Effects of protein kinase inhibitors on chronic lymphocytic leukemia (CLL) cells

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B cell Chronic lymphocytic leukemia (B-CLL) is a neoplastic disorder characterized by accumulation of B lymphocytes due to uncontrolled growth and resistance to apoptosis. Src family kinases (SFKs) are non receptor tyrosine kinases present in the cytosol, which couple with downstream B cell receptor signaling and thus mediate growth, survival, proliferation and antiapoptosis. In CLL cells SFKs are remarkably overexpressed, especially Lyn kinase. This gives the rational to use SFKs inhibitor to treat CLL.

Addition of the specific pharmacological inhibitors of SFKs, bosutinib and saracatinib, inhibited the global tyrosine phosphorylation as well as the basal auto-phosphorylation of SFKs. Mechanistically, inhibition of SFKs is coupled to apoptosis induction via decreased protein levels of the anti-apoptotic proteins Bcl-2, Mcl-1 and survivin, which were demonstrated by Western blotting. To assess apoptosis induction, annexin V binding to freshly isolated CLL cells with or without treatment with kinase inhibitors was measured flow cytometrically. Using the inhibitors at a concentration of 10 µM the average percentages of annexin V-positive, apoptotic cells in 11 CLL samples increased from 24 % in untreated controls to 55 %, 45 % and 37 % after treatment with bosutinib, saracatinib and dasatinib, respectively. The response to each of the inhibitors showed a high but comparable degree of variation among the investigated CLL samples. On the average bosutinib induced apoptosis with significantly higher efficiency than dasatinib, which calls for further investigation of its pre-clinical potential for treatment of CLL.
INTRODUCTION

CLL is the most frequent leukemia in the Western world. This neoplasia of phenotypically mature B lymphocytes occurs mostly at an advanced age and affects more men than women. Despite continuous progress in its management, the disease remains incurable and new therapies are highly warranted. Apart from traditional chemotherapeutic agents targeted therapies are increasingly being developed. Since CLL cells easily can be obtained at high purity from the peripheral blood of CLL patients, it seems straightforward to use such samples for assessing new drug candidates with potential for treating CLL. Freshly isolated CLL cells can be kept in culture for a couple of weeks, but in vitro do not show substantial proliferation nor the apoptosis resistance observed in vivo due to lack of micro-environmental stimuli.

For the survival of CLL cells the micro-environment in different compartments like bone marrow, lymph nodes and peripheral blood plays a pivotal role as exemplified by the poor survival of isolated CLL cells in vitro and by the difficulties encountered in establishing CLL cell lines. Among these, B cell receptor signaling, which determines B cell fate, is particularly important. This is reflected by the importance of immunoglobulin heavy chain variable region (IgVH) mutation status as a prognostic marker used for stratification of the vastly heterogeneous disease. Expression of the non-receptor kinase ZAP-70 and the cell surface protein CD38 are used as surrogate markers. The indolent and aggressive subgroups of CLL correspond to mutated and unmutated IgVH genes, ZAP70- or CD38-positive and negative or BCR responsive and unresponsive cases.

For the transduction of micro-environmental signals via surface receptors on CLL cells, non-receptor kinases are required, since several receptor types are devoid of intrinsic enzyme activity. Src family kinases are known to interact with a variety of surface receptor types, representatives of which are expressed on CLL cells and are stimulated by soluble factors and cell-cell interactions in their microenvironment (Fig. 1). For instance, the Src family kinase Lyn is known to bind to the intracellular parts of BCR subunits and to initiate pro-survival and anti-apoptotic signaling cascades in B cells. Interestingly Lyn is aberrantly expressed in CLL cells and its pharmacological inhibition leads to apoptosis induction. Therefore CLL may be included among the conditions, where Src-kinase inhibition may constitute a useful therapeutic strategy.
The ATP-competitive tyrosine kinase inhibitor imatinib is capable of keeping Bcr-Abl positive leukemias under control that are driven by the de-regulated kinase activity of the BCR-Abl fusion gene resulting from the Philadelphia translocation [14]. Following the success of imatinib, a number of second generation Abl inhibitors with activity against some imatinib-resistant forms of Bcr Abl have been developed [15]. Chemically these inhibitors are based on different scaffolds (*Table I*) and some of them are dual-specific Src-Abl inhibitors [16], which enhances their effect on CML cells [17]. The lead compounds from which dasatinib and bosutinib were developed were originally identified due to their inhibition of Src kinases [18,19] and Saracatinib shows Src kinase inhibition in solid tumors [20,21]. Dasatinib was the first of these second generation dual-specific Src/Abl inhibitors that was pre-clinically characterized [22] and has been approved for treatment of imatinib-resistant Bcr-Abl-positive leukemias since 2006. Bosutinib (SKI-606) followed [23] and phase II trials for the same diseases are scheduled from 2006-2010. The Src/Abl inhibitor Saracatinib (AZD0530) [24,25] is under investigation in phase II trials for treatment of various solid tumors, which have been completed for prostate cancer [26]. The design of selective small molecule kinase inhibitors integrating structural information is a huge challenge with tremendous potential for cancer therapy [27].
Table I: Characteristics of the investigated ATP-competitive kinase inhibitors

<table>
<thead>
<tr>
<th>Structure Formula</th>
<th>Compound (Company)</th>
<th>Chemical Class</th>
<th>Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Imatinib" /></td>
<td>Imatinib, Gleevec, STI571, CGP57148B (Novartis)</td>
<td>2-phenylamino-4-pyridino-pyrimidine</td>
<td>Abl, c-kit, PDGFR</td>
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<tr>
<td><img src="image" alt="Dasatinib" /></td>
<td>Dasatinib, Sprycel, BMS 354825 (Bristol-Myers Squibb)</td>
<td>2-aminothiazole</td>
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<tr>
<td><img src="image" alt="Bosutinib" /></td>
<td>Bosutinib, SKI-606 (Wyeth)</td>
<td>4-anilino-3-carbonitriolo-quinoline</td>
<td>Src family kinases, Abl, Axl</td>
</tr>
<tr>
<td><img src="image" alt="Saracatinib" /></td>
<td>Saracatinib, AZD-0530 (Astra-Zeneca)</td>
<td>4-anilino-quinazoline</td>
<td>Abl, Src family kinases,</td>
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</table>

The success of tyrosine kinase inhibitors in the therapy of Bcr-Abl-positive leukemias prompted pre-clinical investigations of these compounds on primary CLL cells. While imatinib, which targets Abl but not the SFK Lyn or Lck, in CLL cells enhances the activity of the chemotherapeutic agent chlorambucil without exhibiting substantial reduction of viability of its own, dasatinib was found to reduce basal SFK activation and to induce apoptosis in primary CLL cells and was reported to show clinical efficiency. Apoptosis induction by dasatinib is stronger in CLL samples with unmutated IgV\_H genes or high ZAP-70 expression, but the CLL patients that eventually will profit from dasatinib treatment are difficult to predict. Moreover, the preclinical effects at pharmacologically achievable dasatinib concentrations are low in the majority of patient samples. Therefore, the objective of the current investigation is to compare the biological effects on CLL cells of dasatinib and two additional Src/Abl inhibitors that are currently being clinically investigated for treatment of CML and solid tumors.
MATERIALS AND METHODS

Inhibitors

Information on the inhibitors used in this study is summarized in Table I. Pure substance of dasatinib and bosutinib was kindly provided by Bristol Myers Squibb and Wyeth, respectively. AZD0530 was purchased from Chemietek. The compounds were dissolved in DMSO and 100 mM stock solutions stored at -20°C. An equal DMSO concentration of 0.1 % was maintained at all inhibitor doses and the solvent control.

Patient samples

Peripheral blood samples were obtained from patients who were previously diagnosed for CLL according to standard criteria 1. The studies were performed in accordance with the local ethics committee of the University of Cologne (approval no. 01-163) and after informed written consent had been obtained from all patients included in the study. The clinical and biochemical characteristics of samples from 11 patients (90 % male, median age 67 years, 55 % untreated) are summarized in Table II. Characterization and stratification of patient samples according to molecular prognostic markers of CLL was performed as described previously 29.

Table II: Characteristics of the studied patient samples

<table>
<thead>
<tr>
<th>ID no.</th>
<th>Age/sex</th>
<th>Stage (Binet)</th>
<th>Treatment</th>
<th>Cytogenetic aberrations</th>
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<th>ZAP70 3</th>
<th>CD38 3</th>
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<td>U</td>
<td>del13q14</td>
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<td>n</td>
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<td>p</td>
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<tr>
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<td>p</td>
<td>p</td>
</tr>
<tr>
<td>9</td>
<td>m / 60</td>
<td>A</td>
<td>U</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>10</td>
<td>m / 69</td>
<td>n.d.</td>
<td>U</td>
<td>n.d</td>
<td>n.d</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>12</td>
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<td>P</td>
<td>p</td>
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<td>f / 54</td>
<td>A</td>
<td>U</td>
<td>normal</td>
<td>mut</td>
<td>N</td>
<td>n</td>
</tr>
</tbody>
</table>

1: u (untreated) / t (treated): never treated / with prior treatment (more than three months ago); n.d.: not determined.
2: UNM (unmutated) / mut (mutated): <2 % / >2 % sequence divergence with the closest germline gene.
3: p (positive) / n (negative): percentage of cells with higher fluorescence after staining with specific antibody than with isotype antibody >20 % / < 20 %.
Cell isolation and culture

The CLL-derived cell line Mec-1 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Peripheral blood mononuclear cells were isolated from heparinized blood samples by Ficoll-Plaque Plus sedimentation (GE Healthcare, Freiburg, Germany). To obtain pure CLL cells, the Ficoll gradient was preceded by incubation of whole blood with the Rosette Sep B-cell purification antibody cocktail (Stem Cell Technologies) to aggregate unwanted cells with erythrocytes. The purity of CLL cell population was determined by flow cytometry using FITC-labeled antiCD-5 and PE labeled antiCD-19 antibodies (BD Biosciences, Heidelberg). Freshly prepared CLL cells and all cell lines were cultured in RPMI medium (Gibco, Karlsruhe, Germany) supplemented with 10% heat inactivated fetal calf serum (PAN Biotech, Nürnberg Germany) and 1% antibiotics (Gibco) at 37°C in a humidified atmosphere containing 5% CO₂.

Proliferation and apoptosis assays

The XTT assay of cellular metabolic activity was performed according to the instructions of the supplier (Roche, Mannheim, Germany) in order to assess cellular respiration with or without inhibitors. NADH produced by the mitochondria of metabolically active cells reduces the modified tetrazolium salt XTT to a water soluble formazan. Three to 4 x 10⁴ cells per well in a volume 100 µL were seeded into 96 well plates and incubated with or without the inhibitors for a specified period of time. After addition of 50µL of XTT solution to each well the assay plates were incubated for another four hours at 37°C to allow formation of the orange colored formazane reduction product. The treated dying cells will be metabolically less active than the healthy untreated cells and consequently less absorbance values will be shown. Absorbance values at 490nm with reference measurements at 650nm were recorded using a Dynatech MR5000 plate reader. The XTT assay results were analyzed and survival fractions were expressed by the mean absorbance of drug treated cells over the mean absorbance of untreated cells.

For apoptosis assays, 5 x 10⁵ cells were collected and washed once before the determination of annexin V–binding or mitochondrial membrane potential. The cells were stained with FITC-labeled annexin V and 7-amino-actinomycin (7AAD; BD Biosciences), and analyzed using a FACS-Canto flow cytometer (BD Biosciences). The percentages of nonapoptotic cells relative to control were calculated from the percentages of viable (i.e. annexin V–negative) inhibitor-treated and control cells.
**Western blot assay**

Intracellular protein and phosphorylation levels were examined after treatment of primary CLL cells with 10 µM of inhibitors for 30 min, 2 or 4 hours. Cells were lysed by incubation for 30 min on ice in 50 mM Tris (pH 7.5), 0.15 M NaCl, 2 mM EDTA, 1% deoxycholic acid, 1% each of protease and phosphatase inhibitors cocktail (Sigma, Steinheim Germany). Equal amounts of protein samples were loaded in the slots of the stacking gel and subjected to electrophoresis on 10% sodium dodecyl-sulfate (SDS)-polyacrylamide gels and blotted on nitrocellulose membranes. The following antibodies were used: anti-phosphotyrosine (PY99), anti-Lyn (H-6 and 44), anti-pTyr20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-pSrc (Y416), anti-actin, (Cell Signaling Technology, Beverly, MA) and anti Mcl1 (S19), anti-Bcl2 (BD Biosciences). After staining with IRDye 800CW-labeled goat anti-mouse IgG (Licor Biosciences, Bad Homburg, Germany) as the secondary antibody, proteins on the blotting membrane were detected by scanning with an Odyssey Near Infrared Imager (Licor).

**Statistics**

Differences between treatment conditions were analyzed by two-sided Student’s t-test. For p<0.05 differences were considered as significant.
RESULTS

SFK inhibition in primary CLL patient cells

Aberrant expression and activity of the SFK Lyn has been linked to enhanced survival of CLL cells \(^{12}\) and treatment with 0.1 µM of the second generation dual-specific Abl/SFK inhibitor dasatinib resulted in a clear reduction of overall tyrosine phosphorylation in CLL cells and particularly in a sharp decline of the basal phosphorylation at the activating phosphotyrosine of SFK \(^{29}\). In the current study we examined the effects of two additional second generation Src kinase inhibitors, namely bosutinib and saracatinib on global tyrosine phosphorylation and Src kinase autophosphorylation by Western Blotting with subsequent quantification of the specific signals (Fig. 3). For this purpose freshly isolated CLL patient cells were cultivated either in the presence of DMSO or in the presence of 10 µM of the tyrosine kinase inhibitors bosutinib and saracatinib. In these experiments tyrosine phosphorylated proteins in CLL cell lysates were detected on Western blots by means of phosphotyrosine-specific antibodies. In addition, phosphorylation of the positively regulatory tyrosine residue in SFKs was probed by a pan-phospho-Src antibody and controlled by the determination of the amounts of Lyn, which is by far the predominantly expressed among SFK in CLL cells.

Fig. 2: Inhibition of Src family kinases in CLL cells by ATP competitive tyrosine kinase inhibitors. Freshly isolated CLL cells from patients no. 4 (A, B) and 12 (C, D) were analyzed for global tyrosine phosphorylation (A, C) or activating autophosphorylation of Src family kinases (B, D) after treatment with 10 µM bosutinib (Bo) or saracatinib (Sa) or the corresponding solvent controls (Ctr). Lanes, in which molecular weight standards were separated are labeled M. The sizes of the standards are indicated in kilodaltons. For the quantification of inhibitor effects the signals obtained with primary antibodies against actin (A, C) or Lyn (B, D) were used as standards and the treatment-dependent reduction of tyrosine phosphorylation at the double band representing SFK was calculated relative to untreated controls.
With both primary antibodies a clear reduction of the tyrosine phosphorylation levels of SFK by treatment with the kinase inhibitors was detected. With the exception of blot C the basal phosphorylation level of SFK in CLL cells was reduced stronger by bosutinib than by saracatinib, namely by about 80 % or 60 %, respectively.

**Reduction of the levels of anti-apoptotic Bcl-2 proteins in CLL cells by bosutinib and saracatinib**

Signaling cascades in CLL cells that involve SFK in their initial steps, including B cell receptor signaling, result in anti-apoptosis e.g. by regulating the protein levels of pro- and antiapoptotic Bcl-2 proteins in the mitochondrial membrane via transcriptional regulation and in some instances also directly by phosphorylation that results in modulation of protein stability. Pharmacological inhibition of SFK is therefore expected to result in the reverse changes. In order to investigate the mechanism of kinase inhibitor-induced apoptosis induction we examined the protein levels of the anti-apoptotic Bcl-2 proteins Bcl-2 and Mcl-1 that are involved in the regulation of cytochrome C release from mitochondria, which in turn triggers caspase cleavage cascades. The levels of the anti-apoptotic proteins Bcl-2 and Mcl-1 were compared in three CLL samples without and with addition of bosutinib and saracatinib (Fig. 3). Quantification relative to constantly expressed actin showed substantial decreases of anti-apoptotic Bcl-2 proteins after treatment with bosutinib and saracatinib.

![Fig. 3: Reduction of anti-apoptotic Bcl-2 protein levels in CLL cells by SFK inhibitors. Freshly isolated CLL cells from patients no. 12 (A), 13 (B) and 14 (C) were analyzed for relative protein levels of Bcl-2, Survivin and Mcl-1 with and without treatment by 10 µM of the inhibitors.](image)

**Biological effects of second generation Src-Abl inhibitors on CLL cells**

In order to compare the biological effects of tyrosine kinase inhibitors in a quantitative manner we assessed the dose dependent induction of phosphatidylserine exposure and reduction of metabolic activity in the cell line Mec1 and in a primary CLL sample (Fig. 4).
Fig. 4: Biological effects of second generation Src/Abl inhibitors on CLL cells. Mec1 cells (A, B) or freshly isolated CLL cells from patient 10 were incubated with the indicated concentrations of bosutinib, saracatinib or dasatinib and analyzed for phosphatidylserine exposure (A, C) or metabolic activity (B, D) after 24 or 72 hours of incubation respectively.

The XTT assay of metabolic activity indicated the inhibitor’s effect more sensitively than the annexin V binding assay, particularly for the strongly proliferating CLL-derived cell line Mec1, in which inhibitor-induced phosphatidylserine exposure was hardly observed at all. Bosutinib showed stronger biological effects than saracatinib and dasatinib, except for the lower inhibitor concentrations in the case of patient sample no. 10.

Dose-dependent induction of annexin V-binding by bosutinib and saracatinib

Dose-dependent effects of kinase inhibitors on freshly isolated CLL cells were recorded for several CLL samples (Fig. 5). As these examples show, there is considerable variation of inhibitor sensitivity among individual CLL samples. Bosutinib tended to induce more apoptosis than saracatinib. While the effects of bosutinib and saracatinib continued to increase in the micromolar range, dasatinib appeared to induce apoptosis to the degree achievable with this drug already at the lowest concentration tested of 0.3 µM.
**Fig. 5:** Dose-dependent induction of annexin V-binding by SFK inhibitors in four primary CLL samples.

**Fig. 6:**

**Differential inhibitor effects among primary CLL samples**

The effects of 10 µM bosutinib, saracatinib and dasatinib on eleven primary CLL samples were compared (*Fig. 6*). The percentages of annexin V-binding cells were significantly increased after treatment with all three kinase inhibitors (*Fig. 6A*). On the average 24 % of untreated cells showed phosphatidylserine exposure. This percentage was increased most pronouncedly by bosutinib to 55 %, followed by saracatinib (45 %) and dasatinib (37 %). The response to each of the inhibitors showed a high degree of variation among the investigated CLL samples and bosutinib on the average induced apoptosis with significantly higher efficiency than dasatinib (*Fig. 6B*).
Fig. 6: **Apoptosis induction by second generation Src-Abl inhibitors in CLL samples.**

**A:** The percentages of annexin V-negative cells after 24 h treatment with 10 µM dasatinib, bosutinib and saracatinib were determined by flow cytometry. The percentages of viable cells with and without kinase inhibitor treatment were compared by paired Student's T-test.

**B:** The quartiles of the relative loss of viability due to kinase inhibitor treatment show a highly variable response of individual CLL samples to each of the inhibitors. Bosutinib in contrast to saracatinib shows significantly better efficiency than dasatinib according to unpaired Student's T-test.
DISCUSSION

Treatment of Bcr-Abl positive leukemias has been revolutionized by the advent of efficient specific tyrosine kinase inhibitors \(^{14}\) and second generation Abl inhibitors have been developed to override kinase inhibitors resistance \(^{15}\). Although the pathogenesis of CLL in contrast is multifactorial, the dependence of this disease on stimuli from the microenvironment forms a rationale for Src family kinases as a therapeutic target (Fig. 1). Accordingly dasatinib was found to induce apoptosis in primary CLL cells \(^{29}\) and was reported to show clinical efficiency \(^{30}\). The preclinical effects at a pharmacologically achievable concentration of 0.1 µM were, however, low in the majority of patient samples, so that the target group that might eventually profit from dasatinib treatment is difficult to define and expected to be quite small. Therefore it was the goal of the present pre-clinical investigation to compare the potential of dasatinib with two additional second generation Src/Abl kinase inhibitors, namely bosutinib and saracatinib. To varying degrees all three inhibitors were shown to inhibit SFK auto-phosphorylation, to reduce the levels of anti-apoptotic Bcl-2 proteins and to induce apoptosis in CLL cells.

Since the kinase inhibitors investigated here have already been used in humans, information about pharmacologically achievable plasma concentrations is available. The rough concentrations found in phase I trials are in the order of 0.1 µM for dasatinib \(^{32,33}\), 0.2 µM for bosutinib \(^{34}\) and 0.4 µM for saracatinib \(^{25}\). Although the biological effects observed with 10 µM of saracatinib and bosutinib are superior to those with dasatinib (Fig. 6), this may be irrelevant with regard to the attainable plasma concentrations. Moreover, the dose response curves of saracatinib and bosutinib display a different shape than that of dasatinib (Fig. 5D), so that it is uncertain whether bosutinib and saracatinib are superior to dasatinib also at these low concentrations.

Dose-dependent cell killing by bosutinib was much less effective in CLL cells (Figs. 4, 5) than in CML cell lines \(^{35}\). Apoptosis induction by saracatinib was observed to varying degrees in primary CLL cells (0.5 % to 68 %, Fig. 6B) but did not occur in Mec1 cells (Fig. 4A). This is compatible with the observation that apoptosis is efficiently induced by saracatinib in the B cell lymphoma cell line DOHH-2 but not in Raji or Jurkat cells \(^{24}\).

The inter-sample variation of inhibitor sensitivity is about equally wide for the three inhibitors, but the distribution of drug responsiveness among CLL samples does not follow any common patterns for the three inhibitors with identical postulated primary tyrosine kinase
targets. Similarly it was impossible to detect any correlations between inhibitor responsiveness and prognostic subgroups of CLL due to insufficient sample number.

As compared to the described dasatinib effects on CLL cells \(^{29}\), the reduction of SFK autophosphorylation by bosutinib and saracatinib appears less efficient, since it was obtained at much higher inhibitor concentrations. SFK inhibition by dasatinib in CLL cells occurs independently of the concomittant dasatinib-induced apoptosis, while the phosphorylation of the direct SFK substrates Syk and PLC-\(\gamma\) are correlated with the apoptotic response to dasatinib \(^{36}\). The reduction of SFK auto-phosphorylation by bosutinib was stronger than that by saracatinib to a similar degree than the average apoptosis induction by these inhibitors in eleven CLL samples, but this is not a basis for concluding that these effects are causatively related.

Small molecule-kinase interaction maps have been compiled on the basis of binding affinities \(^{37}\) and are available for dasatinib \(^{38}\) and bosutinib \(^{39}\). Apart from inhibition of SFK, other target kinases may be of therapeutic use for treating CLL, e.g. the receptor tyrosine kinase Axl, which is inhibited by bosutinib and expressed in CLL cells and bone marrow stromal cells \(^{40}\).

In summary the present comparison of Src-Abl kinase inhibitor effects on CLL cells \textit{ex vivo} suggests, with the mentioned limitations, that bosutinib and to a lesser degree also saracatinib may exhibit potentially superior efficiency than dasatinib, but this trend needs to be substantiated in a higher number of samples and as a direct comparison at pharmacologically achievable inhibitor concentrations, preferably also incorporating stimuli from the microenvironment of CLL cells and extended analysis of signaling pathways.
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