Chapter 3

Raf-1 is overexpressed in chronic lymphocytic leukemia and promotes cell survival by phosphorylation of ERK and Bad

Miao Wang¹, Philip Kluin¹, Stefano Rosati¹, Marjan Luinge¹, Simon Daenen², Lydia Visser¹

¹Department of Pathology & Laboratory Medicine, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands. ²Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

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Abstract:

Raf-1 kinase plays a protective role in many cell types, but its expression and function in chronic lymphocytic leukemia (CLL) has not been studied in detail. By using qRT-PCR and western blot, we identified a significantly higher expression of Raf-1 in CLL cells than in non-germinal center B-cells isolated from normal tonsils. Constitutively active p-Raf-1 (Ser338) was detected in all CLL cases (N=45) and 4 CLL cell lines. Furthermore, p-Raf-1 and p-ERK1/2 (Thr202/Tyr204) were upregulated after IgM treatment in ZAP-70 positive CLL samples, whereas p-Bad (Ser112, Ser136, and Ser155) was constitutively expressed in all CLL cases. By immunoprecipitation and confocal studies, we demonstrated that Raf-1 co-localizes with Bcl-2, which may account for Bad phosphorylation. In CLL cell lines the inhibition of p-Raf-1 by three different inhibitors (GW5074, YC 137 and Geldanamycin) led to the downregulation of p-ERK and p-Bad, as well as a diminished cell growth. Furthermore, Raf-1 inhibition altered the cell cycle by downregulating cyclin D3 and cyclin E expressions, which are important for G0/G1 transition. Moreover, the Raf-1 inhibitor GW5074 induced apoptosis in all CLL cases. In conclusion, our study identified Raf-1 as a critical anti-apoptotic and cell cycle regulating kinase in CLL.
Introduction

CLL is characterized by a relatively low rate of proliferation and a high anti-apoptotic activity as exemplified by the very high expression of Bcl-2. In addition B-cell receptor (BCR) signaling plays an essential role in survival and expansion of CLL cells, as illustrated by numerous studies on the significance of immunoglobulin gene usage, somatic hypermutations and ZAP-70 expression in these cells. Several kinases have been investigated for their possible roles in tumor cell survival of lymphoma, with Syk, ERK and Akt receiving the most attention to date. In cancer other than lymphomas, many treatment modalities that target the Raf/ERK pathway have been developed. However, the function of Raf-1 in B cells and B cell activation is still incompletely characterized.

Raf-1 (a MAP kinase kinase kinase - MAP3K) was originally described as an important target of Ras in receptor tyrosine kinase signaling. In this pathway, activated Raf-1 initiates a signaling cascade involving the phosphorylation of numerous downstream molecules including the kinases MEK and ERK1/2, which leads to cell proliferation. Based on this notion, it has been proposed that Raf-1 is an important therapeutic target in cancer.

In addition, there is a unique ERK-independent function for Raf-1 in cell survival. Raf-1 and Bcl-2 can combine to cooperate in the suppression of apoptosis. After being recruited by Bcl-2 to the mitochondrial membrane, Raf-1 can directly phosphorylate and inactivate Bad, leading to an anti-apoptotic effect. Therefore, we hypothesized that Raf-1 may play a key role in CLL cell growth not only by the Raf-1/ERK pathway but also by combining with Bcl-2.

We examined Raf-1 and Bcl-2 expression in CLL cases by qRT-PCR, western blot and immunohistochemistry. We checked the activity of Raf-1 in CLL cases and cell lines. By using immunoprecipitation and confocal microscopy in CLL cell lines that overexpress Bcl-2, we analyzed the possible colocalization of Raf-1 and Bcl-2. We also investigated cell growth, apoptosis and cell cycle changes by using specific inhibitors of Raf-1 and Bcl-2.

Material and Methods

Samples

45 blood cell suspensions and 23 paraffin-embedded tissue samples of CLL were obtained from patients diagnosed with CLL in the departments of Hematology and Pathology, University Medicine Center Groningen. Tonsil cell suspensions of 5 hyperplastic tonsils were used as control B cells. All protocols for obtaining and studying human tissues and cells were approved by the institution’s review board for
human subject research.

CLL cell lines JVM-3, MEC-1 and MEC-2 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The CLL cell line MO1043 was a gift from Dr. Ricardo Dalla-Favera (Columbia University, New York, NY). JVM-3 and MO1043 cells were propagated in RPMI 1640 medium containing 10% fetal bovine serum. MEC1 and MEC2 cells were propagated in Iscove’s modified Dulbecco’s medium containing 10% fetal bovine serum.

qRT-PCR

RNA was extracted by using TRIZOL. Turbo DNAse treatment and qRT-PCR were performed as described by the manufacturer (Ambion, Foster City, CA, USA). GAPDH was used for normalization ($\Delta Ct = Ct_{gene} - Ct_{GAPDH}$) and to check the quality of the samples, i.e. only cases with a cycle threshold (Ct) value lower than 35 for GAPDH were used. Probes and primers were obtained from Applied Biosystems (Foster City CA, USA). Raf-1, Bcl-2, Syk and ZAP-70 mRNA expression were analyzed by qRT-PCR. Relative expression levels were determined by using the formula $2^{-\Delta Ct}$.

Antibodies

Rabbit monoclonal antibodies to Raf-1 and Bcl-2 (for immunohistochemistry and western blot) were purchased from Epitomics (Burlingame, CA, USA). Mouse monoclonal antibody reacting with phospho (p)-Raf-1(Ser338) was purchased from Upstate (Millipore, Billerica, MA, USA). P-ERK1/2 (Thr202/Tyr204), ERK1/2, HSP90 (E289), p-Bad (Ser112), p-Bad (Ser136), p-Bad (Ser155), p-p38 MAPK (Thr180/Tyr182), p21 (DCS60), survivin (71G4B7E), Bcl-xL, p27 and cyclin D3 antibodies were purchased from Cell Signaling Technology (Boston, MA). Mouse monoclonal antibodies to Bad and cyclin E were purchased from BD Bioscience (Rockaway, NJ, USA). Mouse monoclonal Bcl-2 antibody (for confocal microscopy), Ki-67, HRP-labeled rabbit anti mouse antibody, HRP-labeled goat anti rabbit antibody and FITC-conjugated swine anti rabbit antibody were purchased from DAKO (Glostrup, Denmark). TRITC-conjugated goat anti mouse antibody was purchased from Southern Biotechnology Associates (SBA, Birmingham, AL, USA).

Cell purification and treatment

CLL and tonsil cells were grown 4 hours in RPMI-1640 medium with 10% FBS to let the cells recover. For T cell depletion in CLL samples, cells were incubated with anti-CD3 (OKT3, LCG, Middlesex, UK) and depleted with Dynal magnetic beads (Invitrogen, Breda, The Netherlands). For tonsil B cell isolation, cells were incubated with anti-CD3 and anti-CD38, to remove both T cells and most of the germinal center B cells. Treatment with IgM was performed as previously described 3. In short, $1 \times 10^7$ cells /
ml cells were incubated with 10 µg/mL biotinylated goat anti human immunoglobulin M (IgM) F(ab’)2 (SBA) on ice for 10 minutes. Avidin (Sigma, St Louis, MO, USA) was added in a final concentration of 10 µg/mL and the cell mixture was incubated at 37°C for 10 minutes to have an effect on BCR signaling. For Western blotting cell pellets were lysed in 1x Sample Buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue).

For MTT assays and cell cycle analysis cell lines were cultured at 1x 10^6/ml during 48 hours in 6-well plates in the presence of varying concentrations of GW5074 (a selective Raf-1 inhibitor), YC 137 (a selective Bcl-2 inhibitor) (Merck, Darmstadt, Germany), ERK1/2 inhibitor (3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione hydrochloride, Biaffin Gmbh & Co KG, Kassel, Germany) and Geldanamycin (GA, Invivogen, San Diego, CA, USA). Optimal concentrations of all inhibitors were checked by western blot.

**Immunohistochemistry (IHC)**

The slides were deparaffinized and endogenous peroxidase was blocked by incubation with 0.3% H$_2$O$_2$ for 10 minutes. Antigen retrieval was performed according to the various protocols of the manufacturers. Immunostaining was performed using Raf-1, Bcl-2, Ki-67, HSP90, Bad, p-Bad, ERK1/2, pERK1/2 and cyclin D3 at a dilution of 1:50-1:100. Immunostaining was amplified by incubation with the appropriate HRP-conjugated antibodies for 60 min and the reactivity was visualized by diaminobenzidin. Appropriate positive and negative controls were used.

**Western Blot**

Cell lysates were separated on a polyacrylamide gel and electro blotted onto a nitrocellulose membrane. Blots were incubated for 60 min in blocking buffer (TBS-T (TBS, 0.05% Tween 20, pH 7.6) with 5% skimmed milk) to block the membrane, washed by 0.1% TBST and incubated with primary antibodies at 4 ºC overnight. Immunostaining was amplified by incubation with HRP-conjugated antibodies for 60 min. Blots were washed and chemiluminescence was detected with ECL (Pierce, Rockford, USA).

**Immunoprecipitation (IP)**

IP was performed as described by the manufacturer (Epitomics). Cell lysate was diluted to 1 mg/ml total cell protein with PBS. 4 µg of Raf-1 and Bcl-2 antibody was added to 500 µg cell lysate and incubated at 4°C overnight. The immune complex was captured by the addition of Protein G agarose beads (Roche Molecular Biochemicals, GmbH, Mannheim, Germany). After washing with PBS, the beads were resuspended in 2x sample buffer (10 % glycerol, 62.5 mM Tris HCL (pH 6.8), 2 % SDS, 0.03% (w/v) bromophenol blue) and heated at 95-99°C for 5 minutes.
Figure 1: Raf-1 is overexpressed in CLL cells at mRNA and protein levels

A: mRNA expression of Raf-1 and Bcl-2 in purified B cells of 5 reactive tonsils (CD3-CD38- cells) and 45 CLL cases (CD3- cells) was detected by qRT-PCR. Each bar represents mean ± SD. p < 0.01 is significantly different from tonsil. B: Correlation of Raf-1 and Bcl-2 expression by qRT-PCR. Significance of linear correlation: p<0.01. C: Immunohistochemical detection of Raf-1 and Bcl-2 protein expression in reactive tonsils and CLL cases. In tonsil, Raf-1 (I) and Bcl-2 (III) are highly expressed in the mantle zone and interfollicular region. In CLL cases, Raf-1 (II) and Bcl-2 (IV) are highly expressed in all the cells (200x). D: Western blot detection of Raf-1 protein expression in 5 tonsillar B cells (CD3-CD38-) and 25 CLL B cells (CD3-); for both groups 1 representative case is shown. Actin was used as a loading control. Raf-1 expression level as measured by western blot (Raf-1/actin ratio) in CLL cells compared to tonsil B cells (significance of difference p<0.01).
SDS-PAGE and immunoblot analysis were performed on a sample of the supernatant fraction. Protein G agarose beads without the addition of antibodies to the cell lysates were used as negative controls.

**Cell growth measurements**

The MTT assay was performed as previously described\(^\text{18}\). 20 μl of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) in medium was added to 200 μl cell suspension and incubated for 4 hours at 37°C to let the cells absorb the MTT. The cells were centrifuged at 1000 rpm for 10 minutes and the supernatant was removed. 150 μl of DMSO (Sigma) per well was added to dissolve the pellet completely. The absorption was measured at 540nm in an ELISA reader.

**Cell cycle analysis**

Cell cycle profiles were analyzed by flowcytometry. Cells (1 \( \times \) 10\(^6\)) were washed in phosphate-buffered saline with 0.1% BSA. Hypotonic DNA staining buffer (0.1% Sodium citrate; 0.3% Triton–x 100; 0.01% Propidium iodide, 0.002% Ribonuclease A) was added to the pellet and mixed well. The percentage of cells in different departments of the cell cycle was analyzed by ModFit LT3.

**Apoptosis measurements**

Annexin V staining (IQ products, Groningen, the Netherlands) and analysis by flowcytometry was used to determine the percentage of apoptotic cells in cell lines after incubation with inhibitors for 4 and 24 hours. Samples of CLL cases were treated for 4 hours with inhibitors and a double staining with CD19 (BD Biosciences) was used to measure the percentage of apoptotic B cells.

**Statistical analysis**

All data were derived from at least three independent experiments. Quantity one software was used to quantify western blot bands. Statistical analyses were conducted using Prism 5 software, and values were presented as means ± SD. Significant differences between the groups were determined using Student’s t-test.

**Results:**

**Raf-1 is overexpressed in CLL cells at the mRNA and protein level**

Firstly, we examined the gene expression of Raf-1 and Bcl-2 in the purified B cells of 45 CLL cases (CD3- B cells) and 5 reactive tonsils (CD3-CD38- B cells) by qRT-PCR. Our results revealed a significantly higher expression of Raf-1 (about 6 fold) and Bcl-2 (about 12 fold) in CLL cases than the normal B cells (P<0.01) (Figure 1A).
Figure 2: Raf-1 is activated in CLL cases and cell lines and co-localizes with Bcl-2
A: Western blot analysis of p-Raf-1(Ser338), p-ERK1/2 (Thr202/Tyr204) and p-Bad (Ser112, Ser136, or Ser155) before and after IgM stimulation in 25 CLL cases of purified B cells and 4 CLL cell lines. Actin is used as a loading control. Two cases are shown as representative examples for the response and no response groups. MEC-2 is shown as a representative cell line. Similar results were found in all four cell lines. B: 14 CLL cases in the response group have a significantly higher ZAP-70/SYK ratio compared to the no response group (p<0.01). Each point represents the ZAP-70/SYK mRNA ratio of the samples. C: Western blot analysis for Raf-1, Bcl-2 and HSP90 after immunoprecipitation of Raf-1 and Bcl-2 proteins. Antibody used for IP in columns, antibody used for detection (IB) in rows. MEC-2 is shown as a representative cell line. D: Confocal microscopy for Raf-1 and Bcl-2 in the MEC-2 cell line (200x). P: Phosphorylation; IP: immunoprecipitation; IB: immunoblot.
Furthermore, there was a significantly linear correlation between Raf-1 and Bcl-2 gene expression levels (p<0.01, R^2=0.8698) (Figure 1B).

Secondly, we analyzed the protein expression of Raf-1 and Bcl-2 by immunohistochemistry (Figure 1C). In normal tonsils, Raf-1 and Bcl-2 were highly expressed in mantle zone B cells and interfollicular T cells, whereas they were weakly expressed or negative in germinal center B cells. In CLL cases, Raf-1 and Bcl-2 were highly expressed in all cells with a somewhat stronger expression of Raf-1 in the cells of proliferation centers.

Thirdly, we performed western blot analysis for Raf-1 and Bcl-2 on isolated B cells (CD3- cells) from 25 CLL cases and 5 reactive tonsils (CD3-CD38- cells) to analyze the protein expression level. There was higher expression of Raf-1 protein in CLL cells than tonsil B cells. This was semiquantitatively confirmed by densitometry: Raf-1 was significantly higher expressed in CLL cells compared to tonsil B cells (p<0.01) (Figure 1D). When measuring protein levels by western blot analysis no correlation between Bcl-2 and Raf-1 expression was found.

In conclusion, our results showed that Raf-1 is overexpressed in CLL cells at both the mRNA level and protein level.

**Raf-1 is highly activated after IgM stimulation in ZAP-70 positive CLL cases**

Two groups of CLL cases were identified by the reaction to IgM stimulation. 14 CLL cases were named the “response group” in which cells were highly upregulating p-Raf-1 and p-ERK expression upon IgM stimulation; 11 CLL cases were designated as the “no response group” in which there was no obvious change of p-Raf-1 and p-ERK expression after IgM stimulation (Figure 2A, supplementary data). Of note the p-Raf-1 and p-ERK expression in the no response group was already high before stimulation and thus, the no response group seems to be constitutively active. To study the correlation of ZAP-70 expression with this response, we used the ZAP-70/ SYK mRNA expression ratio levels instead of the ZAP-70 levels\(^{19}\). A higher ZAP-70/ SYK ratio was observed in the response group than in the no response group (p<0.01) (Figure 2B). This means that most ZAP-70 positive cases as defined by a ZAP-70/Syk ratio of more than 0.2 have an inducible Raf-1 / ERK pathway.

A recent report\(^{20}\) showed that co-localization of Raf-1 and Bcl-2 can lead to phosphorylation of Bad. CLL is characterized by a very high expression of Bcl-2 protein\(^{21}\) and as presently shown also Raf-1. In line with these findings and regardless of IgM stimulation, all CLL cases and all four CLL cell lines (MEC-1, MEC-2, MO1043 and JVM-3) showed a high expression of p-Bad (Ser112, Ser136 and Ser155; Figure 2A). We investigated co-localization of these proteins and of HSP90, an important 
Figure 3: Raf-1 inhibition leads to changes in cell growth and induction of apoptosis by downregulation of p-ERK and p-Bad.

A: Cell lines were treated with different inhibitors (10 μM GW5074, 10 μM GA, 10 μM YC 137 or 10 μM ERK1/2 inhibitor) for 24 hours and cell growth was analyzed with the MTT assay. Results are expressed as the percentage cell growth compared to control (untreated) cells.

B: Cell lines were treated with different concentrations of inhibitors (GW5074, YC137) or inhibitor combinations (GW5074 and YC137) for 24 hours. The percentage of cell growth was detected by MTT.

A-B: Each bar represents mean ± SD of difference between the four cell lines. *p < 0.01 is significantly different from control.

C: Western blot analysis for p-Raf-1 (Ser338), p-ERK1/2 (Thr202/Tyr204), p-Bad (Ser112, Ser136 and Ser155) and p-p38 (Thr180/Tyr182) in the 4 CLL cell lines. Total levels of Raf-1, Bcl-2, Bad, HSP90, survivin and BCL-xL proteins were also analyzed. Actin was used as a loading control.

D: Flow cytometry for Annexin V in the 4 CLL cell lines. On the x-axis the signal for Annexin V FITC is shown, on the y-axis the cell counts.
chaperon of Raf-1 and Bcl-2 in the CLL cell lines. Using immunoprecipitation of Raf-1 or Bcl-2 and western blot analysis for Raf-1, Bcl-2 and HSP90 (Figure 2C), we found that part of the Raf-1 protein co-localizes with Bcl-2. Moreover, HSP90 protein co-precipitated efficiently with both Raf-1 and Bcl-2. We also performed confocal microscopy showing co-localization of Raf-1 and Bcl-2 in cytocentrifuge preparations of all 4 CLL cell lines (Figure 2D).

Together, these data imply that Raf-1 does not only act as an upstream activator of ERK but is also involved in the Bad/Bcl-2 pathway.

**Raf-1 kinase inhibition leads to changes in cell growth and apoptosis by downregulating p-ERK and p-Bad**

Based on the findings that Raf-1 is constitutively active and can colocalize with Bcl-2, we studied changes in cell growth and cell signaling by using the Raf-1 inhibitor GW5074, the Raf-1 destabilizer GA, the Bcl-2 inhibitor YC 137 and the ERK1/2 inhibitor. Optimal concentrations were chosen using western blot analysis. Cell lines were treated up to 2 days and analyzed for cell growth with the MTT assay. As shown in Figure 3A, 10 μM GW5074, 10 μM GA or 10 μM YC 137 significantly blocked the cell growth in all cell lines. Furthermore, there was an additive effect of the Raf-1 inhibitor when combined with the Bcl-2 inhibitor (Figure 3B). The ERK1/2 inhibitor (10 μM) did not significantly affect cell growth, although phosphorylation of ERK1/2 was inhibited.

Next, we studied p-Raf-1 (Ser338), p-ERK1/2 (Thr202/Tyr204) and p-Bad (Ser112, Ser136 and Ser155) levels before and after treatment with the inhibitors (GW5074, GA and YC 137) for 24 hours. All three inhibitors led to downregulation of p-Raf-1, p-ERK and p-Bad with all three inhibitors (Figure 3C). In contrast, no downregulation of p-p38 was observed, except for some downregulation after GA treatment. We also determined total protein content in western blots before and after treatment during 24 hours. Raf-1 was slightly downregulated after GW5074 and YC 137 treatment and obviously decreased after GA treatment. There was a reduction in Bcl-2 and Bad proteins after YC137 or GA, but no change in HSP90, survivin and BCL-xL (Figure 3C). Thus, Raf-1 inhibition by the inhibitors GW5074, GA and YC 137 can lead to both p-ERK1/2 and p-Bad inhibition, which may account for the observed diminished cell growth. At the same time, these inhibitors also reduce the amount of Raf-1, Bad and Bcl-2, possibly by enhanced protein degradation.

Thirdly, using Annexin V staining in the CLL cell lines we analyzed if the inhibitors can lead to apoptosis. Cells were cultured for 4 and 24 hours. Apoptosis could be induced in all four cell lines after treatment with GW5074, GA and YC 137, but there was no obvious effect of the ERK1/2 inhibitor (Figure 3D). We also examined if these inhibitors
Figure 4: Raf-1 and ERK inhibition leads to CLL apoptosis. 22 cell suspensions of CLL cases were treated with 10 μM GW5074, 10 μM GA, 10 μM YC 137 or 10 μM ERK1/2 inhibitor for 4 hours. Shown are the percentages of apoptotic B cells (Annexin V positive CD19 positive cells) induced after treatment. A: Different inhibitors are represented on the x-axis and the percentage of apoptotic cells is represented on the y-axis. B: The effect on apoptosis of GW5074 and the ERK 1/2 inhibitor in individual cases. Case numbers are represented on the x-axis (cases are ordered based on increasing effect of ERK1/2 inhibitor) and the percentage of apoptotic cells is represented on the y-axis.
can cause apoptosis in patient samples after culturing for 4 hours. This short period was chosen to exclude nonspecific effects. In all 22 tested CLL samples, GW5074 induced apoptosis in 3 to 30% of the CD19 positive cells. Different from the cell lines, the ERK1/2 inhibitor did cause apoptosis in only a part of CLL cases; no apoptosis was detected after YC137 or GA treatment in the CLL cases (Figure 4A). Interestingly, the cases more sensitive to the Raf-1 inhibitor seemed to be less sensitive to the ERK1/2 inhibitor and vice versa (Figure 4B, supplementary data). No correlation was found between the amount of apoptosis and the p-ERK or p-Raf-1 status as measured by western blot (supplementary data).

In summary, Raf-1 kinase inhibition can block cell growth and can lead to apoptosis by downregulating p-ERK and p-Bad.

Raf-1 kinase inhibition leads to G0/G1 cell cycle arrest by downregulating cyclin D3 and cyclin E expression

To determine whether the inhibitors can alter the cell cycle distribution, CLL cell lines were treated with the inhibitors GW5074, YC 137, GA or ERK1/2 inhibitor for 24 hours and analyzed by DNA flowcytometry. No cell cycle change was detected with the ERK1/2 inhibitor, but different cell cycle changes were observed with the treatment of the other inhibitors. GW5074 mainly led to G0/G1 arrest whereas YC 137 mainly led to G2/M cell cycle arrest. GA decreased the percentage of cells in S phase, with an increased percentage of cells in both the G0/G1 phase and G2/M phase (Figure 5A, 5B).

To further investigate other proteins that could be involved in cell cycle changes, we studied expression of various cell cycle regulators before and after inhibitor treatment for 24 hours. We observed a pronounced reduction of cyclin D3 and cyclin E protein levels in cells treated with GW5074, YC 137 or GA. p21 downregulation was only observed after YC 137 treatment. There was an obvious reduction of p27 after YC 137 or GA treatment (Figure 5C).

Based on the notion that there is higher expression of Raf-1 in the cells within the proliferation centers (PCs) of CLL tissues, we speculated that there is higher expression of the cell cycle markers in this structure as well. Immunohistochemistry was performed for Ki-67, cyclin D3 and cyclin E in 23 paraffin-embedded tissue samples of CLL. Our results revealed that Cyclin D3 is specifically overexpressed in PCs (Figure 5D). Unfortunately, the staining of cyclin E failed.

In conclusion, Raf-1 kinase plays a role in cyclin D3 and cylin E expression, which is important for G0/G1 cell cycle transition.

Discussion
Figure 5: Raf-1 inhibition leads to changes in the cell cycle by downregulating cyclin D3 and cyclin E expression. A-B: The 4 CLL cell lines were treated with 10 μM GW5074, YC 137, GA and ERK1/2 inhibitor for 24 hours and analyzed by flow cytometry after propidium iodide staining. MEC-2 is shown as a representative example. Similar results were found in all four cell lines. DNA content is represented on the x-axis and the cell count is represented on the y-axis (A). Different inhibitors are represented on the x-axis and the percentage of cells in various phases of the cell cycle as determined by flow cytometry is represented on the y-axis (B). C: Total cell lysates were prepared in the presence or absence of inhibitors for 24 hours followed by western blot analysis for specific cell cycle signaling proteins. Actin was used as a loading control. MEC-2 is shown as a representative example. Similar results were found in all four cell lines. D: Immunostaining was performed in 23 paraffin-embedded tissue samples of CLL. One case is shown as a representative example. Ki-67 staining was used (I) as a marker for proliferation centers. Cyclin D3 (II) is specifically overexpressed in proliferation centers of CLL cases (200x).
The objectives of the present study were to determine the expression and function of Raf-1 kinase in CLL. Our study showed four major findings. First, we found a strong overexpression of Raf-1 in CLL cells at mRNA and protein levels. Secondly, we found that the p-Raf-1 and p-ERK response to IgM stimulation is correlated with the ZAP-70 status. Thirdly, Raf-1 inhibition by GW5074 can lead to cell apoptosis in all CLL cases. Fourthly, Raf-1 kinase plays an important role in cell cycle regulation by affecting the expression of cyclin D3 and cyclin E.

Our results showed a high expression of Raf-1 in CLL cells at mRNA and protein level. Raf-1 activity is controlled by its phosphorylation status, especially at serine 338, which contributes to ERK1/2 activation, the mitochondrial location of Raf-1 and phosphorylation of Bad. The Ras-Raf-mitogen-activated protein kinase cascade mediates pro-survival signals in a broad spectrum of human tumors and is considered as an attractive target for anticancer therapies. The methods of targeting Raf include the use of antisense oligodeoxynucleotides (ASON), Raf-1 inhibitors such as GW5074 or Raf-1 destabilizers such as GA. The anti-tumor activity of the Raf-1 antisense ISIS 5132 has been evaluated in phase II studies in patients with ovarian, small-cell lung and non–small-cell lung carcinomas. GW5074 is a benzylidine oxindole derivative that inhibits the Raf/MEK/ERK kinase cascade by blocking the kinase activity of Raf-1. GA is a benzoquinone ansamycin antibiotic that binds to HSP90 (Heat Shock Protein 90) and leads to destabilization of HSP90 client proteins. Piatelli et al reported that GA inhibits the Raf-1/ERK pathway by proteasome mediated degradation of Raf-1. Therefore, disruption of Raf-1 activity by specific or more indirect inhibitors might open a novel therapeutic modality in B cell malignancies.

By using IgM stimulation and specific inhibition of Raf-1, we demonstrated that p-Raf-1 plays a key role in cell survival by regulating p-ERK and p-Bad activity. Our results showed that p-ERK was constitutively phosphorylated in part of the CLL cases and all cell lines. The constitutively activated ERK1/2 in all CLL cell lines may be accounted for by EBV infection. Muzio et al also found that unresponsive CLL cases constitutively express ERK1/2 and that this should be considered as a molecular imprint of an anergic state. We confirmed these data and extended them, showing that both the p-Raf-1 and p-ERK1/2 response to IgM stimulation correlated with the ZAP-70/SYK mRNA expression ratio, which is a solid marker of the mutational status of the IgH genes and prognosis. Many of our CLL cases within the responsive CLL group did not show any detectable p-ERK expression without IgM treatment and ERK1/2 inhibitor did not lead to apoptosis in CLL cell lines, which suggests that there is also a p-ERK independent cell survival pathway. Our results show that p-Bad (Ser112, Ser136 and Ser155) is constitutively activated in all CLL cases and cell lines irrespective of IgM treatment. Bad is a pro-apoptotic member of the Bcl-2 family that promotes cell death by displacing...
Bax from binding to Bcl-2 and Bcl-xL. Survival factors such as cytokines or epidermal growth factor, can inhibit the apoptotic activity of Bad by activating intracellular signaling pathways that result in its phosphorylation at Ser112, Ser136, or Ser155. Phosphorylation at these sites prevents an association between Bad with Bcl-2 or Bcl-xL and thus contributes to cell survival.

Our results suggested that Raf-1 is involved in Bad phosphorylation by colocalizing with Bcl-2. Wang et al reported that Bcl-2 can be co-immunoprecipitated with Raf-1 when transfected in the mammalian hematopoietic cell line 32D.3. Raf-1 is targeted to the outer mitochondrial membrane after co-localization with Bcl-2 where it regulates apoptosis through phosphorylation of Bad. By transfection of Raf-1 or Bcl-2, Moye et al found that Bcl-2 can synergize with the activated Raf-1 to abrogate the cytokine dependency of the murine FDC-P1 hematopoietic cell line. Moreover, a recent report demonstrated that the interaction between Raf-1 and Bcl-2 is regulated by Raf-1 phosphorylation at Ser 338/9 by using a S338/9A mutant transfection. Our results show that both the Bcl-2 inhibitor (YC137) and Raf-1 inhibitor (GW5074) can lead to downregulation of p-Raf-1 and p-BAD, which is in agreement with these reports. Moreover, they show that inhibition of Raf-1 by GW5074 treatment leads to immediate apoptosis in all CLL cases, which is independent of the p-ERK status.

Raf-1 kinase inhibition led to cell cycle deregulation by downregulating cyclin D3 and cyclin E in all cell lines, which is in agreement with a recent report that showed Raf-1 activation increased the expression of cyclin D and cyclin E in Raf-1 transfected human TF-1 hematopoietic cells. Cyclin D3 plays a prominent role in differentiation and proliferation and is also important for G1/S transition and highly expressed in various kinds of cancers. Our IHC results showed that cyclin D3 is specifically overexpressed in PCs of CLL. In lymph nodes and the bone marrow involved with CLL, PCs are the hallmark of this lymphoproliferative disorder. Tumor cells in PCs showed a distinct antigen expression profile (Ki-67+, CD69+, CD71+, CD38+) and active BCR signaling compared to the surrounding tumor cells. Moller et al reported that cyclin D3 overexpression might identify a subpopulation of patients with indolent B cell lymphoma with adverse clinical features and poor outcome. Furthermore, a recent study showed that downregulation of cyclin D3 enhances fludarabin mediated killing of WSU-CLL cells by increasing the number of cells undergoing apoptosis. In normal dividing cells, cyclin E regulates the transition from the G1 phase to the S phase and a high level of cyclin E protein accelerates the transition through the G1 phase. By analyzing the cyclin E expression with western blot, Bogner et al reported that cyclin E was differentially expressed in ZAP 70-positive and ZAP 70-negative B-CLL cells, which may reflect a larger proliferating compartment in ZAP 70-positive patients. This suggests that cyclin E might add prognostic information for patients with B-CLL, but might also provide a promising target for treatment in CLL.
Collectively, our studies identify Raf-1 as a critical anti-apoptotic kinase via ERK and Bad phosphorylation and a key player in cell cycle progression. Raf-1 may therefore be considered as a potential target for therapy in CLL.

References

Chapter 3


Supplementary data 1

General information of CLL samples

<table>
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<tr>
<th>Sample</th>
<th>Ref-1</th>
<th>Bcl-2</th>
<th>Syk</th>
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1: Relative expression levels were determined by using the formula 2−ΔΔCt. CT = CT gene – CT GAPDH.
2. ZAP-70/SYK mRNA level ratio.
3. The pixel density of the unstimulated samples of actin, phospho-ERK and phospho-Raf-1 are analyzed by Quantitative One software.
4: Response to IgM stimulation detected by western blot. N: no response; Y: there is response
5. Response to inhibitors detected by Annexin V after inhibition with inhibitors for 4 hours. ND:not done.