	59	
PDGFRβ	84	32		
<b>EPH receptors</b>				
EPHA2	8	4		
EPHA3	28	6		
EPHA5	2	1		
EPHA8	6	2		
EPHB1	14	2		
EPHB2	47	5		
<b>Other</b>				
BMX	46	3		
c-RAF	1	2	181	482
DDR2	11	7	220	225
FMS	9	8	204	147
PTK5 (FRK)	3	12		

Activity remaining (%) at 1 and 10 μM INNO-406 is shown for all kinases inhibited by at least 50% at 1 μM. For comparison, values for the remaining SFKs and wild-type c-KIT and PDGFRα/β are also shown. For the complete list, see Supplementary Table S4. IC<sub>50</sub> values (nM) were determined for both INNO-406 (INNO) and imatinib (IMA) for those kinases, which were most likely to be inhibited by both drugs. Published values are referenced accordingly.

The mixed-lineage kinase ZAK was also identified as a specific target of INNO-406 as compared to imatinib. Indeed, we found that, using biotinylated myelin basic protein as a substrate, INNO-406 inhibited ZAK activity with an IC<sub>50</sub> of 73 nM (Figure 5a). Imatinib, on the other hand, did not significantly inhibit ZAK at concentrations as high as 10 μM, which is consistent with its absence in imatinib drug pull-downs.<sup>7,8</sup> ZAK is a MAP3K that activates p38 and JNK proapoptotic signaling pathways following ribotoxic stress (for example, exposure to anisomycin, Shiga toxin and ricin) or UV radiation.<sup>27</sup> However, in addition to being involved in hypertrophic growth of cardiomyoblast cells, an effect often associated with heart failure, evidence also suggests that ZAK may be involved in cancer development and therefore



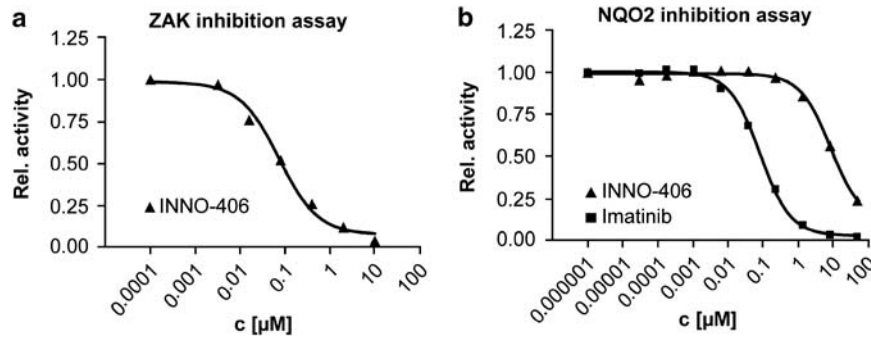
**Figure 3** DDR1, but not NAD(P)H/quinone oxidoreductase (NQO2), is a novel specific interactor of INNO-406. K562 lysate was either untreated (-) or pretreated (+) with 20 μM free INNO-406 before exposure to the drug matrix. Known INNO-406 targets, ABL and LYN, were also shown to be significantly competed by free INNO-406 pretreatment. Proteins, which bind to the affinity matrix, are enriched compared to the total cell lysate (TCL).



**Figure 4** Cellular inhibition of PDGFRα phosphorylation. INNO-406 inhibits PDGFRα phosphorylation in Chinese hamster ovary (CHO) cells that are transiently transfected with wild-type PDGFRα, D842V and V561D in a concentration-dependent manner, albeit with different apparent potencies.

might serve as an attractive new therapeutic target of INNO-406.<sup>28,29</sup>

An important difference of INNO-406 compared to the other BCR-ABL kinase inhibitors is its distinct selectivity profile with regard to the SRC and TEC family kinases (Table 2). As intended, INNO-406 potently inhibits LYN (IC<sub>50</sub> = 51 nM). In addition, it also inhibits LCK, which is essential for T-cell signaling. Thus, INNO-406-treated patients might have the potential to develop immune system-related side effects. FYN, HCK and the SRC-related kinase FRK are also inhibited, although to a lesser extent. Nonetheless, INNO-406 is neither a pan-SFK inhibitor nor does it affect the TEC family kinase BTK, which is important for B-cell function, as do dasatinib and bosutinib.<sup>7-9,11</sup> BMX is the only



**Figure 5** ZAK and NAD(P)H/quinone oxidoreductase (NQO2) inhibition assays for INNO-406 and imatinib. **(a)** An *in vitro* kinase assay with recombinant ZAK and biotinylated myelin basic protein as substrate illustrates potent concentration-dependent ZAK inhibition by INNO-406, with an IC<sub>50</sub> of 73 nM. Imatinib did not inhibit ZAK at 10 μM. **(b)** Measuring NQO2 activity with menadione as substrate using a continuous spectroscopic method monitoring MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) reduction<sup>14</sup> revealed a 100-fold reduction in inhibitory potential of INNO-406 (triangles) versus imatinib (squares) (IC<sub>50</sub> = 8.5 or 0.08 μM, respectively). IC<sub>50</sub>s are based on the nonlinear regression best-fit curve as determined using GraphPad Prism 4.00. Data are representative of two independent experiments.

TEC kinase targeted, and that only weakly. Thus, INNO-406 represents a finely tuned compromise between the rather specific drugs imatinib and nilotinib and the multikinase inhibitors dasatinib and bosutinib, and might therefore confer the by and large advantageous activity of a dual ABL/LYN inhibitor without most of the caveats of a spectrum-selective compound.

The NAD(P)H/quinone oxidoreductase (NQO2) is the first nonkinase target of imatinib and nilotinib.<sup>7,8</sup> Considering the strong structural similarity between imatinib, nilotinib and INNO-406 (Figure 1), a surprising finding of this study was that INNO-406 did not compete for binding of NQO2 to the INNO-406 affinity matrix nor did it inhibit NQO2 activity (Figures 3 and 5b; Supplementary Table S2). Therefore, NQO2 should not be considered a significant target of INNO-406. This could be due to the nonplanarity of the terminal dimethylaminopyrrolidine ring of INNO-406 as well as reduced flexibility of rotation of this ring due to the presence of the trifluoromethyl substituent on the adjacent ring, as has been similarly suggested for explaining the slight reduction in activity of nilotinib toward NQO2.<sup>14,30</sup>

## Conclusion

By applying a two-tiered approach, we describe here the global target profile of the kinase inhibitor INNO-406, which, together with the profiles of imatinib, nilotinib, dasatinib and bosutinib, provides a basis for patient-specific use of such kinase inhibitors as single agents or in combination therapy against CML. In addition to the cognate targets BCR-ABL and LYN, novel targets of INNO-406, such as ZAK, DDR1/2 and several ephrin receptor kinases, were identified. These may serve as intervention points for the use of INNO-406 in other diseases. We also confirmed the specificity of INNO-406 for the c-KIT V560G mutant over wild-type c-KIT, which might indicate efficacy of INNO-406 for also treating these cases of GIST. For the PDGFRα mutants D842V and V561D, however, INNO-406 did not seem to provide a significant advantage over imatinib. In contrast to dasatinib and bosutinib, which, among many other kinases, potently inhibit LCK and BTK and thereby possibly impair both T- and B-cell functions, respectively, INNO-406 inhibits LCK, but not BTK, and therefore might show milder immune-related side effects. Thus, given

the improved efficacy against imatinib-resistant CML cells through its potent inhibition of LYN in addition to wild-type BCR-ABL and most of its clinically relevant mutants, INNO-406 represents an attractive additional therapeutic for treating CML.

## Conflict of interest

The authors declare no conflict of interest.

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