

The Kinase Inhibitor PCI-32765 Demonstrates Activity In Chronic Lymphocytic Leukemia Cells Independent of Microenvironmental Survival Signals

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Background

Bruton's tyrosine kinase (BTK), a member of the Tec family kinases, has a well characterized role in B-cell receptor signaling and activation. BTK is activated by Src-family kinases and leads to activation of essential cell survival pathways such as NF- κ B and MAPK. Although BTK is expressed in multiple hematopoietic cells, the primary defect in BTK^{-/-} mice is B-cell specific, suggesting a more selective B-cell function. BTK mutations in humans give rise to X-linked agammaglobulinemia, an inherited disorder characterized by severe B-cell-specific defects including decreased immunoglobulin production and absence of B-cells, further underscoring the importance and selectivity of BTK to B-cells. Although the functional role of the BCR in chronic lymphocytic leukemia (CLL) pathogenesis is still unclear, BTK was identified in an unbiased screen as an essential signaling kinase for survival of certain lymphomas such as diffuse large B-cell lymphoma.

We hypothesized that therapeutic targeting of BTK would have a B-cell selective impact due to their dependence on BTK for normal cellular function. Recently, a novel selective irreversible inhibitor of BTK, PCI-32765, has been described and has shown objective responses in dogs with B-cell malignancies. Given the potential importance of BCR signaling in CLL tumor cell development and survival, we investigated PCI-32765 for its effect on CLL cells with respect to promoting apoptosis, inhibiting proliferation, and disrupting external survival stimuli provided by the microenvironment. Collectively, these studies strongly support the development of PCI-32765 as a therapeutic agent for the treatment of CLL and other lymphoproliferative diseases.

Materials & Methods

Immunoblot analysis. Antibodies included anti-AKT, anti-phospho-AKT (Ser⁴⁷³), anti-PARP, anti-phospho-ERK (Thr²⁰²/Tyr²⁰⁴), anti-ERK (Cell Signaling, Danvers, MA), anti-BTK (Epitomics, Burlingame CA), and anti-GAPDH (Millipore, Billerica, MA).

Quantitative RT-PCR. RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was prepared using a SuperScript First-Strand Synthesis System (Invitrogen). Real-Time RT-PCR was performed using pre-designed TaqMan® Gene Expression Assays and ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA).

Viability Studies. Standard MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO) assays were performed to determine cytotoxicity. Cell viability was also measured using annexin/PI flow cytometry.

Enzymatic Caspase Assay. The presence of active caspase enzymes was determined by the amino trifluoromethyl coumarin assay (AFC). Release of free AFC was determined using a Beckman Coulter DTX 880 multimode detector (Filters: excitation; 405/10 nm, emission; 535/25 nm).

Proliferation Assay. Proliferation of CLL cells was measured by incorporation of radiolabeled thymidine. CLL cells were stimulated with a CpG oligonucleotide (CpG685) for 120 hours in RPMI 1640 containing 10% HS. Cells were then treated with various concentrations of PCI-32765, then were pulsed with [³H]-thymidine (1.0 μ Ci/well). [³H]-thymidine incorporation was measured after 18 hours.

Statistical analysis. All statistical evaluations were performed by the Center for Biostatistics at the OSU Comprehensive Cancer Center. P-values at $\alpha=0.05$ for single comparisons or after adjustment for multiple comparisons are considered significant.

Disclosures:

A. Hamdy and J. Buggy are employees of Pharmacyclics Inc. and have financial interest in this company.

All others have no relevant conflicts of interest to disclose.

Figures

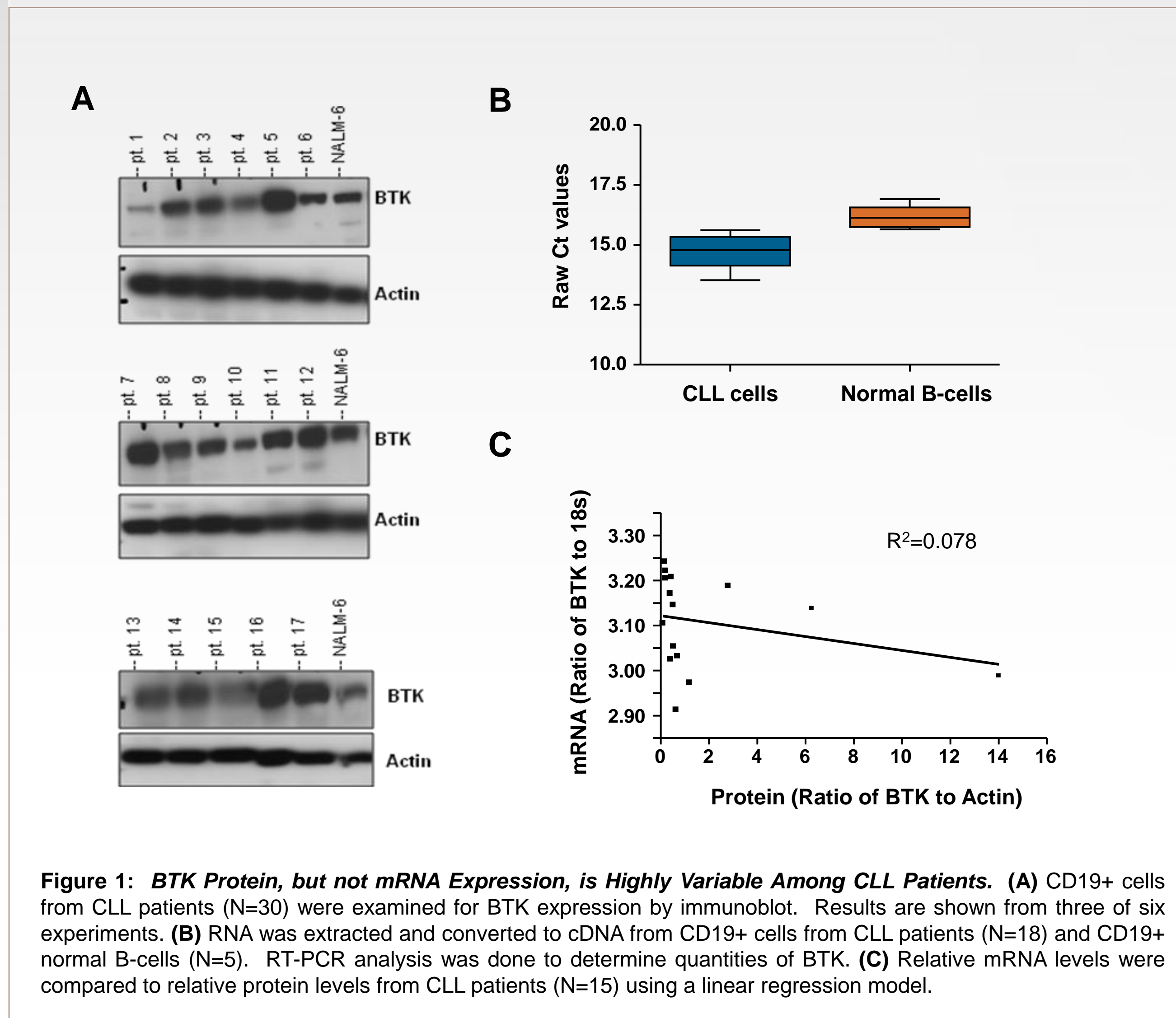


Figure 1: BTK Protein, but not mRNA Expression, is Highly Variable Among CLL Patients. (A) CD19+ cells from CLL patients (N=30) were examined for BTK expression by immunoblot. Results are shown from three of six experiments. (B) RNA was extracted and converted to cDNA from CD19+ cells from CLL patients (N=18) and CD19+ normal B-cells (N=5). RT-PCR analysis was done to determine quantities of BTK. (C) Relative mRNA levels were compared to relative protein levels from CLL patients (N=15) using a linear regression model.

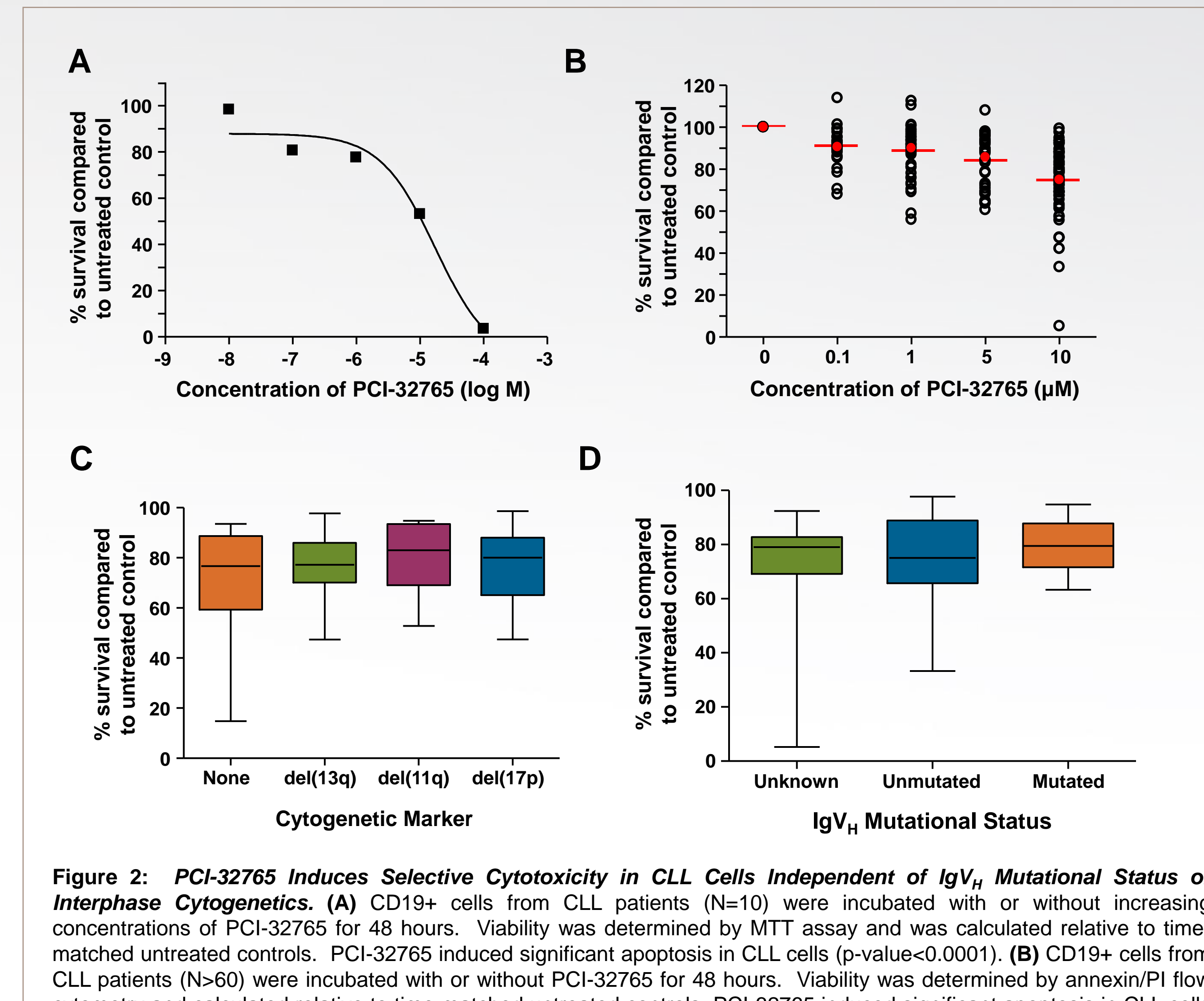


Figure 2: PCI-32765 Induces Selective Cytotoxicity in CLL Cells Independent of IgV_H, Mutational Status or Interphase Cytogenetics. (A) CD19+ cells from CLL patients (N=10) were incubated with or without increasing concentrations of PCI-32765 for 48 hours. Viability was determined by MTT assay and was calculated relative to time-matched untreated controls. PCI-32765 induced significant apoptosis in CLL cells (p-value<0.0001). (B) CD19+ cells from CLL patients (N=60) were incubated with or without PCI-32765 for 48 hours. Viability was determined by annexin/PI flow cytometry and calculated relative to time-matched untreated controls. PCI-32765 induced significant apoptosis in CLL cells (p-value<0.0001). (C, D) CD19+ cells from CLL patients (N=60; minimum 10 per group) were incubated with or without 10 μ M PCI-32765 for 48 hours. Viability was determined by annexin/PI flow cytometry, and was calculated relative to time-matched untreated controls. Differences were not significant.

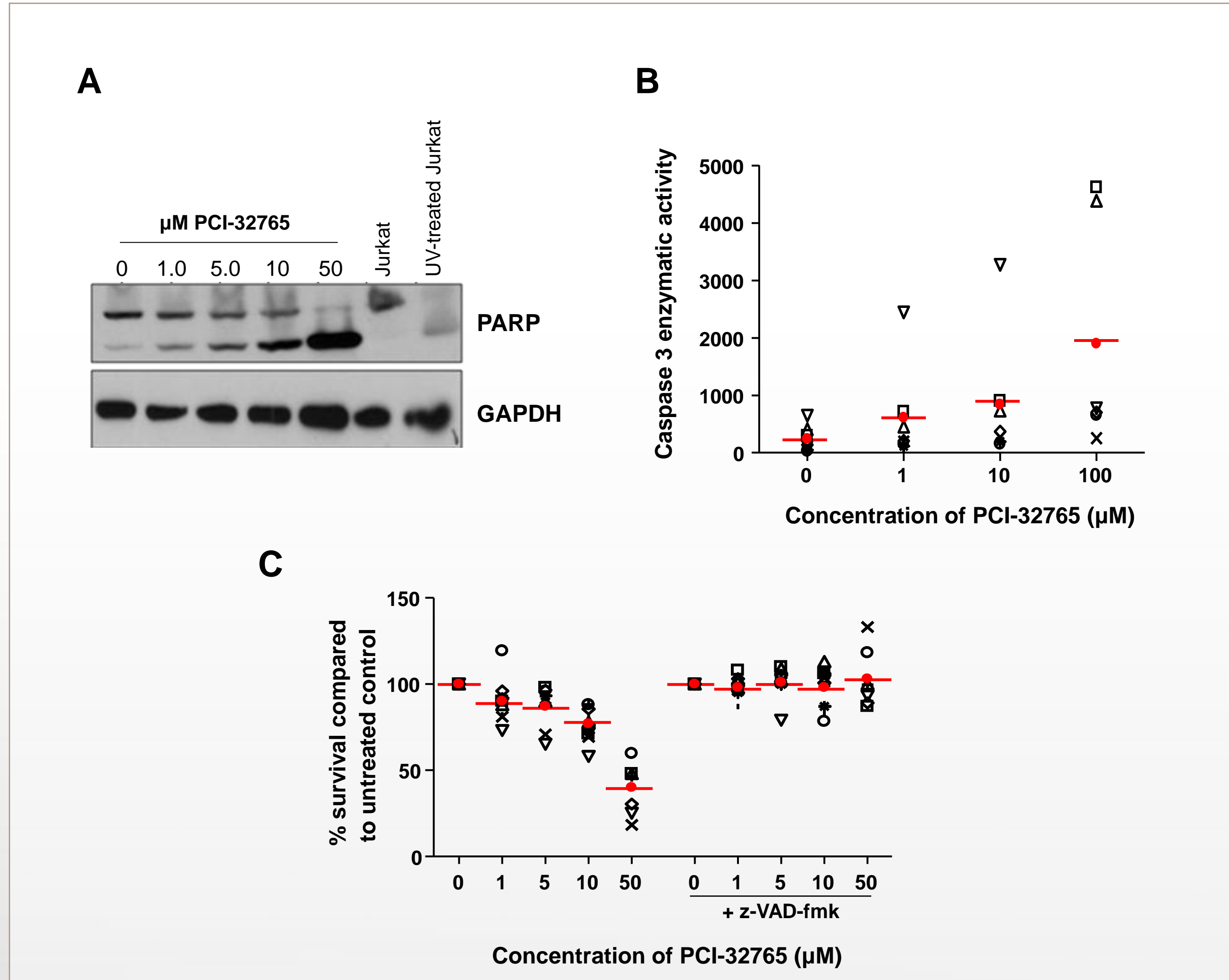


Figure 3: PCI-32765 Cytotoxicity Against CLL Cells is Dependent on Caspase Pathway Activation. (A) CD19+ cells from CLL patients (N=4) were incubated with PCI-32765 for 8 hours and PARP cleavage was assessed by immunoblot. Results are shown from one of four experiments. (B) CD19+ cells from CLL patients (N=7) were incubated with PCI-32765 for 8 hours. Caspase activity was determined by AFC assay. (C) CD19+ cells from CLL patients (N=10) were incubated with PCI-32765 and/or 100 μ M z-VAD-fmk for 48 hours. Viability was determined by annexin/PI flow cytometry, and is shown relative to time-matched untreated controls. z-VAD prevented the significant apoptosis seen with PCI-32765 treatment (p-value<0.0001).

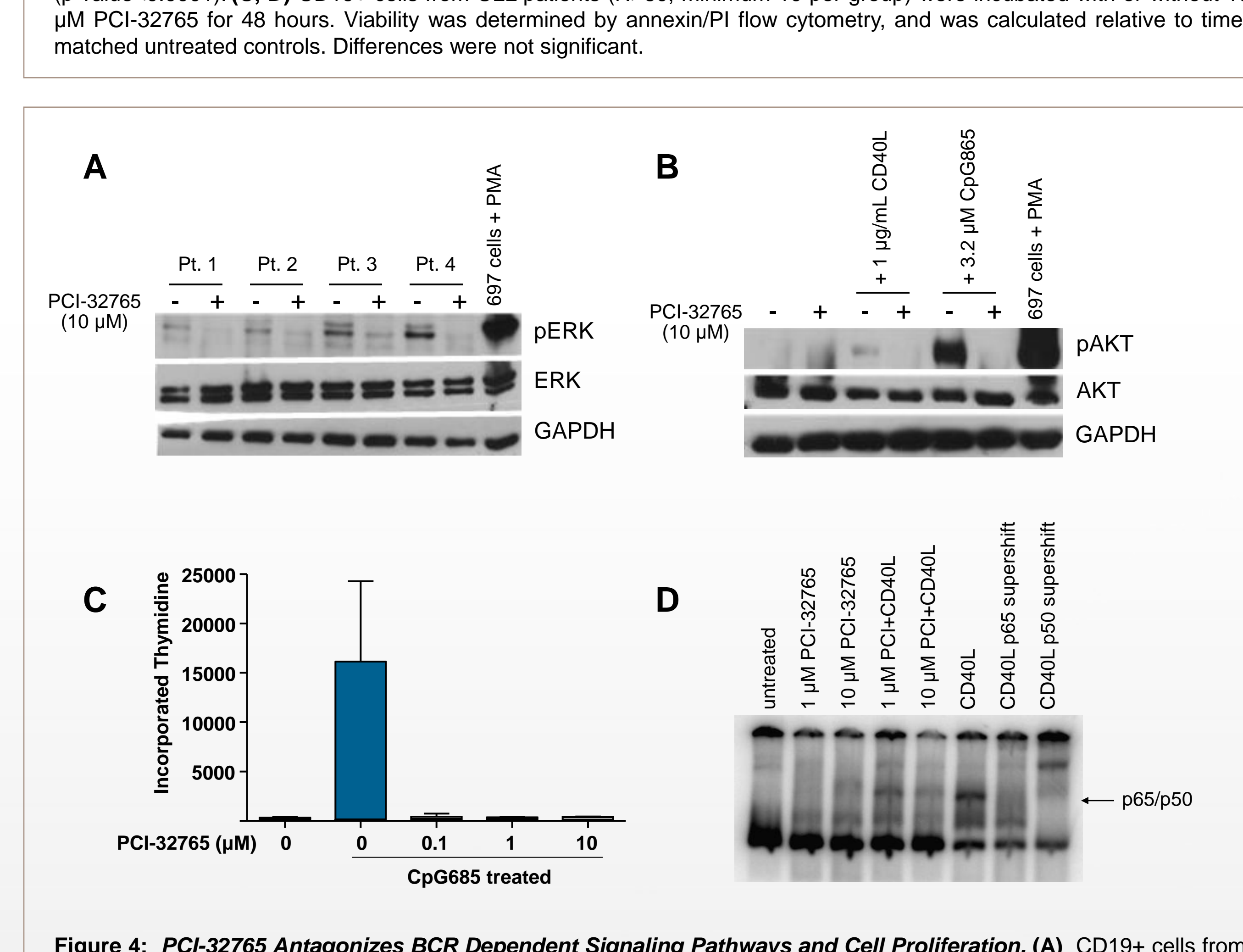


Figure 4: PCI-32765 Antagonizes BCR Dependent Signaling Pathways and Cell Proliferation. (A) CD19+ cells from CLL patients (N=7) were incubated with 10 μ M PCI-32765 for 1 hour. ERK phosphorylation at Thr²⁰²/Tyr²⁰⁴ was assessed by immunoblot. (B) CD19+ cells from CLL patients (N=3) were incubated with 10 μ M PCI-32765 and 3.2 μ M CpG685 or 1 μ g/mL CD40L for 1 hour. AKT phosphorylation at Ser⁴⁷³ was assessed by immunoblot. Results are shown from one of three experiments. (C) CD19+ cells from CLL patients (N=7) were incubated with or without PCI-32765 and 3.2 μ M CpG685 for 120 hours. Proliferation was assessed by [³H]-thymidine incorporation. (D) CD19+ cells from CLL patients (N=5) were incubated with 1 or 10 μ M PCI-32765 and/or 1 μ g/mL CD40L. Electrophoretic mobility shift analysis was done using a consensus NF- κ B oligonucleotide. Antibody shifts were performed from CD40L treated sample incubated with antibodies specific to the NF- κ B p65 or p50 subunits.

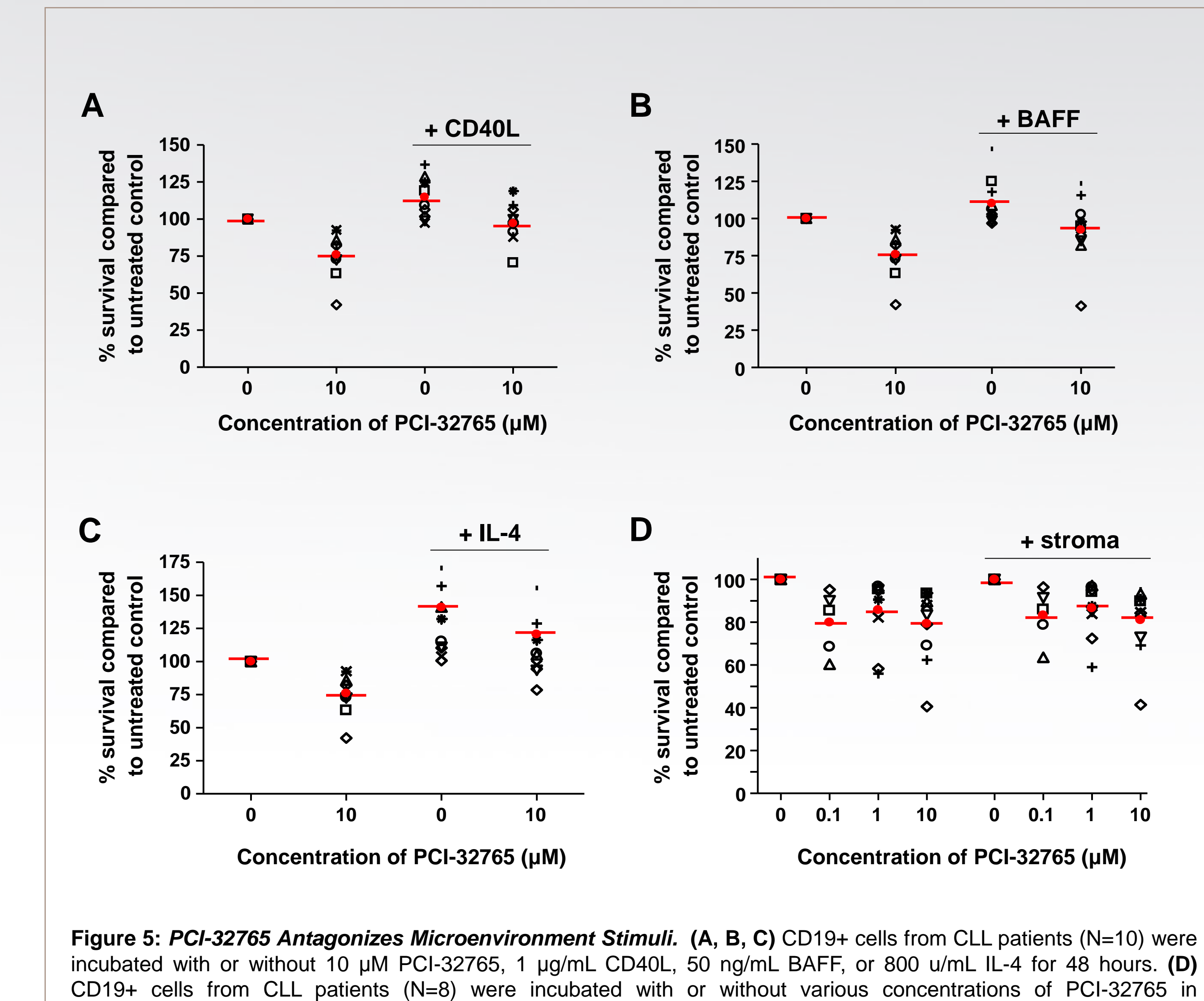


Figure 5: PCI-32765 Antagonizes Microenvironment Stimuli. (A, B, C) CD19+ cells from CLL patients (N=10) were incubated with or without 10 μ M PCI-32765, 1 μ g/mL CD40L, 50 ng/mL BAFF, or 800 u/mL IL-4 for 48 hours. (D) CD19+ cells from CLL patients (N=8) were incubated with or without various concentrations of PCI-32765 in suspension or on an HS-5 stromal cell layer for 48 hours. In all experiments, viability was determined by annexin/PI flow cytometry and is shown relative to time-matched untreated controls for each group. Red lines represent averages. None of the survival signals prevented PCI-32765-induced apoptosis (all p-values > 0.09).

Results and Conclusions

- All CLL samples evaluated show BTK expression at both the mRNA and protein levels.
- PCI-32765-induced apoptosis in primary CLL cells is independent of deletions of 11q22 or 17p13 and IgV_H gene mutational status, prognostic factors that influence CLL progression and response to therapy.
- Cytotoxicity observed with PCI-32765 is accompanied by PARP cleavage and induction of caspase 3 activity, and is blocked by caspase inhibition. These results indicate that PCI-32765-mediated cytotoxicity in CLL cells is caspase-dependent.
- PCI-32765 alters both ERK and AKT signaling as well as NF- κ B DNA binding activity, suggesting that treatment decreases BCR-induced survival signals.
- PCI-32765 treatment prevented CpG oligonucleotide-induced CLL cell proliferation.
- PCI-32765 blocks the protective effects elicited by pro-survival microenvironmental factors such as CD40L and BAFF.
- Co-culture of CLL cells on a stromal layer did not significantly affect the cytotoxic properties of PCI-32765, suggesting that this agent is able to overcome stroma-mediated protection.
- Together, these data suggest that PCI-32765 acts directly on CLL cells to induce apoptosis, but may also act on the surrounding microenvironment to prevent the creation of a survival niche for the CLL cells.