Published in final edited form as:

Leukemia. 2016 June ; 30(6): 1431–1436. doi:10.1038/leu.2015.323.

Axl Activates Fibroblast Growth Factor Receptor Pathway to Potentiate Survival Signals in B-Cell Chronic Lymphocytic Leukemia Cells

Sutapa Sinha¹, Justin Boysen¹, Michael Nelson¹, Steven L. Warner², David Bearss², Neil E. Kay¹, and Asish K. Ghosh^{*,3}

¹Division of Hematology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905

²Tolero Pharmaceuticals, Inc., Salt Lake City, UT 84070

³Stephenson Cancer Center, Department of Pathology, The University of Oklahoma, Oklahoma City, OK 73104

Letter to the Editor

The fibroblast growth factor (FGF) family and their four receptors, FGFR1/2/3/4, mediate multiple physiologic processes, including cell migration, proliferation, survival, and differentiation. FGFRs are expressed on nearly every cell type of hematopoietic origin, and the deregulation of FGFR gene expression and/or gene mutation has been found in hematologic malignancies¹. Given this, we and others have detected that CLL B-cells spontaneously produce the basicFGF (bFGF) and CLL plasma contain elevated levels of bFGF². However, the precise role of FGF/FGFR signaling pathway in CLL B-cell survival and apoptotic resistance remains undefined.

To explore whether bFGF plays a role in CLL B-cell survival, we first examined the expression profile of FGFR1/R2/R3/R4 in CLL B-cells by Western blot analysis using specific antibodies. We find that CLL B-cells overexpress FGFR3 significantly (**Fig. 1A**). While a low level expression of FGFR1/R2/R4 was noted in CLL B-cells, these levels were no significantly different than those detected in normal B-cells (**Supplementary Fig. 1A**). It appears that CLL B-cells predominantly express two splice variants of FGFR3 with molecular weights of ~100/125kDa in Western blots. Thus, the banding pattern of FGFR3 as shown in **Fig. 1A** was further confirmed using a different antibody to FGFR3

***Correspondence:** Asish K. Ghosh, Ph.D., Stephenson Cancer Center, Department of Pathology, The University of Oklahoma, 975 NE 10th Street, BRC North 364, Oklahoma City, OK 73104; Phone: 405-271-8001 Ext. 48998; Fax: 405-271-2141; ; Email: Asish-Ghosh@ouhsc.edu

Authorship

Contribution: S.S. performed experiments, analyzed data and created figures; J.B. and M.N. performed experiments; S.W. and D.B. provided access to the Axl inhibitor TP-0903, and edited manuscript; N.E.K. edited the manuscript; A.K.G. conceived and supervised the project, designed the research, analyzed data and wrote the manuscript; and all authors read and approved the final manuscript.

Conflicts of Interest

Authors declare no potential conflict of interest.

Supplementary information is available at *Leukemia's* website

(**Supplementary Fig. 1B**). Although several splice variants are known to exist for each member of the FGFR family³, the mechanism of their regulation(s) is largely undefined.

To validate our findings that CLL B-cells primarily express FGFR3, individual FGFRs were immunoprecipitated from equal amount of lysates from CLL B-cells or normal B-cells followed by Western blot analyses to detect FGFR1/R2/R3/R4. As expected, we detected significantly elevated levels of FGFR3 in CLL B-cells as compared to normal B-cells (**Fig. 1D**). Although we were able to detect FGFR1 (**Fig. 1B**), FGFR2 (**Fig. 1C**) and FGFR4 (**Fig. 1E**) in CLL B-cells, the level of expression was comparable with those in normal B-cells. Furthermore, significantly higher levels of FGFR3 were also detected on CLL B-cells vs. normal B-cells in flow cytometric analysis (**Fig. 1F&G**). Finally, FGFR3 transcript was detected in CLL B-cells by semi-quantitative RT-PCR (**Supplementary Fig. 2**) using specific primers (see **Supplementary Methods**) and confirmed by sequencing the PCR products. Of interest, we also found that exons 8 and 9 of FGFR3 are largely absent in CLL B-cells as reverse primers designed for exon-8 or -9 could not amplify the transcript using the forward primer from exon-6, while the reverse primer for exon-11 and forward primer at exon-6 amplified FGFR3 transcript (**Supplementary Fig. 2**). Deletions of FGFR3 exons-8–10 have been reported in multiple human malignancies including breast, squamous and osteosarcoma⁴. However, an in-depth study is needed to define more clearly the nature of FGFR3 regulation in CLL B-cells. In total, our results suggest that CLL B-cells overexpress primarily FGFR3.

Phosphorylation at tyrosine residues 653 and 654 (Y653/654) in the kinase domain is important for catalytic activity of the activated FGFRs and its downstream signaling⁵. To that end we detected that FGFRs in CLL B-cells remain constitutively phosphorylated at Y653/654 tyrosine residues (**Fig. 1H**); indicating that the FGFR signaling pathway is catalytically active. Of interest, we have also detected that CLL B-cells co-express both P-FGFR and P-Axl (**Fig. 1I**) suggesting that there may exist a possible functional link between these two RTKs. However, as CLL B-cells overexpress FGFR3, we hypothesized that FGFR3 remains as the constitutively active FGFR. Indeed FGFR3 displays elevated levels of phosphorylation at Y653/654 residues (**Fig. 1J**). Further analysis demonstrates that FGFR3 in CLL B-cells remains as a highly phosphorylated RTK (**Fig. 1K**). Together, these findings suggest that (i) FGFR signaling is a constitutively active pathway in CLL B-cells and that (ii), the heavily phosphorylated FGFR3 likely drives the FGFR signal in CLL B-cells.

To define the mechanism of constitutive phosphorylation on FGFRs, CLL B-cells were treated with recombinant-bFGF or a bFGF-neutralizing antibody and analyzed the cells for alteration of P-FGFR levels. We found that neutralizing antibody treatment or recombinant-bFGF addition to CLL B-cell culture could not alter the phosphorylation levels on FGFRs from the basal level (**Supplementary Fig. 3**); suggesting that FGFR phosphorylation in CLL B-cells is likely independent of any autocrine/paracrine loop. Of interest, a recent report suggests that Axl, which remains as a highly active RTK in CLL B-cells^{6,7}, can also crosstalk with the epidermal growth factor receptor (EGFR) and exists in a complex with the latter RTK in cetuximab (targets EGFR)-resistant non-small cell lung cancer cells⁸. These information and our findings that CLL B-cells co-express P-FGFR (**Fig. 1H**) and P-Axl (**Fig. 1I**) prompted us to investigate whether Axl is also involved in a crosstalk with the

FGFR signaling in CLL B-cells. To address this, we first inhibited Axl phosphorylation in CLL B-cells using a high-affinity Axl inhibitor TP-0903⁹ and determined P-Axl/P-FGFR levels. Results demonstrate that TP-0903 mediated inhibition of P-Axl in CLL B-cells (**Fig. 1L**) resulted in reduced levels of P-FGFR (**Fig. 1M**). On the other hand, treatment of CLL B-cells with the FGFR-inhibitor TKI-258¹⁰ could not alter Axl phosphorylation (**Fig. 1N, bottom panel**), while the reduction of PFGFR levels was evident (**upper panel**), suggesting that Axl is likely an upstream regulator of the FGFR signaling. Next, to rule out the possibility that TP-0903-mediated inhibition of P-FGFR was not an off-target effect, Axl in CLL B-cells was targeted using an Axl-specific siRNA. Indeed, a partial depletion of Axl in CLL B-cells resulted into reduction of P-FGFR levels (**Fig. 1O, Supplementary Fig. 4**). As primary CLL B-cells are difficult to transfect, we chose a breast cancer cell line MDA-MB-231 reported to express both Axl and FGFR1/3^{11, 12} as a model to further confirm Axl/FGFR functional relationship. As expected, siRNA-mediated depletion of Axl in MDA-MB-231 cells significantly reduced P-FGFR level (**Fig. 1P**), corroborating well with the findings in primary CLL B-cells described above (**Fig. 1; panels L,M,O**). Furthermore, induced activation of Axl in MDA-MB-231 cells by Gas6-ligation resulted into increased phosphorylation on FGFR (**Fig. 1Q**). Interestingly, Gas6-ligation did not alter significantly the P-Axl levels in CLL B-cells (**Supplementary Fig. 5A**), while BCR stimulation augmented Axl phosphorylation in CLL B-cells expressing low basal level of P-Axl (**Supplementary Fig. 5B**). However, mechanism of Axl activation by BCR signal remains unknown.

Next, we wished to interrogate whether Axl forms a complex with FGFR3 which we have found predominantly expressed and heavily phosphorylated FGFR in CLL B-cells (**Fig. 1; panels A,D,J,K**). Using IP/Western blot techniques we detected that Axl and FGFR3 indeed form a complex in CLL B-cells (**Fig. 2A,B**). To confirm the specificity, Axl was depleted in MDA-MB-231 cells using an Axl-specific siRNA to detect Axl/FGFR3 association. We did find co-precipitation of FGFR3 in the immunocomplex of Axl obtained from the control cells however the level of co-precipitated FGFR3 was reduced significantly in the immunocomplex upon depletion of Axl in MDA-MB-231 cells (**Fig. 2C**); while it appears that complex formation between Axl and FGFR3 may not depend on phosphorylation status of Axl (**Supplementary Fig. 6**). We also found that Axl and FGFR3 co-localize in CLL B-cells (**Fig. 2D, panel d**) where a punctate plasma membrane distribution of FGFR3 (red) and Axl (green) was observed. Further analysis of images using Image-pro premier software suggests that Axl and FGFR3 were highly co-localized in CLL B-cells with an average overlap coefficient of 0.83 (**Fig. 2E**). Together, these findings suggest that Axl forms a complex with FGFR3, co-localize in the cells and regulates FGFR signaling pathway in human malignant cells.

Depending on cellular context, several pathways are activated by FGFRs including the p38 MAPK, Jun N-terminal kinase, AKT, phospholipase C γ and Stat pathways. However, the Ras-MAPK and Stat are the major pathways mediating the oncogenic effects of FGFR3¹³. Given this, a TKI-258-mediated inhibition of the constitutively active FGFR signal induced significant levels of apoptosis in CLL B-cells from previously untreated CLL patients (n=12; **Supplementary Table 1**) in a time- and dose-dependent manner (**Fig. 2F**). Relevant to this,

analysis of the TKI-258 treated CLL B-cell lysates used in **Fig. 1N** demonstrates that inhibition of P-FGFR in CLL B-cells reduces phosphorylation of c-Src, Stat3, and Erk1/2, but not of AKT (**Fig. 2G**), and expression of Mcl-1 but not XIAP or Bcl-2 (**Fig. 2G**). In addition, a partial depletion of FGFR3 also reduces phosphorylation on Erk1/2, Stat3, c-Src and expression of Mcl-1 in CLL B-cells (**Fig. 2H**) thus ruled out any off target effect of TKI-258. However, CLL bone marrow stromal cells showed a significant level of protection of the leukemic B-cells from TKI-258-induced apoptosis (**Fig. 2I**). Recently, overexpression of FGFR3 has been reported in Waldenstrom macroglobulinemia (WM) and that inhibition of FGFR3 induces apoptosis in WM cells and overcomes stromal protection¹⁴. Of interest, we also found that while *in vitro* combined treatment of CLL B-cells with TKI-258 and ibrutinib produced synergistic effects in 5 of 7 cases (**Fig. 2J**), combination of TKI-258 with TP-0903 was mostly antagonistic (**Fig. 2K**) in augmenting apoptosis levels. Collectively, these findings suggest that FGFR-signaling is constitutive and may play an important role in regulating CLL B-cell survival.

In summary, we report that CLL B-cells express high levels of FGFR3 which remains as a constitutively active RTK. Through a series of experiments, we, for the first time, report that Axl regulates FGFR signaling via complex formation with FGFR3. Crosstalk between RTKs is widespread; for example, ErbB3 robustly couples to the PI3-kinase pathway and it is used by EGFR and ErbB2 as well as other RTKs to activate this pathway¹⁵. Most recently, Axl was found to activate EGFR in cetuximab-resistant clones via complex formation, previously reported in triple-negative breast cancer cells⁸. We found that FGFR signaling is a downstream target of Axl and a novel addition to the growing list of RTKs in the Axl signaling network. Thus, targeting Axl signaling axis either alone or in combination with other signal inhibitors warrants further attention as a maneuver to more effectively induce apoptosis in CLL B-cells, particularly in the relapsed/refractory setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by a research fund from the National Cancer Institute CA170006 to AKG. Collection, processing and deposition of CLL samples into the CLL tissue bank were supported by the Predolin Foundation grant. We also wish to acknowledge Tolero Pharmaceuticals and Novartis for providing the inhibitors to Axl and FGFR, respectively, with relevant information and the excellent secretarial help of Ms. Tammy Hughes.

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Figure1. Expression profile and regulation of FGFR signaling in CLL B-cells. (A) CLL B-cells overexpress FGFR3

Lysates from normal B- and CLL B-cells were analyzed for the expression of FGFR3 by Western blots using a specific antibody. MDA-MB-231 breast cancer cell lysate was used as a positive control. Actin was used as loading control. Normal subjects (N1 – N3) or CLL patients (P1 – P8) are indicated by assigning arbitrary numbers. **(B) Detection of FGFR1 in CLL B-cells.** FGFR1 was immunoprecipitated from normal B- or CLL B-cell lysates, followed by Western blot analysis of the immune complex to detect FGFR1. Normal rabbit Immunoglobulin G (IgG) was included as an antibody control for the IP using CLL B-cell

lysates (P4). IgG heavy chain (HC) was used as loading control. “NS” indicates non-specific band. **(C) Detection of FGFR2 in CLL B-cells.** Similarly, FGFR2 was also immunoprecipitated from the same lysates used above, followed by Western blot analysis of the immune complex to detect FGFR2. Normal rabbit IgG was included as an antibody control for the IP using CLL B-cell lysates (P3). IgG HC was used as loading control. “NS” indicates non-specific band. **(D) Detection of FGFR3 in CLL B-cells.** FGFR3 was immunoprecipitated from the above normal B- or CLL B-cell lysates, followed by Western blot analysis to detect FGFR3. Normal rabbit IgG was included as an antibody control for the IP using CLL B-cell lysates (P3). IgG HC was used as loading control. **(E) Detection of FGFR4 in CLL B-cells.** FGFR4 was immunoprecipitated from the same set of normal B- or CLL B-cell lysates used above, followed by Western blot analysis to detect FGFR4. Normal rabbit IgG was included as an antibody control for the IP using CLL B-cell lysates (P4). IgG HC was used as loading control. Cell lysates from normal subjects (N1 – N4) or CLL patients (P1 – P4) used in the panels B – E are indicated by assigning arbitrary numbers. **(F)** Detection of FGFR3 on CLL B-cell surface by flow cytometry. Expression of FGFR3 on normal B-cells (n=10) and CLL B-cells (n=122) was determined by flow cytometric analysis using specific antibody. Results are presented as mean fluorescent intensity (MFI) after normalizing the values with the isotype control. Significantly ($p<0.0001$) higher level expression of FGFR3 was detectable on CLL B-cells as compared to normal B-cells. **(G)** Presentation of FGFR3 expression levels by histogram overlay. Expression of FGFR3 on normal B-cells from a representative healthy individual (blue), CLL B-cells with low (orange line), medium (green) and high (red) FGFR3 levels from representative CLL patients as determined by flow cytometry in **panel F** are presented by histogram overlay. The results were normalized with the isotype controls. **(H)** FGFRs in CLL B-cells are phosphorylated at the catalytic site. Cell lysates from normal B- or CLL B-cells were analyzed for the FGFR phosphorylation status at the catalytic tyrosine residues (Y653/654) by Western blots using a phospho-specific antibody. Actin was used as loading control. Normal subjects (N1 – N3) or CLL patients (P1 – P9) are indicated by assigning arbitrary numbers. **(I)** Expression levels of phosphorylated Axl in CLL B-cells. Axl was immunoprecipitated from equal amounts of the same CLL B-cell lysates used above (P1 – P9) in **panel H**, followed by Western blot analysis using a phosphotyrosine specific antibody (4G10) to detect phosphorylation status on Axl. The blot was stripped and reprobed with anti-Axl antibody to detect total Axl in the immune complex. IgG HC was used as loading control. **(J)** FGFR3 remains phosphorylated at the catalytic site. FGFR3 was immunoprecipitated from equal amounts of purified normal B- or CLL B-cell lysates, followed by Western blot analysis using a phospho-FGFR specific antibody to detect Y653/654 phosphorylation on FGFR3. Normal rabbit IgG was included as an antibody control for the IP using CLL B-cell lysates (P4). The blot was stripped and reprobed with an antibody to FGFR3 to detect total FGFR3 in the immune complex. IgG HC was used as loading control. Cell lysates from normal subjects (N1, N2) or CLL patients (P1 – P4) are indicated by assigning arbitrary numbers. **(K)** Detection of total tyrosine phosphorylation level on FGFR3 in CLL B-cells. FGFR3 was immunoprecipitated from the lysates of normal B- and CLL B-cells including those (N1, N2, P1 – P4) used in the above experiment (**panel J**) and analyzed for total phosphorylation levels on FGFR3 in Western blot using a P-Tyr (4G10) antibody. Normal rabbit IgG was included as an antibody control for the IP using

CLL B-cell lysates (P6). The blot was stripped and reprobed for the detection of immunoprecipitated FGFR3. IgG HC was used as loading control. Cell lysates from normal subjects (N1, N2) or CLL patients (P1 – P6) are indicated by assigning arbitrary numbers. (L) High-affinity Axl inhibitor TP-0903 reduces Axl phosphorylation. Total Axl was immunoprecipitated from lysates of CLL B-cells treated with a sub-lethal dose (0.1 μ M for 16 hours) of a high-affinity Axl inhibitor TP-0903, followed by Western blot analysis using a P-Tyr (4G10) antibody. The blot was stripped and reprobed to detect immunoprecipitated Axl. IgG HC was used as loading control. CLL patients (P1 – P3) are indicated by arbitrary numbers. (M) Inhibition of P-Axl reduces FGFR phosphorylation. TP-0903 treated CLL B-cell lysates used above were further analyzed for the status of FGFR phosphorylation by Western blot using a phospho-specific (Y653/654) FGFR antibody. Actin was used as loading control. (N) Inhibition of P-FGFR could not alter Axl phosphorylation. CLL B-cells were treated with a sub-lethal dose of TKI-258 or left untreated and phosphorylation status of FGFRs was analyzed by Western blots using a phospho-specific (Y653/654) FGFR antibody. Actin was used as loading control. Axl was also immunoprecipitated from the same CLL B-cell lysates and phosphorylation status of Axl was examined by Western blot analysis using the 4G10 antibody. IgG HC was used as loading control. CLL patients (P1 – P3) are indicated by arbitrary numbers. (O) Depletion of Axl reduces FGFR phosphorylation in CLL B-cells. Purified primary CLL B-cells from two representative CLL patients (P1, P2) were transfected with the control sc-siRNA (indicated by “-”) or Axl-specific siRNA using the Lipofectamine 2000 lipid reagent, and cell lysates were analyzed for the status of Axl expression and P-FGFR (Y653/654) levels by Western blots using specific antibodies. Actin was used as loading control. Fold changes of Axl and P-FGFR after siRNA transfection were determined by densitometric analysis and indicated. (P) Targeting Axl in MDA-MB-231 cells reduces FGFR phosphorylation level. In a similar experiment, MDA-MB-231 breast cancer cells were transfected with sc-siRNA (indicated by “-”) or Axl-specific siRNA and cell lysates were analyzed for the status of Axl expression and P-FGFR (Y653/654) by Western blots using specific antibodies. Actin was used as loading control. (Q) Enforced activation of Axl increases FGFR phosphorylation. MDA-MB-231 cells were serum starved and treated with Gas6 (200ng/ml) for 0, 5, 10 and 20 min. Cell lysates were then analyzed for the phosphorylation status of Axl at Y702 and FGFR at Y653/654 using specific antibodies. The blot showing P-Axl was stripped and reprobed to detect Axl expression. Actin was used as loading control.

Figure 2. FGFR3 remains in a complex with Axl and could be a potential therapeutic target in CLL. (A & B) Axl and FGFR3 exist in a complex in CLL B-cells
 FGFR3 or Axl was immunoprecipitated from the same lysates of purified CLL B-cells, followed by Western blot analyses to detect Axl (**panel A**) or FGFR3 (**panel B**), respectively using specific antibodies as indicated. Blots were stripped and reprobed to detect immunoprecipitated FGFR3 and Axl, respectively (bottom panels). IgG HC was used as loading control. CLL patients (P1 – P6) are indicated by arbitrary numbers. Normal species-specific IgG was included as an antibody control for the IP using CLL B-cell lysates (P6). (C) Axl and FGFR3 exist in a complex in breast cancer cells. MDA-MB-231 cells were

transfected with sc-siRNA (indicated by “-”) or Axl-siRNA for 48 hours. Cell lysates were prepared and Axl was immunoprecipitated, followed by Western blot analysis to detect FGFR3 in the immune complex. The blot was stripped and reprobed to detect immunoprecipitated Axl. IgG HC was used as loading control. **(D) Axl co-localizes with FGFR3.** Purified CLL B-cells were fixed and immunostained with a mouse monoclonal antibody to Axl and a rabbit antibody to FGFR3, followed by incubation with chromogen-conjugated secondary antibodies as appropriate. Cells were counterstained with the nuclear dye DAPI and visualized the cells under confocal microscope. Membrane distribution of FGFR3 (red; panel a) and Axl (green; panel b) was discernible in CLL B-cells. Co-localization (yellow; panel d) of FGFR3 and Axl in CLL B-cells was evident when the panels exhibiting expression of FGFR3 and Axl were merged. Horizontal lines indicate size of the cells. (E) Quantitation of Axl and FGFR3 co-localization in CLL B-cells. Degree of co-localization of Axl and FGFR3 in CLL B-cells was quantified using the Image-pro premier software (version 9.1: Media Cybernetics). Average of overlap coefficient (coloc overlap) from 10 different fields is shown by bar diagram where “0” indicates no co-localization and “1” indicates highest degree of co-localization. (F) Inhibition of FGFR in CLL B-cells induces apoptosis. CLL B-cells from previously untreated CLL patients (n=12) were treated with increasing doses of TKI-258 as indicated for 24, 48 and 72 hours or left untreated (DMSO control). Induction of apoptosis was determined by flow cytometric analysis after staining the cells with annexin V/PI. The dotted blue line indicates the average *in vitro* LD₅₀ dose of TKI-258 at 72-hour. (G) TKI-258 treatment modulates downstream targets of FGFR signaling pathway in CLL B-cells. Lysates of purified CLL B-cells treated with a sub-lethal dose of TKI-258 or left untreated (DMSO) used in **Fig. 1N**, were further analyzed for the activation status of Stat3, AKT, c-Src (SFK; Src-family kinases) and Erk1/2 (p42/44) by Western blots using phospho-specific antibodies. The blots were stripped and reprobed to detect total Stat3, AKT, and Erk1/2 (p42/44), respectively. Cell lysates were also examined for the expression of the anti-apoptotic proteins Mcl-1, Bcl-2 and XIAP by Western blot analyses using specific antibodies. Actin was used as loading control. CLL patients (P1 – P3) are indicated by arbitrary numbers. (H) Impact of endogenous FGFR3 depletion by RNA interference on FGFR signaling in CLL B-cells. CLL B-cells from 3 different patients transfected with sc-siRNA (indicated by “-”) or FGFR3-specific siRNA using the Amaxa nucleofection reagent, and cell lysates were analyzed for the status of FGFR3 expression and the phosphorylation status of Erk1/2 (p42/44), Stat3 and c-Src by Western blots using phospho-specific antibodies. The blots were stripped and reprobed to detect total Erk1/2(p42/44) and Stat3, respectively. Cell lysates were also examined for the expression of the anti-apoptotic proteins Mcl-1. Actin was used as loading control. Results from a representative CLL patient are shown. (I) Impact of TKI-258 on CLL B-cell survival in co-culture with CLL BMSCs. Purified CLL B-cells from previously untreated CLL patients (n=4) were cultured alone or co-cultured with CLL BMSCs (n=3) and treated with the average LD₅₀ dose of TKI-258 for 72 hours. Induction of apoptosis in CLL B-cells was determined by flow cytometric analysis using annexin V/PI staining and presented as percent viability. (J) Combined treatment of CLL B-cells with TKI-258 and ibrutinib shows synergistic effect. CLL B-cells from 7 previously untreated high-risk CLL patients with 17p-/11q-deletion were treated with increasing doses of TKI-258 or ibrutinib as a single agent or in combination of TKI-258 with ibrutinib (1:1) for 72 hours. Cells were harvested

and induction of apoptosis was determined as described elsewhere. Combination index (CI) of the results obtained from individual CLL samples following treatment with TKI-258 and ibrutinib was calculated following the method of Chou and Talalay. CI values <1 indicate a synergistic effect, CI value of 1 indicates additive effects and values >1 indicate antagonistic effects of combined treatment. (K) Combined treatment of CLL B-cells with TKI-258 and TP-0903 shows antagonistic effect. CLL B-cells from the same 7 CLL patients used above (**panel J**) were treated with increasing doses of TKI-258 or TP-0903 as a single agent or in combination of TKI-258 with TP-0903 (1:0.1). After 72 hours, induction of apoptosis in CLL B-cells was determined and the Combination Index (CI) values were calculated as described above. Combination of these two agents shows antagonistic effects in CLL B-cells from 5 of 7 CLL patients tested.