Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets

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The BCR-ABL tyrosine kinase inhibitor imatinib represents the current frontline therapy in chronic myeloid leukemia. Because many patients develop imatinib resistance, 2 second-generation drugs, nilotinib and dasatinib, displaying increased potency against BCR-ABL were developed. To predict potential side effects and novel medical uses, we generated comprehensive drug-protein interaction profiles by chemical proteomics for all 3 drugs. Our studies yielded 4 major findings: (1) The interaction profiles of the 3 drugs displayed strong differences and only a small overlap covering the ABL kinases. (2) Dasatinib bound in excess of 30 Tyr and Ser/Thr kinases, including major regulators of the immune system, suggesting that dasatinib might have a particular impact on immune function. (3) Despite the high specificity of nilotinib, the receptor tyrosine kinase DDR1 was identified and validated as an additional major target. (4) The oxidoreductase NQO2 was bound and inhibited by imatinib and nilotinib at physiologically relevant drug concentrations, representing the first nonkinase target of these drugs. (Blood. 2007;110:4055-4063)

Introduction

Chronic myeloid leukemia (CML) is a stem cell disease, in which neoplastic cells express the Philadelphia chromosome and the related fusion oncoprotein BCR-ABL. Deregulated tyrosine kinase activity of BCR-ABL is the biochemical hallmark of CML and drives several proliferative and antiapoptotic pathways.1,2 The central role of BCR-ABL in the pathogenesis of CML made it a suitable drug target and cumulated in the discovery of imatinib (Gleevec, STI-571, Figure 1A), a 2-phenylaminopyrimidine–type tyrosine kinase inhibitor, which is currently the frontline CML therapy and considered the paradigm for targeted therapy.3 The remarkable specificity of imatinib for its major targets ABL, KIT, and PDGFR has been studied in great detail and has in part been uncovered by X-ray crystallography revealing a counterintuitive preference of imatinib for the inactive conformation of ABL.4,5

Although most patients show excellent responses to imatinib treatment, clinical resistance may develop predominantly caused by point mutations in the ABL kinase domain.6,7 Two potent second-generation BCR-ABL inhibitors have been recently developed, nilotinib and dasatinib. Nilotinib (Tasigna, AMN107) is a close analog of imatinib with approximately 20-fold higher potency regarding BCR-ABL kinase inhibition.8 Nilotinib has good clinical efficacy in imatinib-resistant patients and is a well-tolerated drug.9 Dasatinib (Sprycel, BMS-354825), a structurally distinct drug with yet approximately 10-fold increased potency compared to nilotinib,10 has already been approved for the treatment of patients with imatinib-resistant CML and of patients with BCR-ABL–positive acute lymphoblastic leukemia (ALL). Both compounds inhibit most of the imatinib-resistant mutants of BCR-ABL, the exception being in both cases the T315I (gatekeeper) mutant.6,8,11

An interesting phenomenon is that imatinib-resistant CML patients who develop resistance against nilotinib may still show a response to dasatinib, and less frequently, patients with resistance against dasatinib may still respond to nilotinib.12,13 Another remarkable aspect is that in contrast to nilotinib, dasatinib, when applied at approved doses, exhibits a number of clinically relevant side effects including cytopenias and pleural effusions.14 All these observations point to major differences of the 3 therapeutics regarding their molecular mechanism of action and target profiles in pathological as well as normal cells.

Previous biochemical studies have already revealed pronounced differences between the 3 BCR-ABL inhibitors with regard to their selectivity. Nilotinib is thought to display similar specificity toward BCR-ABL, c-ABL, c-KIT, and PDGFR compared with imatinib, although with improved selectivity for BCR-ABL/ABL.8 Dasatinib, on the other hand, has been developed as a dual-specificity ABL- and SRC-family kinase inhibitor.10 A recent approach to describe the target profiles of 2 of these drugs, imatinib and dasatinib, done by binding assays with kinase panels expressed as recombinant phage fusion particles, suggested dasatinib to be a much more promiscuous kinase inhibitor in vitro.15,16 To expand on these findings and address selectivity in an unbiased, more physiological context, we embarked on a global chemical proteomics approach.17,18 We used all 3 BCR-ABL inhibitors, imatinib, nilotinib, and dasatinib, as affinity matrices, in parallel to probe the entire expressed kinomes/proteomes of K562 and primary CML cells. Thus, we were able to identify novel kinase and nonkinase targets for the 3 drugs. Among these, we demonstrated inhibition of the receptor tyrosine kinase DDR1 by nilotinib and dasatinib and of...
the oxidoreductase NQO2 by imatinib and nilotinib. The latter represents the first nonkinase enzymatic target of these drugs. Imatinib and nilotinib displayed much more specific target profiles compared with dasatinib, which targets a large number of Tyr and Ser/Thr kinases, many of which play important roles in immune cell signaling. Dasatinib is therefore likely to have a strong impact on multiple biologic processes, which may help to explain the profound antileukemic activity and some of its unique side effects.

Materials and methods

Biologic material

K562 cell pellets were generated by Cilbiotech (Mons, Belgium). Unfractionated peripheral blood leukocytes (buffy coat) were obtained from an untreated patient in chronic-phase CML. After separation, leukocytes \( (1.5 \times 10^9) \) were washed in NaCl. Studies performed with CML leukocytes were approved by the institutional review board (Medical University of Vienna). Written informed consent was obtained in accordance with the Declaration of Helsinki before blood donation.

Antibodies used were rabbit polyclonal anti-ABL K12, rabbit polyclonal anti-DDR1 (C20), and mouse monoclonal anti-BTK (E9) (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-phosphotyrosine 4G10 and rabbit polyclonal anti-TEC (Upstate Biotechnology, Lake Placid, NY); and IRDye 800 donkey anti-rabbit IgG and Alexa Fluor 680 goat anti-mouse IgG. NQO2, mitomycin C, and NADH were purchased from Sigma-Aldrich (St Louis, MO).

Compounds and immobilization

Imatinib (97% pure), nilotinib (97% pure), and dasatinib (99% pure), as well as c-dasatinib (96% pure) and c-imatinib1, were synthesized by WuXi PharmaTech (Shanghai, China), c-imatinib2 was generated in-house by partial synthesis, c-nilotinib (95% pure) was generated by Albany Molecular Research (Albany, NY), and c-imatinib3 was purchased from Gateway Pharma (Freeland, United Kingdom). Final purification of c-imatinib3 (97% pure) was accomplished by Boc-protection of the amino group, high-performance liquid chromatography (HPLC) on C-18 reversed-phase silica, and subsequent deprotection with trifluoroacetic acid. Compounds were immobilized on NHS-activated Sepharose 4 Fast Flow via their amino functionalities as follows: Beads were washed with dimethyl sulfoxide (DMSO) and incubated overnight at room temperature (RT) with 1 mM compound and 100 mM triethyamine (TEA). After HPLC-mass spectrometry (MS) analysis of supernatant indicated deprotection and therefore complete coupling of the compound, the affinity matrix was blocked with 0.8 M ethanolamine, washed, and stored at 4°C until use on the following day.

Compound acetylation

Compounds were dissolved in anhydrous pyridine under argon atmosphere, and 1.5 Eq TEA and 1.5 Eq acetic anhydride were added. Reaction mixtures were stirred overnight at RT, stopped by addition of methanol, and analyzed by HPLC-MS. Byproducts were removed by evaporation and purification was performed, if necessary, by HPLC on C-18 reversed-phase silica.

Kinase assays

c-imatinib3, c-nilotinib, and c-dasatinib and their N-acetyl derivatives were assayed in parallel with the parent drugs for inhibition of recombinant full-length c-ABL (Upstate Biotechnology) in vitro. Phosphorylation of SFAM-KKGEAIYAPFFA-NH2 was monitored using the IMAP kinase assay kit and a SpectraMax M5 plate reader (both from Molecular Devices, Eugene, OR).

Affinity purification

Affinity chromatography (in duplicate, except for primary CML cells) was performed in parallel for all 3 compounds. Cell lysates were prepared immediately prior to affinity chromatography by suspending cell pellets in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2% NP-40, 0.5% N-dodecyl-β-D-maltoside (DDM), 5% glycerol, 1.5 mM MgCl2, 25 mM NaF, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT), 10 μg/mL TLCK, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 10 μg/mL soybean trypsin inhibitor. Cell suspensions were homogenized and clarified by centrifugation before application to pre-equilibrated affinity matrices. Lysates were incubated with affinity matrices for 2 hours at 4°C, centrifuged, and transferred to spin columns. Columns were drained and washed with lysis buffer. Retained proteins were eluted by heat denaturing with SDS sample buffer.

Sample preparation and mass spectrometry (MS)

Affinity-purified proteins were separated by gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently silver stained. Each lane was cut into slices, washed, reduced, alkylated, and subjected to in-gel digestion with porcine trypsin. Resulting peptide mixtures were extracted and desalted with stage tips. Purified samples were analyzed in a data-dependent acquisition mode by on-line reversed-phase nano-HPLC coupled to a Q-ToF mass spectrometer (Q-ToF Ultima or Praemier XEWS, Manchester, United Kingdom) equipped with a nanoelectrospray source. Acquired data were searched against the human IPI database with the search engine Mascot (Matrix Science, London, United Kingdom). Search parameters were set to a mass tolerance of 20 ppm for the precursor ions and 0.1 g/mol for the fragment ions. Two missed cleavage sites were allowed. Carbamidomethyl cysteine was set as fixed and oxidized methionine was searched as variable modification. The raw datasets are provided as Table S5 (available on the Blood website; see the Supplemental Materials link at the top of the online article).

Bioinformatic analysis

Peptide ion scores of 20 or higher, peptide lengths of 6 or higher, and 2 distinct peptides per protein were required. EpiCenter (Proxcen, Odense, Denmark) was used to further filter by a minimum peptide score difference of 5 and a minimum y score of 50 and to visualize protein identifications. A false-positive detection rate of less than 1% was estimated by searching a reversed database. The number of distinct peptides identified for each protein \( P \) was collected for each sample \( k \), and expression profiles \( (x_{1k},x_{2k},...,x_{nk}) \) were obtained for the peptide count in sample \( k \). The K562 “core proteome” was acquired and compared with pulldown datasets using peptide counts. Proteins detected with a large number of peptides in one sample and no respective peptides in the replicate were removed. A threshold \( T \) was defined by performing a reproducibility analysis on the basis of the core proteome duplicates using a bootstrap procedure. The probability \( P(n/k) \) to detect a protein with \( n \) peptides given it was detected with \( k \) peptides in an independent analysis was estimated such that \( P(0/T) \) for 5% or less, which yielded \( T = 11 \) for our data. Significantly enriched proteins of each pulldown were identified by: (1) applying a clustering method, K-means with Pearson correlation as a metric, and (2) the nonparametric statistical method SAM modified to obtain a one-sided test (ie, more target abundance in the pulldown sample) based on the distribution of the test statistics. The top 4% scoring proteins found by the statistical analysis in addition to proteins identified by the clustering method were selected. Frequently identified proteins from independent chemical proteomics experiments were subtracted.

NQO2 inhibition assay

NQO2 (1 μM) was incubated with mitomycin C (100 μM) as substrate, NaNH4 (4 mM) as cosubstrate, and various drug concentrations as indicated in 100 mM potassium phosphate buffer (pH 5.8) at 27°C for 30 minutes prior to workup and HPLC-MS analysis (positive mode ESI). Hydrocortisone was chosen as internal standard for quantification.

DDR1 inhibition assays

Full-length DDR1 (wild-type and K655M mutant) was transiently expressed in HEK293 cells. Transfected cells were treated with dasatinib and...
nilotinib at the indicated concentrations for 1 hour before preparation of total cell lysates. DDR1 was immunoprecipitated with anti-DDR1 and immunoblotted with anti-pY (4G10) and anti-DDR1 antibodies.

Results

Drug affinity purification leads to identification of BCR-ABL inhibitor target proteins

The chemical proteomics approach we applied to investigate the selectivity profiles of imatinib, nilotinib, and dasatinib required the creation of affinity matrices via covalent attachment to a resin. However, neither imatinib nor nilotinib contain suitable chemical functionalities (Figure 1A). Based on inhibitor cocrystallization studies with the c-ABL kinase domain, we identified the N-methyl-piperazine ring of imatinib, the methyl-imidazole moiety of nilotinib, and the hydroxyethyl group on the piperazine ring of dasatinib as feasible attachment points due to their solvent accessibility when bound to c-ABL. The coupleable analogues c-imatinib, c-nilotinib, and c-dasatinib were synthesized accordingly with primary amino groups suitable for immobilization (Figure 1A).

We compared the inhibitory potential of our coupleable analogues to the parent compounds by measuring the kinase activity of the ABL kinase domain in a drug concentration–dependent manner (Figure 1B). The acetylated analogues mimicking the immobilized compounds did not display significantly altered activities (Figure 1B). The observed IC₅₀ values for c-ABL inhibition by c-nilotinib (28 nM) and c-dasatinib (8 nM) closely resembled those of the parent compounds (45 nM and 9 nM, respectively). In the case of imatinib (IC₅₀ = 0.4 μM), we observed a marked reduction in the kinase inhibitory activity of (IC₅₀ = 28 μM). Two other imatinib analogues that we generated, c-imatinib1 and c-imatinib2, displayed an even more pronounced reduction of potency (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). We therefore decided to use c-imatinib3, accepting the reduced potency as a very important caveat when comparing it with nilotinib and dasatinib.

The 3 drug affinity matrices were incubated with lysates of K562 and CML primary cells, washed, eluted, and separated by SDS-PAGE. Proteins were identified by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) and database search (Figure 2A). We identified the 844 proteins most prevalent in the total lysate of K562 cells representing the core proteome. Based on this, pulldown-specific proteins were identified by comparing pulldown and core proteome datasets applying the SAM statistical test and independent clustering analysis (“Bioinformatic analysis”). In addition, we took an empiric approach to further determine and subtract potential nonspecific interactors (“frequent hitters”) frequently detected in purifications with 7 nonrelated drugs (Table S1). While this combined process is justified by greatly improving the signal-to-noise ratio, it bears the inherent important shortcoming that certain proteins that are very abundant and target specific (eg, HSP90 binding to BCR-ABL) may not be represented in the final list. Thus, we identified the most prominent drug-associated proteins from K562

Figure 1. The coupleable analogues of the BCR-ABL inhibitors retain c-ABL inhibitory potential. (A) Chemical structures of imatinib, nilotinib, dasatinib, and their coupleable analogues c-imatinib, c-nilotinib, and c-dasatinib as well as the acetylated derivatives thereof. (B) Kinase inhibition assays for parent compounds ( ), coupleable analogues ( ), and their N-acetyl derivatives ( ) performed on the c-ABL full-length kinase by IMAP using the AbtTide substrate peptide.
lysates, 11 for imatinib, 14 for nilotinib, and 38 for dasatinib (Figure 2A; Table 1; and Tables S2-S4).

Dasatinib strongly differs from imatinib and nilotinib in its protein profile

Visual analysis of the eluates from the respective drug pull-downs on a silver-stained SDS-PAGE gel revealed pronounced differences in the protein compositions (Figure 2B). In comparison, dasatinib yielded many different bands compared with the nilotinib and imatinib eluates, some of which were particularly prominent, indicating a different and possibly broader target profile. The major bands, which MS analysis showed to correspond mainly to the TEC family kinases BTK and TEC, different SRC family kinase members, the c-SRC tyrosine kinase CSK, and the NAD(P)H:quinone oxidoreductase NQO2, embellish this observation.

BCR-ABL and NQO2 are the most prominent interactors of imatinib

The target table (Table 1) displays identified kinases by decreasing peptide count. The cognate target of imatinib, BCR-ABL, for which we observed peptides mapping to both the BCR-part, as well as to the ABL-part, was identified as the major imatinib interactor. Unexpectedly, the NAD(P)H:quinone oxidoreductase NQO2 (or quinone reductase QR2) was found as one of the foremost interactors of imatinib in both the K562 and the CML patient sample (Table S2). It was recovered with an exceptionally high sequence coverage of more than 70% and high protein identification score. In correlation with this, it also represents the most prevalent band seen by silver staining in the imatinib and nilotinib, but not the dasatinib, purifications (Figure 2B).

BCR-ABL and NQO2 are the most prominent interactors of nilotinib

BCR-ABL was identified in the nilotinib pulldown (Table 1) with a higher protein score and better sequence coverage than seen with imatinib (Table S3). Immunoblotting showed both the presence of c-ABL and BCR-ABL in the nilotinib eluate (data not shown). Furthermore, we also obtained specific peptides mapping to the c-ABL paralogue ARG (ABL2), another target protein of nilotinib. As for imatinib, NQO2 appeared as one of the most prevalent interactors (Table S3). Another major nilotinib interactor was found to be the receptor tyrosine kinase DDR1 (discoidin domain receptor 1). DDR1 is an important mediator of extracellular matrix signaling and has not previously been identified as a nilotinib target. Successful competition of DDR1 binding to the nilotinib matrix by preincubation of the cell lysate with free nilotinib (Figure 3) confirmed the specific nature of the DDR1 interaction with nilotinib.

A large number of kinases interact with dasatinib

The dasatinib pulldown dataset (Table 1) displayed a much higher number of protein kinases, totaling 24 altogether in K562 cells, than seen with imatinib or nilotinib. Again, we found peptides from both parts of BCR-ABL. We also identified unique peptides mapping to the breakpoint region of BCR-ABL. Immunoblotting also showed the presence of both, c-ABL and BCR-ABL, in the dasatinib pulldown (data not shown). As for nilotinib, our analysis yielded DDR1 as a prominent interactor, which is also specifically competed by free dasatinib (Figure 3). Other significant dasatinib-binding proteins, the specificity of which was independently confirmed by immunoblotting, include the TEC family kinases BTK and TEC (Figure 3). Both kinases appeared as particularly prominent interactors as inferred already from SDS-PAGE analysis (Figure 2B). In addition, BTK was detected with high sequence coverage of approximately...
70% and was also identified prominently in the CML patient cell pulldown (20 peptides, Table 1). We have independently shown that the kinase activity of BTK and TEC was inhibited by nanomolar concentrations of dasatinib in vitro and in cultured cells. Noteworthy as a novel dasatinib target candidate is also the mixed-lineage kinase ZAK, possibly involved in stress response and actin organization. In addition, we identified several nonkinase proteins, such as the adaptor protein GRB2, the inositol polyphosphate 5-phosphatase SHIP-2 (INPPL1), the E3 ubiquitin ligases CBL-B and c-CBL, as well as its negative regulator STS-1, which are likely to be interactors of BCR-ABL and/or of other dasatinib kinase targets (Table S4).

Among the 20 most predominant dasatinib interactors are the Ser/Thr kinases GAK, p38α (MAPK14), MAP3K1, and MAP3K4. We hypothesize that part of the dasatinib biologic effects may be contributed by inhibition of Ser/Thr kinases.

 Probably the single most prominently dasatinib-targeted family of protein kinases is the SRC family (Figure 2B). We identified c-SRC, LYN, FYN, YES1, FGR, and FRK, as well as their negative regulators CSK (c-SRC kinase, Figure 2B). Interestingly, all of these were also identified as dasatinib-binding proteins in CML primary cells. Furthermore, the dasatinib pulldown from primary CML cells revealed several further kinases not observed in K562 (Table 1), probably due to differential expression. These include 2 additional SRC kinases, namely HCK and LCK, both of which are known to play roles in hematopoietic malignancies, a third member of the TEC kinases, BMX, and the integrin-linked kinase ILK along with...
The oxidoreductase NQO2 is inhibited by imatinib and nilotinib

Since the oxidoreductase NQO2 was one of the most prominent interactors of imatinib and nilotinib (Tables S2,S3), we further validated this unexpected putative nonkinase target by measuring the in vitro inhibition of NQO2 enzymatic activity. Using the anticancer agent mitomycin C, which in vivo is activated by the in vitro inhibition of NQO2 activity (IC_50 = 1.0 μM and 1.8 μM, respectively; Figure 5B). The observed decline of inhibition for the highest imatinib concentration is likely attributable to solubility issues. In contrast, dasatinib did not inhibit NQO2 activity (Figure 5B). Based on the cocrystal structure of NQO2 with FAD and menadione, one might postulate that imatinib (and nilotinib) may act as a FAD-competitive drug (Figure 5C), although further structural and enzymatic studies will be required.

Discussion

The pharmaceutical industry and the broader biopharmaceutical research communities are in constant search for molecular predictors of drug efficacy, selectivity, and safety. Here, we have addressed the question whether profiling of native targets in disease-relevant cells for 3 clinical compounds targeting BCR-ABL could contribute to the understanding of the molecular mechanism-of-action of these drugs and help predict short- and long-term clinical outcomes and possibly drug side effects. We applied the chemical proteomics technology that represents a refined postgenomic version of the classic affinity purification approach integrating high-end mass spectrometry and bioinformatics. The approach also has some important limitations: (1) the assay addresses binding and not inhibition, (2) it is at best semiquantitative, so no target affinities can be derived directly, (3) binding is reflective of affinity, but also abundance of the target, and (4) proteins present with natural posttranslational modifications may interact, (2) proteins are present at natural abundance levels, (3) proteins occur along with their natural binding partners, and (4) proteins present with natural posttranslational modifications and splice variants. All of these can be important factors that influence drug-protein interactions.

One of the most interesting findings of our study was the identification of the drug-metabolizing oxidoreductase NQO2 to interact with imatinib and nilotinib. Indeed, we have shown that

its nonkinase interaction partners PINCH, PARVIN, and RSU1 (Table S4).32

The receptor tyrosine kinase DDR1 is potently inhibited by nilotinib and dasatinib

Besides the ABL kinases, DDR1 was the only kinase that was identified to interact with nilotinib (Table 1). In addition, DDR1 was also highly enriched in the dasatinib pulldown. To study if dasatinib and nilotinib are inhibiting DDR1 kinase activity, we overexpressed DDR1 in HEK293 cells and measured changes in DDR1 phosphorylation in the presence of different concentrations of dasatinib and nilotinib. Overexpression of DDR1 led to a strong increase in tyrosine phosphorylation in contrast to the kinase-inactive counterpart (DDR1 K655M), suggesting that kinase activity correlates with tyrosine phosphorylation (ie, autophosphorylation, Figure 4A). Dasatinib (10 nM) or nilotinib (100 nM) led to a clear reduction of DDR1 autophosphorylation (Figure 4B). Higher concentrations completely abolished DDR1 autophosphorylation indicating complete inhibition of DDR1 kinase activity by dasatinib at concentrations of 100 nM or higher and by nilotinib at concentrations of 1 μM. Sequence alignment analysis of the DDR1 and ABL catalytic domains showed that the residues known to interact with dasatinib and nilotinib are conserved (11 of 14 identical residues; Figure 4C).
imatinib and nilotinib inhibit the enzymatic activity of NQO2 at therapeutically relevant concentrations. For instance, serum levels of imatinib higher than 1 μM are commonly achieved in patients.37 To the best of our knowledge, this is the first nonkinase target of imatinib and nilotinib ever reported. NQO2 activity was not inhibited by dasatinib. This is consistent with NQO2 being essentially absent in the silver-stained gel of the dasatinib pulldown eluate. Our enzymatic assays indicate that neither BCR-ABL inhibitor is metabolized by NQO2 (data not shown). The precise mechanism of inhibition of NQO2 by imatinib and nilotinib is subject to forthcoming structural and enzymatic studies. Although the functional role of NQO2 is unclear,38 previous RNAi knock-down of NQO2 led to decreased proliferation of K562 cells,33 suggesting that inhibition of NQO2 might contribute to the antiproliferative effect of imatinib and nilotinib in CML cells. Furthermore, it is conceivable that long-term treatment with imatinib and nilotinib may interfere with the metabolism of coadministered drugs by NQO2 and thus lead to unwanted drug-drug interactions.

A second highly interesting finding was the identification of the receptor tyrosine kinase DDR1 as a previously unknown target protein for nilotinib and dasatinib. We have shown that both drugs inhibit DDR1 autophosphorylation in the midnanomolar range. This markedly expands the very narrow target spectrum of nilotinib.8 DDR1 is thought to transduce signals from the extracellular matrix by binding collagen39 to stimulate the NFκB pathway and elicit various cellular responses, such as adhesion, migration, differentiation, and cytokine production.40 DDR1 has been suggested to play an important role in several malignancies, as well as in the generation of immune responses.41,42 In addition, DDR1 has been described to be expressed on bronchial epithelial cells and to play a functional role in pulmonary disorders.43,44 Thus, DDR1 might both serve as a mediator of potential nilotinib- and dasatinib-induced side effects, as well as an intervention point for novel therapies of relevant cancers.

For all 3 drugs under investigation, we have been able to identify the cognate targets, BCR-ABL and c-ABL, which serve as validation of our approach. Likewise, dasatinib prominently bound SRC family kinases. The other well-known targets, c-KIT and PDGFR,8,10 were not observed, because they are not (PDGFR) or too marginally (c-KIT) expressed in K562 cells (data not shown). Thus, the amount of c-KIT in the pulldown eluate may be lower than the detection limit of our mass spectrometers, which we determined to be in the low picomolar range. Nevertheless, we have been able to identify these proteins with dasatinib matrices using other cell types (unpublished observations, U.R., December 2005, September 2006). As described earlier, all our coupleable imatinib analogues showed reduced ABL inhibitory potential, which likely affects other kinase targets as well. It is therefore likely that certain target proteins have eluded detection because of low abundance in the pulldown eluate. Despite this, c-ABL, BCR-ABL, as well as NQO2 were identified from K562 cells using c-imatinib3. Paradoxically, we did not observe ABL kinases from the patient-derived CML cell pulldowns with any of the 3 inhibitor matrices. However, a number of additional targets were identified with dasatinib in primary CML cells compared with K562 cells.

An important aspect of our study was the characterization of the target profiles of the 3 BCR-ABL inhibitors in CML cells. While nilotinib displayed relatively high specificity and affects only few kinase targets, we observed more than 24 different kinases in our dasatinib pulldown experiments with K562 cells and more than...
30 kinases when including primary CML cells. In addition, in the dasatinib pulldowns we identified several nonkinase proteins. Some of these, such as GRB2, SHP-2, c-CBL, and CBL-B, as well as PINCH, PARVIN, and RSU1, have been described previously as complex partners of BCR-ABL and ILK, respectively, and are likely to be “piggy-backing” to these. Among the most prominent targets of dasatinib were the TEC family kinases TEC and BTK, which are particularly interesting proteins due to the crucial roles they play in the signaling pathways of the T- and B-cell receptors and thus the host immune system. In addition, BTK has been identified as a potential target in several other cell types, such as endothelial or pulmonary cells, where it interacts with caveolin-1, and in mast cells, which contain diverse vasoactive (edema-promoting) and immunomodulating substances. These observations are of particular interest as dasatinib, but not the other 2 kinase inhibitors, has been described to cause pleural effusions in a high proportion of patients. Whether this particular side effect of dasatinib, which is often severe and dose-limiting, results from functional interactions between the drug and the targets newly discovered in this report (eg, DDR1 and TEC family kinases) remains to be elucidated. Based on the many different targets of dasatinib, one could hypothesize that the simultaneous inhibition of several of these targets causes some of the (side) effects observed in patients treated with relevant doses of dasatinib. Several pathways of the immune system could be severely affected by continuous high doses of dasatinib, harboring significant risks for immunosuppression of patients treated over a long period of time. In general, however, it may be difficult at our current level of knowledge to transpose molecular profiles, such as described here, into pharmacological predictors in patients.

A striking outcome of this study is that compounds designed to target a particular kinase, in this case BCR-ABL, and administered for the same indication (CML), display such different profiles. The efficacy with which dasatinib appears to target many pathways, and several of these multiple times, may represent an advantage in the treatment of heterogeneous malignancies. Advanced-stage CML and Ph+ B-ALL, in which several SRC family kinases play significant roles, may represent good examples. This consideration is also supported by the fact that in particular dasatinib has been shown to act on imatinib-resistant CML patients in the accelerated and even in the blast phase.

In summary, our study has identified several interesting novel interactors and targets of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib, some of which, like DDR1, bear the potential for therapeutic interventions. The relatively similar efficacy and safety profiles of drugs with such different proteomic and signaling impact show that at our current level of understanding regarding CML, no simple rationale can be construed in support or detraction of highly specific inhibitors such as nilotinib or multiget targets such as dasatinib. However, having characterized the target profiles of each of the 3 drugs in CML, this work may lay the foundation for future rational risk-versus-benefit assessments in individual cases including potential combination therapy.

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Authorship

Contribution: U.R. performed and interpreted the experiments, codesigned the study, and wrote the paper; O.H. performed experiments and contributed to the study design and paper; G.D. contributed to data analysis; L.L.R.R. performed experiments and contributed to paper preparation; M.P., N.V.F., and I.K. performed experiments; K.L.B and P.V. contributed to the study design and the paper and provided CML patient cells; J.C. and T.K. designed the analytical strategy, performed data generation, and contributed to the paper; G.S.-F. designed and supervised the study and wrote the paper. U.R. and O.H. contributed equally to this work.

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Chemical proteomic profiles of the BCR-ABL inhibitors imatinib, nilotinib, and dasatinib reveal novel kinase and nonkinase targets

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