Published in final edited form as:

ChemMedChem. 2018 June 20; 13(12): 1172–1180. doi:10.1002/cmdc.201700829.

Alkynynicotinamide-based compounds as ABL1 inhibitors with potent activities against drug-resistant CML harboring ABL1(T315I) mutant kinase.

Elizabeth A. Larocque^a, Dr. N Naganna^a, Clement Opoku-Temeng^{a,b}, Alyssa M. Lambrecht^a, and Prof. Dr. Herman O. Sintim^a

^aDepartment of Chemistry, Purdue University, West Lafayette, IN, 47906, USA, hsintim@purdue.edu

^bGraduate Program in Biochemistry, University of Maryland, College Park, MD, 20742, USA

Abstract

The introduction of imatinib into the clinical scene revolutionized cancer treatment of chronic myeloid leukemia (CML). The overall 8-year survival rate for CML has increased from about 6% in the 1970s to over 90% in the imatinib era. However, about 20% of CML patients harbor primary or acquired resistance to tyrosine kinase inhibitors. ABL1 point mutations in the BCR-ABL1 fusion protein, such as ABL1(T315I), typically emerge after prolonged kinase inhibitor treatment. Ponatinib (AP24534) is currently the only approved CML drug that is active against the ABL1(T315I) mutation. However, ponatinib has severe cardiovascular toxicities hence there have been efforts to find safer CML drugs that work against ABL1 secondary mutations. We reveal that isoquinoline- or naphthyridine-based compounds, such as HSN431, HSN576, HSN459 and HSN608 potently inhibit the enzymatic activities of ABL1, ABL1(T315I), and ABL1(E255K). These compounds inhibit the proliferation of ABL1-driven CML cell lines, K652 and KCL22 as well as the drug-resistant cell line, KCL22-IR, which harbors the secondary mutated ABL1(T315I) kinase.

Graphical abstract

Conflicts of Interest

HOS is a co-founder of KinaRx LLC, a start-up company interested in developing therapies for malignant neoplastic diseases.

Ponatinib gets serious competitors: Alkynyl aminoisoquinolines and alkynylaminonaphthyridines potently inhibit ABL1(T315I). Consequently, these compounds inhibit imatinib-resistant CML cell line, KCL22-IR

Keywords

Chronic Myeloid Leukemia; BCR-ABL; anticancer agent; kinase; drug resistance

Introduction

When a pluripotent hematopoietic stem cell acquires the Philadelphia (Ph) chromosome, which encodes BCR fused to the ABL1 kinase protein (designated as BCR-ABL1 fusion oncoprotein), the cells gain proliferative advantage over normal stem cells and the uncontrolled expansion of the leukemic stem cells lead to chronic myeloid leukemia (CML).^[1] The overactive BCR-ABL1 protein promotes the expansion of granulocytes, which are most commonly seen in CML.^[2] Approved in 2001, imatinib has had tremendous success in increasing the life expectancy of CML patients. Before 1975, the 8-year survival rate for newly diagnosed CML patient was about 6% but this has steadily increased to over 90% in the imatinib/tyrosine kinase era.^[3] Imatinib binds to the inactive conformation of the BCR-ABL protein and it has a good selectivity profile against human kinases, hence it is relatively non-toxic.^[4] Various kinases adopt similar active conformations but have very different inactive conformations. Therefore, inhibitors that bind to the inactive conformer of kinases, but not the active conformer, can achieve selective kinase binding.^[5] Other FDA-approved kinase inhibitors that target BCR-ABL include nilotinib (Tasigna, Novartis), dasatinib (Sprycel, Bristol-Myers Squibb) and bosutinib (Bosulif, Pfizer), (Figure 1).^[6-9] Unfortunately, ABL1(T315I) and other kinase domain mutations, which are resistant to imatinib, emerge upon prolonged drug treatment.^[10] In the mutant BCR-ABL1(T315I) kinase, a polar uncharged threonine side chain becomes a hydrophobic and more sterically

hindering isoleucine. The T315I mutation is the most problematic due to its altering of the three-dimensional structure of the BCR-ABL1 kinase into the “DFG-in” A-loop conformation, causing a decrease in the sensitivity of most kinase inhibitor drugs that bind the inactive conformer of BCR-ABL1, including imatinib. [11,12]

Ponatinib (AP24534), a pan kinase inhibitor, is currently the only approved CML drug that is able to potently inhibit the BCR-ABL1(T315I) mutant kinase. [13] Over the course of ponatinib's clinical trials 17.1 % of patients taking ponatinib reported both serious and non-serious thrombotic events (this is defined as cardiovascular, cerebrovascular, and peripheral vascular events). [14] Five deaths in the study (n=499) were linked to the use of ponatinib. [14] The accumulation of these adverse effects caused the trial to be put on hold and then canceled. [14] Due to these risks, ponatinib is now used as a last resort drug. [15] Because of the adverse events associated with ponatinib, there is a motivation to develop other potent BCR-ABL1 (T315I) inhibitors. Unfortunately, most preclinical studies use mouse models that may not adequately predict clinical success in humans so the search for safer BCR-ABL1(T315I) inhibitors is a non-trivial task. [16,17]

Our group has been interested in developing alkynyl aminoisoquinolines and alkynylaminonaphthyridine as FLT3 inhibitors for the treatment of acute myeloid leukemia. [18,19] Some of our FLT3 inhibitors, such as HSN286 and compound **1** (see Figure 2) contain aryl alkynyl benzamide moiety, which is also found in ponatinib. Because ponatinib also inhibits ABL1, in addition to FLT3, we wondered if our compounds were also ABL1 inhibitors. We however found out that the mere presence of a piperazine-containing alkynylbenzamide in a molecule did not make such compound an ABL1 inhibitor. For example, ponatinib shares the same piperazine-containing alkynylbenzamide moiety with HSN285, yet 500 nM ponatinib inhibited 99% of the enzymatic activity of ABL1 whereas at the same concentration HSN285 only inhibited ABL1 at 8%. Also compounds **1**, **6** and **8** both contain piperidine-containing amide, yet at 500 nM concentration compound **1** inhibited ABL1 at 90%, compound **8** inhibited ABL1 at 96% whereas compound **6** inhibited ABL1 at only 69%. Based on the excellent ABL1 inhibition profile of compounds **1** and **8**, which contain an aminoisoquinoline or aminonaphthyridine moieties and ponatinib, which contains a piperazine-containing alkynylbenzamide moiety, we focused on making more analogs of **1** and **8** or analogs of HSN356 (which combine the aminonaphthyridine moiety found in **8** and methyl piperazine found in ponatinib). Herein we report alkynyl aminoisoquinolines and aminonaphthyridines analogs that are active against the BCR-ABL1 enzyme, as well as ABL1 with T315I and E255K point mutations. These compounds also inhibit the proliferation of CML cells lines K562, KCL22, and KCL22-IR; an imatinib-resistant cell line. [20–22]

Results and Discussion

Structural analysis of alkynyl aminoisoquinolines and alkynylaminonaphthyridine

We have previously reported that the alkynyl aminoisoquinolines and aminonaphthyridines analogs are privileged kinase inhibitors with potent activities against FLT3-driven acute myeloid leukemia. [18,19] Our success in developing alkynyl aminoisoquinolines and aminonaphthyridines as FLT3 inhibitors prompted us to evaluate our compounds against

other kinases and to endow the compounds with moieties that are commonly found in drugs (Figure 3).

To optimize our library, we wanted to change several aspects of the first-generation compounds, compound **1** and compound **9** (HSN356), to make compounds that bind better to ABL1 and also to improve upon the drug likeness (see Figure 3). Docking of compound **9** (HSN356) and ponatinib to ABL1 protein, using GOLD software^[23], indicated that both compounds bound in the same mode (see Figure 4). Importantly the docking of ponatinib to ABL1 recapitulated the bound pose in the crystallized structure, thereby giving confidence to the docking pose. The imidazo[1,2b] pyridazine moiety found in ponatinib interacts with the hinge region at the back of the binding pocket of ABL1 (Figure 4A). Similarly, according to the docking results, the aminonaphthyridine core found in compound **9** (HSN356) also binds to this hinge region (Figure 4B). The 4-methyl benzamide ring in our first-generation compounds and also in ponatinib could pose a possible metabolic liability, whereby the benzylic hydrogens are oxidized. Ponatinib was found to be hydroxylated by liver enzymes, and some of this hydroxylation could occur at the 4-methyl position on the benzamide ring.^[24] We therefore explored the possibility of removing this potential metabolic liability with a halogen or bulkier alkyl group, such as ethyl (compounds **9–16**, Figure 4). In addition, to improve upon the aqueous solubility of the compounds we added nitrogen to the benzamide ring.

The linker (either the CF₃-bearing aromatic or the aliphatic chain) can interact with hydrophobic residues in the active site of ABL1 (Figure 5). The CF₃ group however adds 69 g/mol to the molecular weight of the compounds. We therefore investigated if other groups, such as -CH₃ (compound **20**), -Cl (compound **21**), or -F (compound **22**), see Figure 6, could replace the -CF₃ group without a great penalty to binding. Due to the lack of activity of the compounds with an aliphatic chain to mutated ABL1(T315I), these analogs were no longer pursued (*vide infra*). The N-methylpiperazine group in ponatinib and our compounds mainly acts as an aqueous solubilizing moiety but adds significant mass unit to the molecule. We explored if this group could be replaced by lower mass unit groups, such as morpholine (compound **17**), pyrrolidine (compound **18**), and a dimethylamine (compound **19**). These units also contain basic nitrogen and could also improve upon the aqueous solubility of the compounds.

Compounds were readily synthesized via an established Sonogashira coupling protocol.^[19] With a vast library of compounds that contain various aromatic and aliphatic side chains, initial screening was done at compound concentration of 500 nM against ABL1, ABL1 (T315I), and ABL1 (E255K).

Aminoisoquinolines and Aminonaphthyridines potently inhibit ABL1 Wild type, ABL1 (T315I), and ABL1(E255K)

Analogues that contained an aliphatic side chain (**1–8**) showed similar inhibition against ABL1 compared to their aromatic side chain counterparts. However, these compounds were poor inhibitors of ABL1(T315I) and ABL1(E255K) (Figure 7A). Compounds containing 1-amino or 3-amino isoquinoline or aminonaphthyridine rings were generally good inhibitors of ABL1

(Figure 7A). Compounds which contained the 4-((4-methylpiperazin-1-yl) methyl)-3-trifluoromethylphenylamine moiety displayed potent enzymatic inhibition against ABL1 kinase (at 500 nM, these compounds inhibited over 90% of the enzymatic activity of the ABL1 enzyme). The 4-((4-Methylpiperazin-1-yl) methyl)-3-trifluoromethylphenylamine-containing compounds were also potent inhibitors of ABL1(T315I) and ABL1(E255K) mutant kinases (Figure 7).

Substituting the 4-position of the benzamide ring with different groups, H, F, Cl, Me, Et and CN, did not affect ABL1 inhibition. However, the ethyl (compound **13**) and cyano (compound **14**) analogs were poor inhibitors of ABL1(T315I) while inhibition of ABL1(E255K) was maintained. This decrease in ABL1(T315I) inhibition could be due to steric clash between the larger R group and the isoleucine at position 315 of the mutant enzyme. Substitution of the piperazine moiety with a morpholine (compound **17**) or pyrrolidine (compound **18**) reduced the ABL1 inhibition. However, when substituted with a dimethylamine (**19**), the ABL1 inhibition remained the same (Figure 7B). Compounds **17–19** were poor inhibitors of the mutant ABL1 forms, ABL1(T315I) and ABL1 (E255K). Compounds **19–22** were designed to investigate the importance of the trifluoromethyl group. All of the compounds in this series, **19–22**, significantly inhibited wild type ABL1. However, with regard to ABL1(E255K), compounds **19** (trifluoro methyl) and **21**(chloro), but not **20** or **22**, were able to moderately inhibit this mutant version of ABL1. All four compounds **19–22**, were poor inhibitors of ABL1 (T315I). Interestingly, the only difference between compound **19** and **15** is that **19** contains a dimethyl amine, which we originally thought functioned as a mere aqueous solubilizing group whereas **15** contains a dimethyl piperazine group, which we also thought functioned as a mere solubilizing group. It appears that these groups, which were put into the molecules to increase aqueous solubility also affect binding to mutant ABL1 kinase, providing a cautionary tale that moieties added to potential drugs could have unintended functions.

IC₅₀ determination of select analogs against ABL1 and ABL1(T315I)

Compound **16** (HSN608), **15** (HSN459), **28** (HSN431), and **29** (HSN576) were chosen as representative isoquinoline or naphthyridine compounds for further characterization. These compounds were chosen due to their good activities against the wild type and mutant ABL1 enzymes. Secondly, these compounds contain the water-soluble nicotinamide moiety (and not the benzamide moiety that is found in ponatinib), which made them more appealing for possible translation. All four compounds and ponatinib inhibit ABL1 wild type with IC₅₀ values that are less than 5 nM (Figure 8A). For mutant ABL1(T315I) however, only ponatinib and compound **15** (HSN459) potently inhibited with IC₅₀ less than 10 nM (1.6 nM and 4.7 nM respectively). Compound **29** (HSN576) and Compound **16** (HSN608) inhibited ABL1(T315I) with IC₅₀ values of 32.8 nM and 40.7 nM respectively. Compound **28** (HSN431) was the least sensitive of the four analogs towards ABL1(T315I) with IC₅₀ value of 507 nM (Figure 8B).

Aminoisoquinolines and Aminonaphthyridines potently inhibit the proliferation of ABL1-driven CML cell lines

Having established that the compounds were good ABL1 inhibitors, we proceeded to test the activities of our library against three CML cell lines, K562, KCL22 and KCL22-IR. K562 is a CML line which is positive for the Bcr-Abl1 fusion gene. KCL22 is an imatinib-sensitive CML cell line and KCL22-IR is an imatinib-resistant cell line, which contains the T315I mutation. [22]

In general, there is a positive correlation between the percent inhibition of ABL1 enzymatic activity and BCR-ABL-driven CML cell line proliferation inhibition. The 4-((4-Methylpiperazin-1-yl) methyl)-3-trifluoromethylphenylamine-containing compounds are all potent inhibitors of CML cell lines with IC₅₀ values in the sub-nanomolar or single digit nanomolar range. Excitingly, many compounds (**9–13**, **15**, **16**, **19**, **23**, **25**, **27–29**) maintained good potency (IC₅₀ less than 10 nM) against the KCL22-IR cell line, which contains the T315I mutation (Table 1). A few compounds did not display potent inhibition against ABL1(T315I) *in vitro* yet inhibited the proliferation of KCL22-IR cell line with reasonably low IC₅₀ values. For example, *in vitro*, the IC₅₀ of compound **28** against ABL1(T315I) enzyme is 507 nM (Figure 8B) and thus did not appear to be a potent inhibitor of ABL1(T315I). However, Compound **28** inhibited the fusion kinase BCR-ABL(T315I) in cells with an IC₅₀ of 54.7 nM, (*vide infra*), which is an order of magnitude less than the IC₅₀ against ABL1(T315I) kinase domain *in vitro*. Caution must be taken in directly assuming that IC₅₀ values obtained *in vitro* will translate directly into cellular assays. The *in vitro* IC₅₀ value will depend on the enzyme and ATP concentrations used in the *in vitro* assay, which may be different in the cellular context. IC₅₀ values should therefore really be used in ranking order. Also, the ABL1 kinase, which is used in the *in vitro* experiment, does not contain the fusion BCR partner whereas ABL1 is fused to BCR to form BCR-ABL1 fusion protein in CML cells. Therefore, conformational differences between the isolated ABL1 kinase domain and the BCR-ABL fusion protein that is found in the cell could give rise to differences in compound sensitivities. In fact, others have also noted differences in sensitivities between ABL1 and the fusion protein against other inhibitors. [25–27] There are also BCR-ABL1-independent mechanisms that makes KCL22-IR and other imatinib-resistant CML cell lines resistant to imatinib and it is possible that some of our compounds are also affecting these pathways. Unfortunately, the BCR-ABL1-independent mechanisms of resistance in CML have been poorly characterized. [28–31] Finally the activity of a compound against a cell line does not depend only on the isolated activity against an enzyme target but also depends on cellular permeation, cellular compartment localization, stability of the compound in cells and promiscuity of compound towards binding to other cellular proteins (both kinase and non-kinase targets). A compound that potently inhibits ABL1 but also binds to other cellular macromolecules and/or localizes into compartments that BCR-ABL1 does not reside will not display the same potency against the target protein as was seen in an *in vitro* assay. Compounds **16** (HSN608) and **15** (HSN459) are of interest because in addition to potent activity against drug-resistant CML cell line, KCL22-IR, they have lower LogP values (2.3 and 2.7 respectively) than the rest of the active compounds and therefore are more drug-like in comparison.

Compound series **19-22** allowed for investigation into the role of the CF₃ group in the inhibition of ABL1 and CML cell proliferation. Compound **19** (contains CF₃ group) inhibited imatinib-sensitive K562 and KCL22 with IC₅₀ values of 0.388 nM and 0.459 nM respectively. Compound **19** also inhibited KCL22-IR with a respectable IC₅₀ of 3.8 nM. Replacing the -CF₃ group with Cl, Me or F reduced the sensitivity of both K562 and KCL22 by two orders of magnitude. For the ABL1(T315I) harboring cell line, compounds **20-22** were inactive against this cell line (IC₅₀ > 1000 nM). It therefore appears that the trifluoromethyl group is critical for the potency of these new alkynyl isoquinoline or naphthyridine compounds.

Compound **25** has an impressive potency against all three CML cell lines tested (sub-nanomolar IC₅₀ values for all cell lines). The logP value for compound **25** is 5 and therefore is not as ideal as other tested compounds.

Changing the 6-methylnicotinamide moiety found in compound **15** (HSN459) or **24** (HSN461) into 1-methyl-1H-pyrazole-3-carboxamide (compounds **23** and **24**) reduced affinity for the tested ABL1 enzymes. In line with the reduced inhibition of ABL1 wild type and mutant forms, both compounds **31** and **32** were ~ 50 to 100 times less potent than the 6-methylnicotinamide analogs at inhibiting the ABL1-driven CML cell lines.

When the N-(3-(trifluoromethyl)phenyl) group was replaced with aliphatic side chains (compounds **1-8**), there was a dramatic decrease in activity against the drug-resistant CML cell line KCL22-IR (IC₅₀ > 1000 nM). The low activity of the aforementioned compounds against KCL22-IR is not surprising because these compounds are poor inhibitors of ABL1(T315I) enzyme (Figure 7A).

Compound 15 (HSN459), Compound 16 (HSN608), Compound 28 (HSN431) and Compound 29 (HSN576) inhibit ABL1 and ABL1(T315I) activities in KCL22 and KCL22-IR cell lines respectively.

We proceeded to investigate the effects of approved drugs, imatinib and ponatinib, and four of the compounds in this study (HSN431, HSN459, HSN576 and HSN608) on ABL1 activity in imatinib-sensitive (KCL22-WT) and imatinib-resistant (KCL22-T315I) cell lines. For this investigation, KinaSense (West Lafayette, IN, USA) performed the cellular ABL1 kinase phosphorylation assay, using their novel phosphorylation detection platform. All tested compounds were able to inhibit the phosphorylation of ABL1 in KCL22-WT but imatinib was the least potent, followed by ponatinib (see Figure 9 and Table 2). For the drug-resistant CML cell line, KCL22-IR (harboring ABL1(T315I)), imatinib was ineffective whereas ponatinib inhibited ABL1 phosphorylation inside KCL22-IR cells with IC₅₀ of 50 nM. Impressively three of our compounds, HSN459, HSN576 and HSN608 inhibited KCL22-IR better than ponatinib (Table 2; compare IC₅₀ of HSN459 (19 nM) with ponatinib (50 nM)). These compounds are therefore good candidates to evaluate further for possible clinical translation against drug-resistant CML.

Conclusions

Here we have reported a new library of compounds that are potent against the ABL1, ABL1 (T315I) and ABL1 (E255K) enzymes. These compounds also inhibit the proliferation of CML cell lines K562, KCL22, and KCL22-IR with IC₅₀ values that are competitive with the FDA-approved ponatinib, the only inhibitor currently approved for CML harboring ABL1(T315I) mutation. Since ponatinib has some toxicity liability and hence not appropriate for patients who have a history of cardiovascular disease, new compounds that are as potent as ponatinib but with less toxic profile are welcomed. Unfortunately, due to the nature of drug development, whereby preclinical studies are done on animal models, which may not be accurate models for humans, many of these side effects cannot be predicted until a drug reaches clinical trials.^[16,17] Therefore, one has to be cognizant of the fact that until such new inhibitors pass the muster of clinical testing, one would never be certain if they are indeed less toxic than ponatinib but the importance of finding a less toxic compound than ponatinib should provide the impetus to persevere in the endeavor of finding new BCR-ABL1 (T315I) inhibitors. Future work, beyond the scope of this report, will investigate the safety profiles and *in vivo* efficacies of the reported compounds to ascertain their translational potential. These future studies will be reported in due course.

Experimental Section

Molecular Docking with GOLD program.

For docking, PDB entry 3OXZ was obtained from RCSB Protein Data Bank (www.rcsb.org). Ponatinib and HSN356 were drawn using ChemDraw Professional software version 16.0 and Chem3D software version 16.0 (PerkinElmer Informatics) to minimize the energy; then saved as a mol2 file. The Hermes visualizer was used for protein preparation and docking. The wizard option added hydrogens, deleted waters and crystalized the ligand. Ponatinib and HSN356 were then docked to the binding site using the default 10 genetic algorithm and the ChemPLP scoring function. PyMOL visualization software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) was then utilized to form images.

General Aromatic Amide Coupling Procedure:

A solution of benzoic acid (1 equiv) and HATU (1.1 equiv.) in DMF (5 mL) was cooled to 0 °C. DIPEA (3 equiv) was then added followed by the amine compound (1 equiv). The temperature was increased to 50 °C and allowed to stir 12 h. The reaction was diluted with EtOAc (50 mL) and washed with water (3 × 20 mL). and brine solution (20 mL). Combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated in vacuo. The pure product was then obtained via flash column chromatography.

General Aliphatic Amide Coupling Procedure:

A solution of benzoic acid (1 equiv) and HCTU (1.1 equiv.) in Dry DCM (5 mL) was cooled to 0 °C. DIPEA (3 equiv) was then added followed by the amine compound (1 equiv). The temperature was increased to room temperature and allowed to stir 12 h. The reaction was diluted with DCM (50 mL) and washed with water (3 × 20 mL). and brine solution (20 mL). Combined organic layers were dried over anhydrous sodium sulphate, filtered and

concentrated in vacuo. The pure product was then obtained via flash column chromatography.

General Sonogashira Coupling Procedure:

A solution of aromatic-halogen compound (1 equiv.), Pd(PPh₃)₂ Cl₂ (10 mol%), CuI (5 mol %) and triphenylphosphine (5 mg) in triethylamine (30 equiv.) was de-oxygenated using Argon gas. A de-oxygenated solution of alkyne (1.2 equiv.) in DMF (3 mL) was added slowly over a period of 10 minutes to the triethylamine solution. After the addition, the reaction temperature was increased to 55 °C and allowed to stir for 12 h. The reaction was quenched by addition of NH₄Cl (5 mL) at room temperature. The crude compound was extracted using EtOAc (3 × 30 mL). Combined organic layers were dried over anhydrous sodium sulphate, filtered and concentrated in vacuo. The final compound was purified via flash column chromatography.

Cell Culture:

KCL22-WT and KCL22-T315I cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium. K562 was routinely cultured in Iscove's Modified Dulbecco's Medium (IMDM). Media were supplemented with 10% fetal bovine serum and 1X penicillin-streptomycin solution.

Cellular proliferation assay:

Cells were seeded into 96 tissue culture-treated plates for up to 24 h. After seeding, DMSO stock solutions of compounds were serially diluted in a 1:3 ratio, first in DMSO. Then, subsequently into appropriate cell culture medium before being added to the cultures, with 10 µM being the highest tested concentration. The plates were incubated for 72 h as above. The CellTitre-Blue assay (Promega, Madison WI) was then added to the cultures and incubated an additional 4 hrs before the fluorescence measured following the manufacturers recommendations. The fluorescence data from compounds were normalized to that DMSO and the resulting data fitted to a non-linear regression equation to obtain IC₅₀ using GraphPad Prism 5.0 Software. Data was done with either duplicate or triplicate.

Evaluation of compounds activity against ABL1 and ABL1(T315I) inside CML cell lines KCL22 and KCL22-IR (by KinaSense, West Lafayette, IN, USA).

To evaluate cellular ABL activity, KCL22-WT or KCL22-T315I cells were seeded at 600,000 cells per well and final volume of 750 µL of media into 1 mL/well AcroPrep Advance 96 Filter Plates from Pall Corporation. Cells were dosed with the indicated kinase inhibitor or vehicle control (0.001% (v/v) DMSO) and incubated for 2 hours at 37°C in a 5% CO₂ humidified atmosphere. Following incubation with TKI, ABL substrate was added and incubated for 10 minutes at 37°C in a 5% CO₂ humidified atmosphere. Following incubation with substrate, cell media was removed from the filter plate using a vacuum manifold and the filter plate was stacked on top of a Neutravidin™ coated 96-well capture plate (Thermo Fisher Scientific) containing 100 µL blocking buffer per well (TBST + 1% BSA). In the upper filter plate, 50 µL of modified RIPA buffer containing protease inhibitor cocktail (Roche), phosphatase inhibitors, and 4 mM EDTA was added to each well. The plate stack

was incubated on a short orbital plate shaker at room temperature for 5 minutes. Following lysis buffer incubation, the plate stack was centrifuged at 1500 ref for 5 minutes to collect lysate in the Neutravidin™ capture plate. After lysate collection, the filter plate was removed, and the capture plate was incubated with shaking for 1 hour at room temperature. The plate was then washed with TBST. 100 µL of mouse 4G10 antibody (Millipore, 1: 5,000 in wash buffer) was added to each well and the plate incubated for 1 hour at room temperature with shaking as described above. Following incubation with 4G10 antibody, the plate was washed with TBST and 100 µL of anti-mouse IgG conjugated to HRP (1: 10,000 in wash buffer) was added to each well and incubated for 1 hour at room temperature with shaking. The plate was washed three times with TBST followed by two washes with phosphate buffer (50 mM Na₂PO₄, pH 7.5). 100 µL of developing reagent (50 mM Na₂HPO₄, 2.3 mM H₂O₂, 98 µM Amplex Red™ reagent, pH 7.4) was added to each well and incubated for 30 minutes with shaking as described above. The plate was read using a Biotek Synergy H1 plate reader using 532 nm excitation and 590 nm emission wavelengths.

Each concentration was normalized to vehicle control and were plotted in GraphPad Prism software. IC₅₀ values were calculated by fitting an unconstrained log(inhibitor) vs. response with variable slope (four parameters) curve.

Each cell line was treated using 10 concentrations of control (imatinib and ponatinib) and experimental compounds, ranging from 0.001 – 10000 nM in 4 technical replicates. As expected, Bcr- ABL activity in the KCL22-WT cell line responded to all inhibitors. The IC₅₀ values derived for ponatinib (IC₅₀ - 19.3 nM) and imatinib (IC₅₀ – 574 nM) are in line with known potencies for both *in vitro* biochemical activity assays as well as growth inhibition phenotypic assays. Biochemical IC₅₀ values of ponatinib for the wild type ABL protein was reported as 0.37 nM and growth inhibition assay at 6 nM in the K-562 CML model after a 42-hour exposure. Biochemical IC₅₀ values of imatinib for the wild type ABL protein was reported as 600 nM and growth inhibition assay at 1 µM in K-562 cells following 72-hour imatinib exposure. Agreement between the ABL activity assay results and reported IC₅₀ values indicate reliable assay performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

NMR and MS data were acquired by facilities supported by NIH P30 CA023168. We thank Prof. Robert Kirken (University of Texas at El Paso) for generously giving us KCL22 and KCL22-IR cell lines.

References:

- [1]. Princel S, Maurizio F, Ferrone M, Tamborini E, Mol. Cancer. Ther 2005, 4, 1167. [PubMed: 16093432]
- [2]. Zhou H, Xu R, Protein Cell. 2015, 6, 403. [PubMed: 25749979]
- [3]. Woessner DW, Lim CS, Deininger MD. Cancer J 2011, 17, 477. [PubMed: 22157291]
- [4]. Lyndon N, Nat. Med 2009, 15, 1153. [PubMed: 19812577]
- [5]. Miller G, Bruno B, Lim C, Biologics. 2014, 8, 243. [PubMed: 25349473]

- [6]. Drunker BJ, Guilhot F, O'Brian SG, Gathmann I, Kantarjian H, Gattermann N, Dieninger MW, Silver TR, Goldman JM, Stone RM, Cervantes F, Hochhaus A, Powell BL, Gabrilove JL, Rousselot P, Nielson JL, Radich JP, Simonsson B, Taylor K, Baccarani M, Si C, Letvak L, Larson RA. *N. Engl. J. Med* 2006, 355, 2408. [PubMed: 17151364]
- [7]. Saglio G, Kim DW, Issaragrisil S, le Coutre P, Etienne G, Labo C, Pasquini R, Clark RE, Hochhuas A, Hughes TP, Gallagher N, Hoenekopp A, Dong M, Haque A, Larson RA, Kantarjian HM. *N. Engl. J. Med* 2010, 362, 2251. [PubMed: 20525993]
- [8]. Kantarjian H, Shah NP, Hochhaus A, Cortes J, Shah S, Ayala M, Moiraghi B, Shen Z, Mayer J, Pasquini R, Nakamae H, Huguet F, Boque C, Chuah C, Bleickardt E, Bradley-Garelik MB, Zhu C, Sztatowski T, Shapiro D, Baccarani M. *N. Engl. J. Med* 2010, 362, 2260. [PubMed: 20525995]
- [9]. Brummendorf TH, Cortes JE, de Souza CA, Guilhot F, Duvillie L, Pavlov D, Gogat K, Countouriotis AM, Gambacorti-Passerini C. *Br. J. Haematol* 2015, 367, 2075.
- [10]. Gainor JF, Shaw AT. *J. Clin. Oncol* 2013, 31, 3987. [PubMed: 24101047]
- [11]. Pemovska T, Johnson E, Kontro M, Repasky GA, Chen J, Wells P, Cronin CN, McTigue M, Kallioniemi O, Porkka K, Murray BW, Wennerberg K. *Nature*. 2015, 519, 102. [PubMed: 25686603]
- [12]. Ai J, Tiu R. *Ther. Adv. Hematol* 2014, 5, 107. [PubMed: 25360237]
- [13]. Gibbons DL, Princel S, Kantarjian H, Cortes J, Quintas-Cardama A. *Cancer*. 2012, 118, 293. [PubMed: 21732333]
- [14]. Cortes JE, Kim D-W, Pinilla-Ibarz J, le Coutre P, Paquette R, Chuah C, Nicolini FE, Apperley JF, Khoury HJ, Talpaz M, DiPersio J, DeAngelo DJ, Abruzzese E, Rea D, Baccarani M, Müller MC, Gambacorti-Passerini C, Wong S, Lustgarten S, Rivera VM, Clackson T, Turner CD, Haluska FG, Guilhot F, Deininger MW, Hochhaus A, Hughes T, Goldman JM, Shah NP, and Kantarjian H. *N. Engl. J. Med* 2013, 369, 1783. [PubMed: 24180494]
- [15]. Gainor JF, Chabner BA. *Oncologist*. 2015, 20, 847. [PubMed: 26173838]
- [16]. Giacomini KM, Krauss RM, Roden DM, Eichelbaum M, Hayden MR, Nakaura Y. *Nature*, 2007, 446, 975. [PubMed: 17460642]
- [17]. Arun B. *J. Bioanal. Biomed* 2009, 1, 50.
- [18]. Larocque E, Naganna N, Ma X, Opoku-Temeng C, Carter-Cooper B, Lapidus RG, Sintim HO. *Future Med. Chem* 2017, 9, 1213. [PubMed: 28490193]
- [19]. Ma X, Zhou J, Wang C, Carter-Cooper B, Yang F, Larocque E, Fine J, Tsuji G, Chopra G, Lapidus RG, Sintim HO. *ACS Med. Chem. Lett* 2017, 8, 492. [PubMed: 28523099]
- [20]. Colavita I, Esposito N, Martinelli R, Catanzano F, Melo JV, Pane F, Ruoppolo M, Salvatore F. *Biochim. Biophys. Acta*. 2010, 1804, 1974. [PubMed: 20417730]
- [21]. Yuan H, Wang Z, Gao C, Chen W, Huang Q, Yee JK, Bhatia R, Chen WY. *J. Biol. Chem* 2010, 285, 5085. [PubMed: 20007699]
- [22]. Oaxaca DM, Yang-Reid SA, Ross JA, Rodriguez G, Staniswalis JG, Kirken RA. *Tumour Biol* 2016, 37, 12643. [PubMed: 27444277]
- [23]. Jones G, Willett P, Glen RC, Leach AR, Taylor R. *J. Mol. Biol.* 1997, 3, 727.
- [24]. Ye YE, Woodward CN, Narasimhan NI. *Cancer Chemother. Pharmacol*. 2017, 79, 507. [PubMed: 28184964]
- [25]. De Keersmaecker K, Versele M, Cools J, Superti-Furga G, Hantschel O. *Leukemia*, 2008, 22, 2208. [PubMed: 18784740]
- [26]. Hantschel O, Superti-Furga G. *Nat. Rev. Mol. Cell Biol* 2004, 5, 33. [PubMed: 14708008]
- [27]. Rausch JL, Boichuk S, Ali AA, Patil SS, Lui L, Lee DM, Brown MF, Makielski KR, Liu Y, Taguchi T, Kuan SF, Duensing A. *Oncotarget*, 2016, 8, 4471.
- [28]. Mahon FX, Belloc F, Lagarde V, Chollet C, Moreau-Gaudry F, Reiffers J, Goldman JM, Melo JV. *Blood*, 2003, 101, 2368. [PubMed: 12609962]
- [29]. Lee F, Fandi A, Voi M. *Int. J. Biochem. Cell. Biol* 2008, 40, 334. [PubMed: 18401881]
- [30]. Quintas-Cardama A, Kantarjian HM, Cortes JE. *Cancer Control*. 2009, 16, 122. [PubMed: 19337198]

- [31]. Colavita I, Esposito N, Quintarelli C, Nirgo E, Pane F, Ruoppolo M, Salvatore F, Proteomics, 2013, 13, 2414 [PubMed: 23754495]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.
Current first and second generation CML drugs

Figure 2.
Initial alkynyl compounds, which inspired the synthesis of new ABL1 inhibitors, see Figure 6.

Figure 3. Optimization strategy to improve drug-like properties and potency of first-generation compounds HSN356 and HSN286

Figure 4. Ponatinib and Compound **9** bound to ABL kinase domain. A) Docked ponatinib bound to the ABL kinase domain. (B) Docked Compound **9** bound to ABL kinase domain. All compounds were docked against PDB entry 3OXZ using GOLD software^[23]. Hinge region shown as green and key T315 residue is colored red

Figure 5.
Abl residues within 6 Å of the bound ponatinib (blue). Key hydrophobic residues have been labeled. T315 is colored red while residues that are part of the hinge region are green

Figure 6.
Alkynyl aminoisoquinolines and aminonaphthyridines tested in study

Figure 7.
Enzymatic inhibition against ABL1 wild type and common mutations T315I and E255K.
Screening of compounds was done as a service at Reaction Biology Corporation.

Figure 8. Compounds **16** (HSN608), **15** (HSN459), **28** (HSN431), and **29** (HSN576) inhibit ABL1 (A) and ABL1(T315I) (B) with nanomolar IC₅₀ values.

Figure 9.

Dose-response curves for inhibition of ABL1 and ABL1(T315I) in KCL22 and KCL22-IR respectively by imatinib, ponatinib, HSN431, HSN459, HSN576 and HSN608. IC₅₀'s values were determined by the company KinaSense (West Lafayette, IN, USA).

Table 1.

Calculated LogP and anti-proliferation activities of alkynyl analogs

Compound	LogP ^[a]	IC ₅₀ (mean ±SD, nm)		
		K562	KCL22	KCL22-IR
1 (HSN248)	4.4	7.01 ± 0.07	6.45 ± 0.13	> 1 µM
2	4.2	25.7 ± 1.7	35.5 ± 1.69	> 1 µM
3	5.3	30.05 ± 1.9	ND	ND
4	4.3	10.1 ± 0.8	111 ± 1.35	> 1 µM
5	4.4	161 ± 10.8	ND	ND
6	3	43.5 ± 1.4	ND	ND
7	4.9	94.5 ± 0.8	ND	ND
8 (HSL45)	3.9	25.3 ± 0.5	17.16 ± 0.3	> 1 µM
9 (HSN356)	3.9	1.85 ± 0.03	0.18 ± 0.004	0.74 ± 0.004
10	4.2	0.587 ± 0.004	0.606 ± 0.606	3.27 ± 0.09
11	4.5	0.856 ± 0.006	0.699 ± 0.003	2.12 ± 0.09
12	4.7	0.292 ± 0.005	1.05 ± 0.02	1.52 ± 0.08
13	4.8	1.18 ± 0.05	1.85 ± 0.04	5.49 ± 0.14
14	3.9	0.833 ± 0.005	1.48 ± 0.05	16.3 ± 0.7
15 (HSN459)	2.7	1.75 ± 0.04	0.303 ± 0.002	2.67 ± 0.07
16 (HSN608)	2.3	0.765 ± 0.005	1.02 ± 0.04	4.14 ± 0.11
17	2.6	20.4 ± 0.3	5.71 ± 0.04	198 ± 11.8
18	3.2	6.26 ± 0.02	1.6 ± 0.4	ND
19 (HSN534)	2.8	0.388 ± 0.001	0.459 ± 0.016	3.8 ± 0.1
20	2.4	56.9 ± 0.6	16.6 ± 0.4	> 1 µM
21	2.6	11.4 ± 0.1	3.79 ± 0.07	> 1 µM
22	2.1	75.5 ± 0.4	29.09 ± 0.82	> 1 µM
23	4.9	0.234 ± 0.004	2.19 ± 0.11	3.2 ± 0.2
24	3.6	1.44 ± 0.06	1.45 ± 0.02	13.3 ± 0.5
25	5	0.216 ± 0.002	0.426 ± 0.001	0.25 ± 0.07
26	5.2	8.44 ± 0.05	2.25 ± 0.03	50.1 ± 2.23
27	4.2	1.75 ± 0.02	0.652 ± 0.002	7.3 ± 0.07
28 (HSN431)	2.9	0.485 ± 0.002	0.325 ± 0.007	4.4 ± 0.05
29 (HSN576)	2.6	0.688 ± 0.003	0.444 ± 0.008	4.1 ± 0.03
30	5.7	6.59 ± 0.01	2.59 ± 0.04	23.3 ± 0.6
31	3.4	6.8 ± 0.08	60.6 ± 0.9	> 1 µM
32	2.5	> 1 µM	> 1 µM	> 1 µM
33	2.3	> 1 µM	731 ± 0.09	> 1 µM

IC ₅₀ (mean ±SD, nm)				
Compound	LogP ^[a]	K562	KCL22	KCL22-IR
Ponatinib	4.2	0.6003 ± 0.0023	0.142 ± 0.003	0.66 ± 0.005
Imatinib	4.36	88.6 ± 0.6	83.7 ± 0.7	> 1 µM

^[a] Calculated using online software at <http://www.molinspiration.com>, ND = not determined See Experimental Section for procedural details

Table 2.

Inhibition of cellular ABL1 activity by imatinib, ponatinib and HSN compounds

Compound	IC ₅₀ (mean ±SD, nm)	
	KCL22	KCL22-IR
Ponatinib	19.3 ± 1.1	49.7 ± 1.1
Imatinib	574 ± 1.2	NA
15 ; HSN459	10.9 ± 1.1	18.9 ± 1.1
16 ; HSN608	9.9 ± 1.1	26.0 ± 1.1
28 ; HSN431	14.6 ± 1.0	54.7 ± 1.0
29 ; HSN576	11.1 ± 1.1	36.4 ± 1.1