Development of kinase inhibitors and activity-based probes

Liu, N.

7 An affinity-based probe for kinase profiling: Fishing for Dasatinib targets

7.1 Introduction

Chronic lymphocytic leukemia (CLL) is the most common hematologic disease occurring among adults in the Western world. It is characterized by the clonal expansion of mature, $CD5^+$-$CD23^+$ B cells in the peripheral blood, secondary lymphoid tissues, and bone marrow.[1] This malignancy is still considered incurable, although new treatment combinations of monoclonal antibodies and chemotherapy appear promising.[2] Two compartments can be distinguished in CLL: the peripheral blood, in which quiescent CLL cells accumulate, and the tumor microenvironment within the lymphoid tissues and bone marrow that contain proliferating B cells and act as tumor reservoirs.[3]

The tissue microenvironment plays a central role in the pathogenesis of CLL. CLL cell proliferation occurs in distinct microanatomical sites termed proliferation centers or pseudofollicles.[4] In these areas, CLL cells are in intimate contact with non-tumoral accessory cells, such as bone marrow stromal cells (BMSCs),[5,6] monocyte-derived nurse-like cells,[7,8,9,10] follicular dendritic cells, endothelial cells[11] and CD40L expressing T cells[12,13] that provide the necessary external signals that drive maintenance of the CLL cells.[14,15,16,17] Among the various external stimuli in the tissue microenvironments, B-cell receptor (BCR) signaling has been recognized as a crucial pathologic mechanism. Activation of BCR triggers a cascade of signaling events that promotes survival and growth of the CLL clone.[18,19] As a consequence, BMSCs create niches that protect CLL cells from cytotoxic anticancer drugs and this in turn causes resistance to current chemotherapeutics.[15,20]

Currently, a variety of novel kinase inhibitors aiming to target various components of the BCR signaling pathway have been designed and are in clinical development for CLL. In previous studies, dasatinib (**1**, Figure 1), an oral Src/c-Abl tyrosine kinase inhibitor, has been demonstrated to induce apoptosis and/or sensitize against cytotoxic drugs.[20,21,22] Initially, dasatinib was approved for treatment of chronic myeloid leukemia and second-line treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia on the basis of Bcr-Abl inhibition[23], but the activity of dasatinib (**1**) against additional kinases provides a rationale for investigating it in other hematologic malignancies, such as CLL. In model systems, such as the chronic myeloid leukemia (CML)-derived cell-line K562, the spectrum of kinases targeted by dasatinib (**1**) has been mapped.[24] A prominent binder besides Abl and Src family kinases was the TEC family kinase BTK.[25] Since these kinases are all implicated in BCR- and or CD40-mediated microenvironmental stimuli, it is interesting to determine the actual targets of dasatinib and their contribution in mediating CLL survival and chemoresistance.

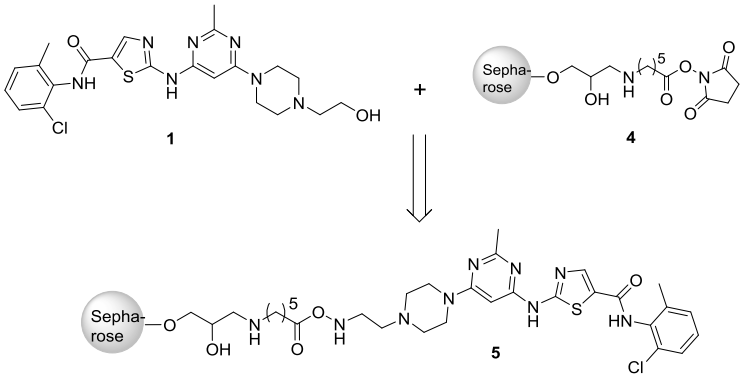


Figure 1. Affinity-based dasatinib probe **5**. Dasatinib **1** is immobilized by coupling it to sepharose beads **4**.

In this study, dasatinib (**1**) is coupled to an affinity matrix **4** to obtain the affinity-based kinase probe **5**, which is used to characterize its spectrum of targets in primary CLL by an affinity-based kinase profiling assay (Figure 2). In this assay, the immobilized dasatinib probe **5** binds noncovalently to its targets after exposure to CLL cell lysates. The interacting kinases are expected to be immobilized by affinity-chromatography, and non-binding proteins will be washed away. Next the retained proteins are washed from the affinity column under protein denaturing conditions and resolved on SDS PAGE. Bands from the gel are next excised, trypsinolysed and identified using mass spectometry (MS).

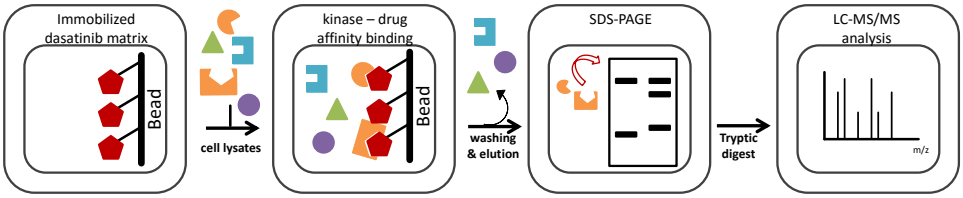
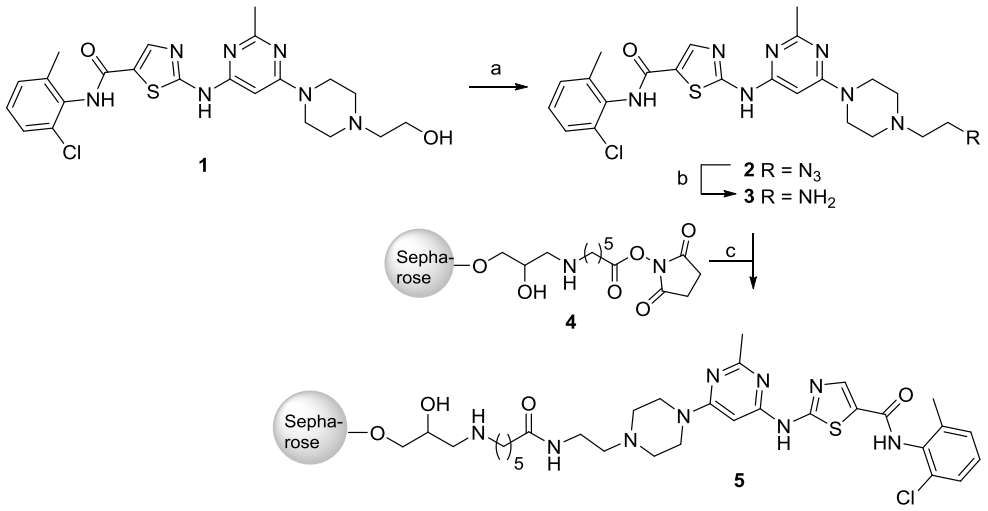


Figure 2. Affinity-based kinase profiling. Immobilized dasatinib matrix (**5**) is noncovalently bound to its cellular targets. After washing and elution, the targets are profiled by SDS-PAGE followed by LCMS for target identification.

7.2 Results and discussion

Synthesis of affinity-based dasatinib probe

In order to identify the interacting kinase(s) of dasatinib (**1**), this drug was modified and immobilized by covalent attachment to NHS-activated sepharose (**4**) via its free amine group (Scheme 1). Compound **3** was prepared via a slightly modified literature procedure of Kim D. *et al.*[26] First, dasatinib (**1**) was extracted from Sprycel^{TM}, which are tablets containing dasatinib, and converted to azide **2** via substitution of the corresponding mesylate with NaN_3. A Staudinger reaction was performed to convert the azide **2** into its amine **3** using PMe_3 in DMF/H_2O. Finally, amine **3** was immobilized on NHS-activated Sepharose 4 Fast Flow beads **4**, which have a ligand density of 16-23 µmol NHS/mL drained gel, to obtain modified dasatinib **5**.



Scheme 1. Synthesis of immobilized dasatinib **3**. Reagents and conditions: a) i) MsCl, TEA, DMF, 0 °C – RT; ii) NaN_3, 41% after RP-HPLC; b) PMe_3, DMF/H_2O (2/1 v/v), 37% after RP-HPLC; c) **4**, DMSO, TEA.

Biological evaluation

In the first instance, the inhibitory potential of dasatinib-amine (**3**) was compared at different concentrations to the parent compound **1** by measuring the inhibitory effect on cell viability of K562 human CML cells, which are dependent on the Bcr-Abl fusion protein for survival[34]. Figure 3 shows that dasatinib-amine (**3**) had identical activity as the parent compound **1**. Thus, it can be assumed that the targets that will be identified with the immobilized dasatinib **5** are identical for the parent compound **1** itself.

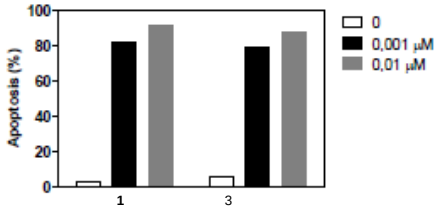


Figure 3. Dasatinib-amine (**3**) induces apoptosis of K562 human CML cells as effectively as the parent compound (**1**). K562 cells were treated with dasatinib (**1**) or dasatinib-amine (**3**) for 48 hr. Cell viability was measured by staining for DiOC6/PI. A representative experiment of two independent experiments is shown.

In the next set of experiments, the immobilized dasatinib **5** was used to identify the drug targets of dasatinib (**1**). Therefore, the immobilized dasatinib **5** was incubated with lysates of CD40 stimulated primary CLL cells. Without SDS-page analysis, the eluted proteins were

identified by liquid chromatography mass spectrometry (LC-MS) with subsequent database search of the analyzed MS spectra. Among the eluted kinases, c-Abl and BTK were identified as important targets of dasatinib (**1**) in the total lysates of CD40 stimulated CLL cells. Furthermore, other identified peptides mapping to the kinases were Lyn, Csk, Fyn, Yes, Src and Lck, which were previously described as specific targets of dasatinib in the CML line K562.[31]

Hereafter, Western blot analysis of pulldown experiments was used to confirm binding of c-Abl and BTK to immobilized dasatinib (**5**) in lysates of CD40-stimulated CLL cells. In agreement with previous studies, Lyn and p38MAPK were also identified as targets of dasatinib[31] (**1**), whereas Syk and ZAP70, although abundantly expressed in CLL, did not bind to dasatinib-sepharose **5** (Figure 4A). As a control for specificity, competition experiments were performed in which lysates were preincubated with free soluble dasatinib **1** prior to exposure to immobilized dasatinib **5**. Western blot analysis confirmed competition of c-Abl, BTK, Lyn and p38MAPK from immobilized dasatinib (**5**) by free dasatinib (**1**) (Figure 4A, lane 4).

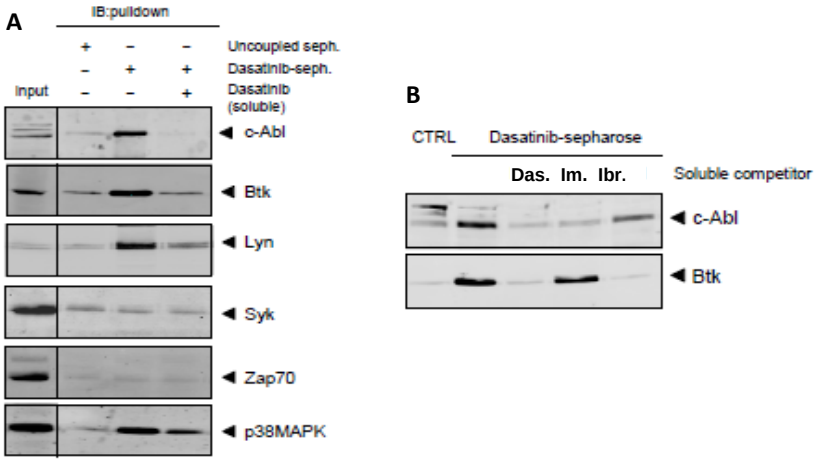


Figure 4. c-Abl and BTK are specific interactors of dasatinib in CD40-stimulated primary CLL. (A) Immunoblots of pull-down experiments with control Sepharose (uncoupled) and immobilized dasatinib **5** from lysates of CLL cells stimulated with CD40L for 48hrs. In lane 4, lysates were pretreated by addition of 10μM soluble dasatinib 30 minutes prior to exposure to dasatinib-sepharose. Western blots of eluates were probed for the presence of the indicated kinases. Input lane designates the total lysate. (B) c-Abl and BTK are specific targets of dasatinib in CD40L stimulated primary CLL. Anti-BTK and anti-Abl immunoblots of CLL pulldown series. Pretreatment was performed by addition of dasatinib (Das.), imatinib (Im.) or ibrutinib (Ibr.) to lysates 30 minutes prior to exposure to drug-affinity matrix. Representative figure is shown of at least 8 independent experiments.

Competition experiments were performed as well to compare the selectivity of the dasatinib (**1**) towards c-Abl and BTK with other CLL drugs imatinib and ibrutinib (Figure 2B). In these experiments CD40L stimulated primary CLL were treated with dasatinib, imatinib or ibrutinib prior to exposure to immobilized dasatinib **5**. Western blot of the eluates provided the kinases that are bound by immobilized dasatinib **5**. Imatinib, which shows greater selectivity than dasatinib for Abl kinase [35-37] in earlier studies, was able to inhibit binding of Abl but not of BTK to immobilized dasatinib **5** in lysates of CD40-stimulated CLL cells (Figure 4B). In a complementary set-up, BTK but not Abl was fully competed by pretreatment with specific BTK-inhibitor ibrutinib prior to pulldown with solid-phase dasatinib.

7.3 Conclusion

In conclusion, an affinity-based probe based on dasatinib has been synthesized and can be used to identify the drug targets that are still unknown for primary CLL. This study has identified c-Abl and BTK as dominant drug targets of dasatinib in CLL upon stimulation with CD40 ligand using the affinity-based dasatinib **5**.

Experimental

General: Tetrahydrofuran (THF) was distilled over $LiAlH_4$ before use. Acetonitrile (ACN), dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol (MeOH) and trifluoroacetic acid (TFA) were of peptide synthesis grade, purchased at Biosolve, and used as received. All general chemicals (Fluka, Acros, Merck, Aldrich, Sigma) were used as received. Traces of water were removed from reagents used in reactions that require anhydrous conditions by coevaporation with toluene. Solvents that were used in reactions were stored over 4Å molecular sieves, except methanol and acetonitrile, which were stored over 3Å molecular sieves. Molecular sieves were flame dried before use. Unless noted otherwise all reactions were performed under an argon atmosphere. Column chromatography was performed on Silicycle Silia-P Flash Silica Gel, with a particle size of 40 – 63 µm. The eluents toluene and ethyl acetate were distilled prior to use. TLC analysis was conducted on Merck aluminium sheets (Silica gel 60 F254). Compounds were visualized by UV absorption (254 nm), by spraying with a solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in 10% sulphuric acid, a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, where appropriate, followed by charring at ca. 150°C. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DMX-400 (400 MHz) or a Bruker DMX-600 (600 MHz) spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane (^{1}H-NMR) or $CDCl_3$ (^{13}C-NMR) as internal standard. Mass spectra were recorded on a PE/Sciex API 165 instrument equipped with an Electrospray Interface (ESI) (Perkin-Elmer). High-resolution MS (HRMS) spectra were recorded with a Finnigan LTQ-FT (Thermo Electron). IR spectra were recorded on a Shimadzu FTIR-8300 and absorptions are given in cm^{-1}. Optical rotations $[\alpha]_D^{23}$ were recorded on a Propol automatic polarimeter at room temperature. LC-MS analysis was performed on a Jasco HPLC system with a Phenomenex Gemini 3 µm C18 50 x 4.6 mm

column (detection simultaneously at 214 and 254 nm), coupled to a PE Sciex API 165 mass spectrometer with ESI. HPLC gradients were 10 → 90%, 0 → 50% or 10 → 50% ACN in 0.1% TFA/H_2O. Chiral HPLC analysis was performed on a Spectroflow 757 system (ABI Analytical Kratos Division, detection at 254 nm) equipped with a Chiralcel OD column (150 x 4.6 mm). The compounds were purified on a Gilson HPLC system coupled to a Phenomenex Gemini 5 µm 250 x 10 mm column and a GX281 fraction collector. The used gradients were either 0 → 30% or 10 → 40% ACN in 0.1% TFA/water, depending on the lipophilicity of the product. Appropriate fractions were pooled, and concentrated in a Christ rotary vacuum concentrator overnight at room temperature at 0.1 mbar.

2-(6-{4-[2-Azidoethyl]piperazin-1-yl}-2-methylpyrimidin-4-ylamino)-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (2).

Compound **1** was obtained by dissolving 20 pulverized Dasatinib tablets (Sprycel™ 70 mg) in H_2O (200 mL) prior to filtration. The residue was washed with DCM/MeOH (1/1 v/v, 5x 150 mL) followed by concentration under reduced pressure to give compound **1** (1.27 g, 91% recovery) as a white solid.

Compound **1** (0.89 g, 1.82 mmol) was dissolved in DMF (20 mL) and cooled to 0 °C. Pre-cooled TEA (3 eq., 0.76 mL, 5.47 mmol) and MsCl (1.5 eq., 0.21 mL, 2.73 mmol) were added dropwise. The reaction mixture was allowed to warm to RT in 5 hrs after which TLC analysis indicated a complete conversion. Hereafter, NaN_3 (10 eq., 1.20 g, 18.2 mmol) was added and the mixture was stirred at RT for 16 hrs until TLC analysis indicated a complete conversion. EtOAc and H_2O were added and the layers were separated. The aqueous layer was extracted with EtOAc (5x), dried over $MgSO_4$ and concentrated *in vacuo*. The title compound was obtained after RP-HPLC purification (gradient: 30% - 70% ACN/0.1% aq. TFA) as a white solid (yield: 0.38 g, 0.75 mmol, 41%). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.64 (br s, 1H), 9.92 (s, 1H), 8.25 (s, 1H), 7.40 (d, *J* = 7.6 Hz, 1H), 7.27 (m, 2H), 6.17 (s, 1H), 3.82 (br s, 2H), 3.55 (m, 8H), 3.28 (m, 2H), 2.45 (s, 3H), 2.24 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 165.44, 162.42, 161.99, 159.91, 157.24, 140.82, 138.83, 133.51, 132.45, 129.06, 128.22, 127.04, 125.96, 83.41, 54.39, 50.76, 45.08, 40.43, 25.56, 18.31. HRMS: calculated for $C_{22}H_{25}ClN_{10}OS$ $[M+H]^+$ 513.16948; found 513.16930.

2-(6-{4-[2-Aminoethyl]piperazin-1-yl}-2-methylpyrimidin-4-ylamino)-N-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (3).

PMe_3 (6 eq., 2.4 mL, 2.4 mmol, 1M in THF) was added to a solution of compound **2** (0.21 g, 0.4 mmol) in DMF/H_2O (1/1/ v/v, 6 mL). The mixture was stirred at RT until TLC analysis indicated complete consumption of starting material after 16 hrs and this was followed by concentration under reduced pressure. Compound **3** was obtained after RP-HPLC purification (20 – 50% ACN/0.1% aq. TFA) as a white solid (yield: 0.43 g, 0.89 mmol, 37%). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 11.63 (br s, 1H), 9.92 (s, 1H), 8.24 (s, 1H), 8.09 (br s, 2H), 7.40 (d, *J* = 7.6 Hz, 1H), 7.27 (m, 2H), 6.17 (s, 1H), 3.76 (m, 6H), 3.17 (m, 6H), 2.44 (s, 3H), 2.24 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 165.38, 162.46, 162.05, 159.92, 157.21, 140.82, 138.84, 133.52, 132.46, 129.07, 128.23, 127.04, 125.94, 83.31, 53.11, 51.24, 41.63, 34.08, 25.57, 18.32. HRMS: calculated for $C_{22}H_{27}ClN_8OS$ $[M+H]^+$ 487.17898; found 487.17883.

Experimental procedures: biochemistry

Patient material

After informed consent, patient material was obtained during diagnostic or follow-up procedures at the Departments of Hematology and Pathology of the Academic Medical Center Amsterdam. This study was approved by the AMC Ethical Review Board and conducted in agreement with the Declaration of Helsinki. PB mononuclear cells of patients with CLL, obtained after Ficoll density gradient centrifugation (Pharmacia Biotech) were frozen and stored as previously described[18]. Expression of CD5 and CD19 (both Beckton Dickinson (BD) Biosciences) on leukemic cells was assessed by flow cytometry (FACScanto; BD Biosciences). CLL samples included in this study contained 81-99% CD5+/CD19+ cells.

Reagents

The pharmacological inhibitor ibrutinib was obtained under a Material Transfer Agreement from Pharmacyclics (Sunnyvale, CA 94085-4521). The kinase inhibitors imatinib and dasatinib were from Novartis (Basel, Switzerland) and Bristol-Myers Squibb (New York, NY), respectively.

Immobilization of dasatinib

The procedure for immobilization of dasatinib was performed as described by Rix et *al*[31]. Compounds were immobilized on NHS-activated Sepharose 4 Fast Flow (GE Healthcare) via their amine functionalities as follows: Beads were washed with dimethyl sulfoxide (DMSO) and incubated overnight at room temperature (RT) with 1 mM compound and 100 mM triethylamine (TEA). After complete coupling of the compound, the affinity matrix was blocked with 0.8 M ethanolamine, washed, and stored at 4°C until use.

Affinity purification

Cell lysates were prepared immediately prior to incubation with immobilized dasatinib in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP-40 (Sigma). Cell suspensions were clarified by centrifugation before incubated with immobilized dasatinib or uncoupled control sepharose overnight at 4°C. Pretreatment was performed by addition of Dasatinib, Imatinib or ibrutinib to lysates 30 minutes prior to exposure to drug-affinity matrix. After incubation, sepharose was washed 3 times with lysis buffer, before beads were drained and retained proteins were eluted by heat denaturing with SDS sample buffer.

Western blot analysis

Western blot analysis was performed using standard techniques[18]. Membranes were probed with anti-Mcl-1 (Cell Signaling), anti-Noxa (Imgenex), anti-Bcl-XL (BD Biosciences), antiserum to β-actin (Santa Cruz Biotechnology), anti-A1/Bfl-1 (Cell Signaling) or Bim (Stressgen Bioreagents Canada). Blots were subsequently incubated with IRDye 680 or 800 labelled secondary antibodies for 1 hour. Odyssey Imager (Li-Cor Biosciences) was used as a detection method according to the manufacturer's protocol.

Statistical analysis

An unpaired two-tailed Student's T test was used to determine the significance of differences between two mean values. The one sample T test was used to determine the significance of differences between means and normalized values (100%). * p <0,05;** p<0,01; *** p<0,001.

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